VYSOKÁ ŠKOLA CHEMICKO-TECHNOLOGICKÁ

V PRAZE

Fakulta potravinářské a biochemické technologie

Ústav biochemie a mikrobiologie

Imunochemická detekce nízkomolekulárních látek

HABILITAČNÍ PRÁCE

PRO OBOR

BIOCHEMIE

Praha 2018 **Ing. Barbora HOLUBOVÁ, Ph.D.**

Tato práce shrnuje mé dosavadní výsledky z oblasti vývoje rychlých imunochemických metod pro detekci nízkomolekulárních látek.

Chtěla bych poděkovat především Prof. Ing. Pavlu Rauchovi, DrSc. a Prof. Ing. Ladislavu Fukalovi, CSc. za jejich cenné rady, všestrannou podporu a spolupráci na této práci. Mé poděkování patří i všem členům laboratoře Bioafinitních metod za vytvoření příjemného, přátelského a klidného pracovního prostředí. V neposlední řadě bych chtěla poděkovat svému manželovi a celé mé rodině za podporu, kterou mi celou dobu poskytují.

Praha 2018 *Barbora Holubová*

Obsah

1 Úvod

Na počátku 20. století se předpokládalo, že se protilátky v organismu mohou tvořit pouze proti látkám považovaným za patogenní, které se vyskytují v našem životním prostředí. Tuto teorii opravil Karl Landsteiner na základě svých experimentů publikovaných ve dvacátých a třicátých letech minulého století. Tento významný imunolog a držitel Nobelovy ceny prokázal, že protilátky mohou být produkovány i proti nízkomolekulárním látkám, které se dokonce ani nemusí v přírodě vyskytovat. Tvorbu protilátek však pozoroval pouze v případě, kdy byly tyto látky kovalentně spojeny („upevněny") na makromolekuly. Landsteiner nazval malé molekuly, které samy o sobě nestimulují imunitní odpověď, hapteny (z řeckého *haptien* = upevňovat). Landsteinerovy experimenty se staly základem výzkumu přírodních i syntetických haptenů, ze kterého vycházel i vývoj imunochemických metod pro jejich detekci.

Imunochemické metody se během uplynulých desetiletí vyvinuly z původní role doplňkového nástroje při analýze vzorků na jednu z alternativ konvenčních metod. Jejich rychlost, jednoduchost a nízká cena se staly předností pro detekci nejrůznějších vysokomolekulárních i nízkomolekulárních látek. Výběr vhodného formátu imunochemické metody závisí na řadě faktorů: účel analýzy, typ cílového analytu, znalost původu vzorku atd. Pokud je cílem stanovit nízkomolekulární látku (hapten), je při vývoji metody kritickým krokem příprava imunizačního konjugátu, který bude použit pro přípravu protilátky, která odpovídá za požadovanou specificitu a citlivost k cílové nízkomolekulární látce. Konečný výsledek analýzy ovlivní také vybraný princip imunochemické metody. Mezi nejčastěji používané metody pro detekci nízkomolekulárních látek patří kompetitivní formát ELISA (z angl. Enzyme Linked Immunosorbent Assay) nebo LFIA (z angl. Lateral Flow Assay). Díky stále se zvyšujícím požadavkům na rychlejší a spolehlivější detekci haptenů se objevují i další imunochemické metody detekce založené např. na nekompetitivních principech nebo kombinující principy imunoanalýzy na pevné fázi s fyzikálně-chemickým převodníkem (imunochemický biosenzor).

Předkládaná práce se věnuje problematice výběru vhodné struktury imunizačního konjugátu a podává přehled imunochemických metod používaných k detekci nízkomolekulárních látek především v potravinách a životním prostředí. V praktické části jsou uvedeny konkrétní imunochemické metody, které byly vyvinuty v naší laboratoři pro detekci vybraných kontaminantů (pesticidů, anabolických androgenních steroidů a nových psychoaktivních látek).

2 Imunochemická detekce molekul typu haptenu

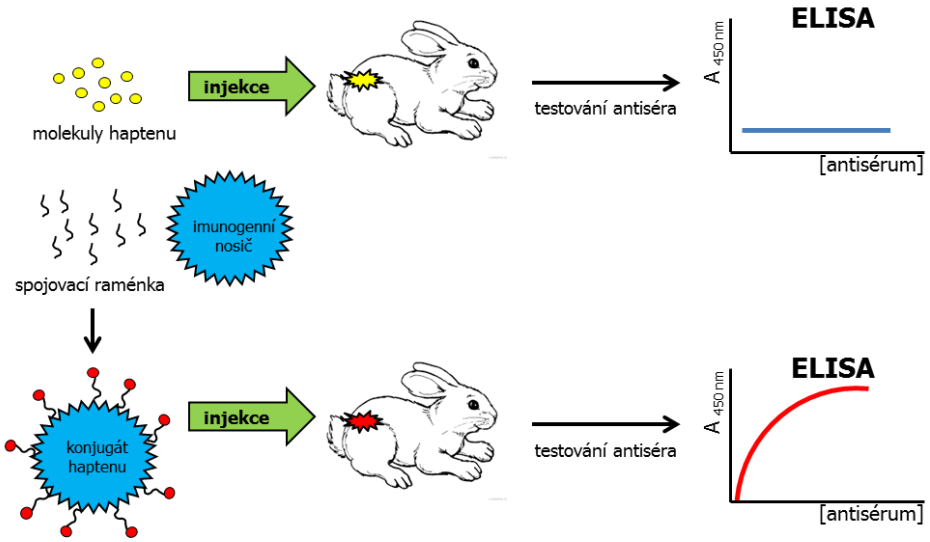
Imunochemické metody využívají k důkazu, stanovení nebo separaci analytu biospecifické interakce s protilátkami. *In vitro* se při nich využívá produkt imunitního systému obratlovců zaměřený původně na specifické rozlišení a zneškodnění cizorodého agens (antigenu) *in vivo*. Tento produkt (specifické protilátky) umožňuje poměrně citlivé a specifické rozpoznání analytu v komplexních směsích biologických vzorků. Podstatou všech imunochemických metod je tedy nekovalentní interakce protilátky s antigenem.

Antigen, který není sám o sobě imunogenní, ale může se vázat na protilátky s příslušnou specificitou, se nazývá hapten. Většina haptenů jsou nízkomolekulární látky (< 3 kDa) (Flaherty, 2012a; Greg, 2013), které můžeme nalézt např. v životním prostředí (pesticidy), zdravotnictví (léky a jejich metabolity, antibiotika), kriminalistice (drogy, anabolické steroidy), aj. V některých případech mohou být jako hapteny označeny i některé makromolekuly; jejich vazba na imunogenní nosič pomáhá T-buňkám při tvorbě protilátek proti nim.

Před začátkem vývoje imunochemické metody pro detekci nízkomolekulární látky je potřeba zvolit typ imunizačního konjugátu, typ protilátky, formát metody, popř. způsob značení imunoreagencií. Výběr typu metody ovlivní konečný výsledek analýzy, tzn., zda metoda bude specifická pro jeden analyt nebo skupinově specifická. Potřebná specifita metody je ovlivněná také výběrem haptenu pro přípravu imunizačního konjugátu. Hapten s výrazným strukturním rysem je vhodný k vývoji specifické metody. Naopak pro skupinovou analýzu je potřeba zvolit hapten s takovým strukturním rysem, který je společný všem strukturám v cílové skupině. Vybraná technika navázání haptenu na nosič by neměla ovlivnit místo pro specifickou vazbu protilátky. V některých případech také výběr typu protilátky (monoklonální, polyklonální, rekombinantní) může umožnit vývoj různých přístupů k analýze nízkomolekulárních látek (Kudlak a Namiesnik, 2011, Spinks, 2000).

2.1 Hapten a jeho konjugace s nosičem

Hapten se může stát imunogenním, když je navázán na vysokomolekulární nosič (Obr. 1). V této souvislosti je třeba zmínit, že pojem „antigenní" je někdy nesprávně používám jako synonymum pro „imunogenní". Pro volný hapten totiž platí, že je „antigenní" ale ne „imunogenní". Po spojení s nosičem se malý hapten může stát antigenním epitopem (determinantou). Jako epitop však bohužel může sloužit i sousední oblast nosiče nebo spojovací raménko s nosičem (Flaherty, 2012a, Greg, 2013, Seppälä a kol., 1998).



Obr. 1: Příprava specifických protilátek proti malým molekulám haptenů. Převzato a upraveno z https://www.abcam.com/primary-antibodies/developing-custom-rabbit-monoclonal-antibodies-against-small-molecule-haptens, VIII/2018.

Nejčastěji používané kovalentní nosiče haptenů jsou imunogenní velké molekuly, zejména proteiny (Tab. 1); mohou se používat i liposomy, syntetické nebo přírodní polymery (dextran, agarosa) nebo synteticky navržené organické molekuly (dendriny), které však nebývají tak imunogenní jako proteiny (Greg, 2013). Při výběru vhodného nosiče je postupováno podle těchto kritérií: 1) potenciál pro poskytnutí imunogenních vlastností; 2) přítomnost vhodných funkčních skupin pro konjugaci; 3) vhodná rozpustnost; 4) netoxičnost *in vivo*. Připravené konjugáty haptenu s nosičem nemusí sloužit jen k imunizaci, ale používají se i během imunochemických analýz jako jeden z imunoreaktantů.

Jak již bylo uvedeno, aby hapteny v organismu vyprovokovaly imunitní systém k produkci protilátek, je nutné je nejprve navázat na vysokomolekulární nosič. V rámci experimentů bylo ověřeno, že čím více haptenů je navázáno na nosič, tím více roste titr protilátek. Ovšem opačný efekt byl pozorován v souvislosti s afinitou protilátek; čím nižší byl poměr hapten-nosič, tím vyšší afinity dosahovaly produkované protilátky. Navíc bylo zjištěno, že vazba haptenu s nosičem přes centrální aminokyselinový zbytek způsobuje vyšší titry protilátek než při použití koncového aminokyselinového zbytku nosiče. Aby bylo dosaženo vhodné hladiny a afinity produkovaných protilátek, je tedy pro přípravu konkrétního imunogenního konjugátu potřeba prověřit různé poměry hapten-nosič a způsoby konjugace (Greg, 2013).

Tab. 1: Přehled používaných nosičů pro hapteny

Název nosiče	**MW** (kDa)	**Příklad haptenů**	**Reference**
Hovězý sérový albumin *(Bovine serum albumin*; *BSA)*	67	Pesticidy, steroidy, drogy, hormony, toxiny	Kato a kol., 2016; Kim a kol., 2007; Al-Dujaili, 2006; Naar a kol., 2002;
Hemocyanin z děrnatky obrovské (*Keyhole limpet hemocyanin; KLH*)	450 – 1,3.10^4	Pesticidy, příprava vakcín pro léčbu rakoviny	Aarntzen a kol., 2012; Curigliano a kol., 2006; Miles a kol., 2011; Kim a kol., 2002
Thyreoglobulin	660	Peptidy, fullereny	Chen a kol., 1998
Ovalbumin	43	Hormony, peptidy	Goda a kol., 2000; Maiken a kol., 2006
Tetanický toxoid	150	Příprava vakcín proti *Streptococcus agalactiae, Neisseria meningitidis, Haemophilus influenzae*	Paolletti a kol., 1994; Dhillon a kol., 2017; McCormick, 2012;
Toxoid záškrtu	63	Příprava vakcín proti *Haemophilus influenzae*	McCormick, 2012
Myoglobin	18	Organické polutanty	Karsten a kol., 2002
Králičí sérový albumin *(Rabbit serum albumin; RSA)*	69	Drogy, pesticidy, fullereny, příprava vakcín	Chen a kol., 1998; Dunbar a kol., 1990; Aoki a kol., 1996
Syntetické polypeptidové řetězce (poly-L-lysin; poly-L-glutamová kyselina)	15 — 30	Hormony, peptidy, příprava vakcín pro léčbu rakoviny	Romestand a kol., 2010; Posnett a kol., 1998; Li, 2002

MW…molekulová hmotnost

Hapten lze na nosič kovalentně navázat přes jeden nebo i více reaktivních atomů v jeho molekule. Přitom výběr vazebného místa na molekule haptenu významně ovlivní specifitu produkovaných protilátek. Lze tak cíleně připravit buď úzce specifické protilátky, které budou interagovat pouze s jediným vybraným zástupcem ze skupiny strukturně podobných haptenů, anebo široce specifické protilátky, reagující se všemi členy haptenové skupiny. Při volbě způsobu přípravy konjugátu nosiče s haptenem se lze řídit empirickými doporučeními, které vyplývají z mnoha dosud provedených experimentů (Mičková a kol., 2004; Fukal a Holubová, 2007):

a) Hapten by měl pomocí krátkého spojovacího raménka (tři – šest atomů) mírně vyčnívat z molekuly nosiče, aby epitopově charakteristické atomy jeho molekuly nebyly stíněny.
b) Místo vazby s nosičem by mělo být co nejvíce vzdáleno od té části haptenové molekuly, proti které chceme vytvořit specifické protilátky.

c) Není vhodné konjugovat hapten přes heteroatom (jiný atom než uhlík) v jeho molekule; tento způsob by mohl vést ke změně polarity a tím i ke změně charakteru epitopu.
d) Vhodné pro konjugaci je alifatické raménko (bez heteroatomů), které by nemělo být výraznějším epitopem než molekula haptenu.
e) Delší raménko může zvýraznit imunitní odpověď na hapten.

Pro konjugaci s nejčastěji používaným nosičem – proteinem – se velmi často využívá metoda smíšeného anhydridu nebo metoda aktivního esteru. Tyto metody lze použít v případě přítomnosti reaktivní karboxylové skupiny v molekule haptenu a aminoskupiny lysinu na proteinovém nosiči. Výtěžnost uvedených technik je obvykle 10–40 %. Po konjugaci je nutné vzniklý konjugát z reakční směsi izolovat a přečistit (Oubiña a kol., 1999, Fukal a Holubová, 2007).

2.2 Přehled imunochemických metod pro detekci nízkomolekulárních látek

Nízkomolekulární látky neprecipitují s protilátkami, a proto se jejich stanovení provádí neprecipitačními imunochemickými metodami. K zesílení a zviditelnění vzniku imunokomplexu se může využít vhodná značka (odd. 2.2.1), která se naváže na jeden z imunoreaktantů, a výrazně tím zvýší citlivost imunochemického stanovení. Podle použité značky jsou jednotlivé imunochemické metody pojmenovány, např. radioimunoanalýza (RIA; radioizotopy: ^{14}C, ^{3}H, ^{32}P, ^{125}I, ^{57}Co), fluoroimunoanalýza (FPIA; fluorofory: fluorescein, umbeliferon, rhodamin), luminiscenční imunoanalýza (luminol a jeho deriváty), enzymová imunoanalýza (ELISA, EMIT; enzym) (Sharma a kol., 2014). V praxi se pro detekci nízkomolekulárních látek nejvíce rozšířilo značení enzymem. V současné době je jedním z cílů při vývoji imunochemických metod sestavit co nejjednodušší systém, kterým by bylo možné pouhou aplikací kapalného vzorku získat výsledek v několika minutách. Vyvíjené metody jsou založeny na principu detekce na membráně a všeobecně známým příkladem je domácí těhotenský test. Neprecipitační imunochemické metody využívající značku nejčastěji dělíme na kompetitivní (odd. 2.2.1.1) a nekompetitivní (odd. 2.2.1.2). Z neprecipitačních technik, které nevyužívají značek, se v oblasti detekce nízkomolekulárních látek uplatnil především imunosenzor (odd. 2.2.2).

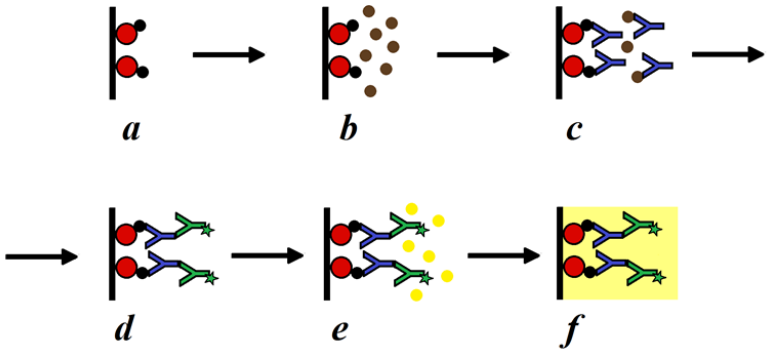
2.2.1 Metody využívající značení imunokomplexu

2.2.1.1 Kompetitivní imunochemické metody

ELISA (z angl. Enzyme Linked ImmunoSorbent Assay) patří do skupiny imunoanalytických metod, které ve fázi detekce nebo kvantifikace používají enzymovou reakci. Pro stanovení nízkomolekulárních látek se používá především kompetitivní formát ve dvou

uspořádáních: a) *na pevné fázi imobilizována protilátka* (značený hapten a hapten ze vzorku soutěží o vazebná místa omezeného množství molekul protilátek; Obr. 2); b) *na pevné fázi imobilizován hapten* (imobilizovaný hapten soutěží s haptenem ve vzorku o vazebná místa omezeného množství molekul protilátek, které jsou také volně v roztoku). Formát, ve kterém se soutěží o vazebná místa enzymem značené specifické protilátky, je označován jako přímá metoda. V nepřímé metodě, která se používá častěji, je protilátka navázaná na imobilizovaný hapten kvantifikována přídavkem protilátky proti protilátce (tzv. druhá protilátka), která byla předem označena enzymem (Obr. 3). Standardní křivka (hodnoty enzymové aktivity, resp. absorbance reakčního roztoku proti logaritmu koncentrace standardu analytu) má ve všech případech sigmoidní tvar se strmou lineární oblastí kolem inflexního bodu (= bod 50% inhibice, I_{50}) (Mičková a kol., 2004).

Obr. 2: Schéma metody ELISA v kompetitivním uspořádání – protilátka imobilizována na pevnou fázi.
a – imobilizace specifické protilátky proti haptenu, **b** – aplikace volného haptenu ve vzorku a haptenu značeného enzymem, **c** – ustanovení rovnováhy, **d** – aplikace substrátu pro enzym a zastavení enzymové reakce



Obr. 3: Schéma metody ELISA v nepřímém kompetitivním uspořádání – hapten vázaný na pevnou fázi. (Horáková, 2015)
a – imobilizace konjugátu haptenu s nosičem, **b** – aplikace volného haptenu ve vzorku, **c** – kompetice o omezené množství vazebných míst na specifické protilátce, **d** – aplikace sekundární značené protilátky enzymem, **e** – aplikace substrátu pro enzym, **f** – zastavení enzymové reakce

Jako pevnou fázi pro ELISA lze použít mikrotitrační destičky, zkumavky, plastové tyčinky (dipstiky) nebo magnetické částice. Výběr se provádí podle účelu a rozsahu prováděných stanovení. Například pro screening většího počtu vzorků v kontrolních, klinických nebo

výzkumných laboratořích je vhodné použít mikrotitrační destičku, která obsahuje více malých reakčních prostorů. Tento formát destičky je vhodný pro aplikaci automatizačních prvků při pipetování reagencií shodných pro všechny reakční prostory, pro promývání těchto prostorů po jednotlivých inkubacích i při finální kvantifikaci absorbance. Ve zkumavkách se analyzují malá množství vzorků. Jako nerozpustný nosič pro imobilizaci jednoho z imunoreaktantů v obou případech tedy slouží stěny plastových zkumavek nebo jamek mikrotitračních destiček. Další možností jsou částice s ferromagnetickým jádrem, které lze separovat od roztoku vnějším magnetem. Kvantitativní analýza vyžaduje u všech variant spektrofotometr. Semikvantitativní hodnocení analýz vystačí se subjektivním porovnáním zbarvení reakčních roztoků při enzymové přeměně přidaného substrátu. Pro uživatele je nejjednodušší semikvantitativní provedení ELISA v podobě tyčinek nebo kartiček s aktivními zónami; po aplikaci imunorektantů a testovaného vzorku je porovnáván odstín zbarvení aktivní zóny se standardní stupnicí. (Mičková a kol., 2004)

Do dnešní doby bylo publikováno velké množství formátů ELISA pro detekci nízkomolekulárních látek v matricích z různorodých oblastí. Příkladem může být detekce herbicidů v půdě nebo vodě (Shankle a kol., 2001, Yazynina a kol., 2002), insekticidů v potravinách (Hemalatha a kol., 2001, Malarkodi a kol., 2017), hormonů ve vodě (Farre´a kol., 2006, Farre´a kol., 2007a), hormonů v biologických matricích (Nara a kol., 2008, Shrivastav a kol., 2011), farmaceutik v mléku nebo medu (Pastor-Navarro a kol., 2007, Samsonova a kol., 2005), fenolů v moči (Nichkova a kol., 2002), drog v tělních tekutinách (Fojtíková a kol., 2018) nebo anabolických steroidů v potravinových doplňcích (Fojtíková a kol., 2016, Sýkorová a kol., 2018). Detekční limity zmíněných metod se většinou pohybují v $\mu g.ml^{-1}$ – $ng.ml^{-1}$ v závislosti na kvalitě protilátky. Velkou výhodou v dnešní době pro běžné uživatele i odbornou veřejnost je možnost zakoupit komerční kity ELISA pro detekci různých nízkomolekulárních látek v potravinách, biologických vzorcích, vzorcích z životního prostředí atd.

V posledních letech byl standardní formát imunoanalýzy modifikován na tzv. multianalytovou enzymovou imunoanalýzu. Výhodou této metody je možnost současné detekce více analytů v testovaném vzorku. Při vývoji imunochemických multianalytových metod se využívá několika strategií. První možností je příprava několika protilátek, které jsou specifické pro jednotlivé cílové analyty v rámci jednoho testu. V této strategii je možnost použít stejný nebo separátní systém značení imunoreagencií (Jiang a kol., 2012, Li a kol., 2017). Příkladem může být simultánní detekce pesticidů v mléce (Zhu a kol., 2011). Ekonomicky výhodnější možností je připravit jednu protilátku, která bude specifická pro více cílových analytů. Ta se pak označuje jako široce specifická nebo skupinově specifická. Lze ji připravit

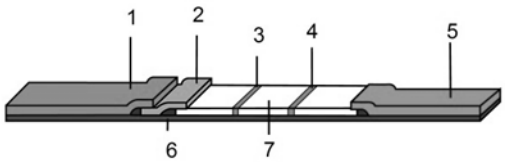
imunizací haptenem s typickou strukturní doménou pro cílovou skupinu analytů nebo konjugátem nosiče s více navázanými typy haptenů. Přínos v této oblasti zaznamenala výroba anti-idiotypových protilátek a techniky molekulového modelování (Jiang a kol., 2012, Sharma a kol., 2014). Velký význam má v oblasti multianalytové detekce nízkomolekulárních látek i modifikace ELISA na miniaturní formát „microarray". Metoda sice využívá stejný protokol jako konvenční multianalytová ELISA, ale výrazně přitom redukuje spotřebu reagencií a vzorků. Tento formát našel své uplatnění např. v detekci toxinů v mléce, pitné vodě nebo obilných výrobcích, v detekci pesticidů v pitné vodě nebo dětských přesnídávkách, v detekci klasických drog v krevním séru (Jenko a kol., 2014, Seidel a Niessner, 2008, Schumacher a Seitz, 2016).

Imunochromatografická metoda (někdy označována jako „lateral flow immunoassay"; LFIA) je nejrozšířenější jednoduchá a rychlá imunochemická technika. Principem je kombinace chromatografie a imunoafinitních interakcí. Zachycení analytu je umožněno díky horizontálnímu (nebo vertikálnímu) toku značeného partnera reakce s analytem ze vzorku membránou přes zónu se zakotveným druhým imunoreaktantem. Detekce analytu je prováděna především vizuálně. K urychlení detekce se jako značka nepoužívá enzym, ale např. nanočástice koloidních kovů; k produkci signálu tak není nutná dodatečná, časově náročná chemické reakce. LFIA není náročná na přístrojové vybavení a umožňuje ve srovnání s metodou ELISA podstatné snížení doby analýzy (Göselová a kol., 2014).

Obecné schéma imunochromatografického testu je na Obr. 4. Tenká a křehká membrána je vyztužena plastovou podložkou. Nejčastěji používaným materiálem pro membránu je nitrocelulosa. Na levé straně membrány (začátek testu) je většinou umístěna podložka pro vzorek a podložka pro konjugát. Tyto podložky slouží k aplikaci vzorku nebo dalších imunoreagencií. Na membráně jsou pak minimálně dvě zóny – kontrolní a testovací linky, na kterých jsou v podobě úzkých příčných linek imobilizovány protilátky nebo konjugáty hapten-protein. Na pravé straně membrány (konec testu) je absorpční podložka, která urychluje tok (Göselová a kol., 2014). Takto připravený test označujeme jako otevřený systém. Pokud je celý test uložen do plastového krytu, označuje se jako uzavřený.

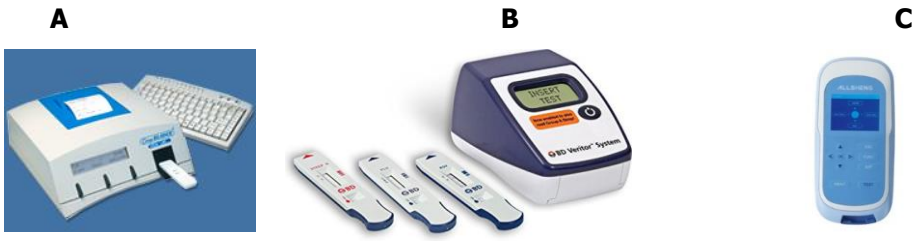
Volba použitého formátu detekce se volí podle typu analytu. Pro nízkomolekulární látky se používají dvě uspořádání tohoto testu: a) *Specifická protilátka je nanesena na testovací linii.* Směs analytu přítomného ve vzorku je aplikována na podložku pro vzorek, značený analyt na podložku pro konjugát a oba typy analytů soutěží při toku přes testovací linii o vazebné místo na specifické imobilizované protilátce. b) *Na testovací linii je nanesen konjugát hapten-protein.* Analyt je aplikován na podložku pro vzorek a značená specifická protilátka na podložku

pro konjugát. V tomto uspořádání při migraci imunoreagencií membránou soutěží o vazbu na značenou protilátku konjugát hapten-protein vázaný na testovací linii s analytem vzorku.



Obr. 4: Schéma imunochromatografického testu (převzato z Fojtíková a kol., 2018).
1 — podložka pro vzorek; **2** — podložka pro konjugát; **3** — testovací linka; **4** — kontrolní linka; **5** — absorpční podložka; **6** — plastová podložka; **7** — nitrocelulosová membrána

V obou případech platí, že čím více je analytu ve vzorku, tím je zbarvení testovací linie slabší. Neobsahuje-li vzorek analyt, nedojde k zeslabení zabarvení testovací linie. Zbarvení kontrolní linky v obou formátech dokazuje správný průběh detekce. Vizuální hodnocení záchytu umožňuje použití barevných značek ve formě nanočástic. Nejčastěji se jako značka používá koloidní zlato, nanočástice uhlíku, barevné latexové nanočástice, liposomy s fluorescenční nebo bioluminiscenční značkou, nanočástice selenu, silikátové nanočástice s chelátem europia, aj. Výsledek získaný pomocí LFIA je možné vyhodnocovat nejen vizuálně (kvalitativně), ale i kvantitativně. Pro kvantitativní hodnocení se detekční proužek skenuje ve stupních intenzity zabarvení, a následně jsou změřené hodnoty zpracovány. Například program TotalLab 224 TL100 software (Nonlinear USA Inc., NC, USA) umožňuje sytost zbarvení v oblasti kontrolní a testovací linie převést na číselné hodnoty, které jsou dále zpracovány v programu MS Excel. Pro provedení LFIA testu i mimo laboratoře jsou komerčně dodávány čtečky imunochromatografických testů (Obr. 5) (Dzantiev a kol, 2014, Göselová a kol., 2014).



Obr. 5: Příklady čteček imunochromatografických testů.
A — VEDALAB EASY READER (převzato z http://www.baghaffar.com/rapid.html**,** VIII/2018); **B** — BD VERIKTOR SYSTÉM (převzato z https://www.amdnext.com/stainless-steel-blade-sterile-size-21-50-bx-3-bx-cs-not-available-for-sale-in-canada.html#.W3_4qc4za70, VIII/2018); **C** — TSR-200 Test Strip Reader (převzato z http://www.allsheng.com/Products/TSR200.html, VIII/2018)

Do dnešní doby bylo publikováno velké množství původních sdělení o detekci nízkomolekulárních látek pomocí imunochromatografického testu v jednoanalytovém

i multianalytovém uspořádání. Pro některé látky jsou testy vyráběny i komerčně. Na internetu jsou dostupné především komerční soupravy na detekci klasických drog v různých biologických matricích (Obr. 6).

Obr. 6: Příklady komerčně dostupných imunochromatografických testů pro detekci drog v různých biologických matricích.
A — Test na detekci marihuany z moči (otevřený formát); **B** — Multianalytový test na 10 drog z moči (uzavřený formát); **C** — Multidrogový test ze slin na 3 drogy (uzavřený formát); **D** – Drogový test Dipro Wipe ze slin, potu a pevných látek na 5 drog (uzavřený formát) (převzato z https://www.drogovy-test.cz/2-drogove-testy, VIII/2018)

Příkladem jednoanalytových testů může být detekce organofosforových pesticidů v potravinách (Malarkodi a kol., 2017), detekce anabolických steroidů v potravinových doplňcích (Fojtíková a kol., 2016; Sýkorová a kol., 2018), detekce glykosidu leptosperinu v medu (Kato a kol., 2016), detekce benzimidazolů v mléce (Guo a kol., 2018) nebo detekce nových syntetických drog v tělních tekutinách (Fojtíková a kol., 2018).

Multianalytové testy jsou sestavovány pro analýzu několika analytů současně. Na rozdíl od jednoanalytových testů obsahuje proužek membrány více testovacích linií (dvě až pět linií) (Blažková, 2017, Dzantiev a kol., 2014). Vzhledem k rozměrům proužku je složité sestavit test s více než pěti liniemi. Tento problém je možné obejít použitím spojených proužků (Obr. 6). V multianalytové detekci se využívá také možnost různě barevně značit jednotlivé analyty a tím zpřehlednit výsledek analýzy. Velký potenciál do budoucna má i test ve formě bodově uspořádaných testovacích zón – tzv. „microarray", kdy jsou imunoreagencie imobilizovány na membránu pomocí dávkovací hlavice s jehličkami (Blažková, 2017). Multianalytový test byl vyvinut například pro detekci antibiotik (Wang a kol., 2017).

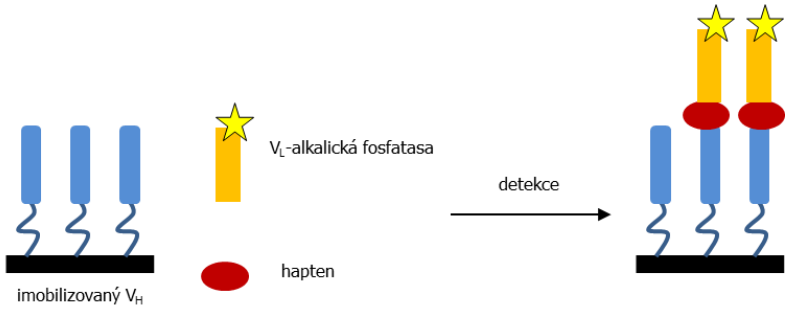
2.3.1.2 Nekompetitivní imunochemické metody

Další významnou skupinou neprecipitačních imunochemických technik jsou nekompetitivní metody. Podle publikovaných informací vykazuje tento typ metod oproti kompetitivním technikám nižší křížové interakce, vyšší citlivost, přesnost a širší oblast linearity

(Anfossi a kol., 2002, Liu a kol., 2012). Přesto u látek s nízkou molekulovou hmotností s pouze jedním dostupným epitopem je dominantní použití kompetitivních imunoanalýz.

Běžně používaný nekompetitivní (tzv. sendvičový) formát stanoví pouze analyt, který má alespoň dva přístupné epitopy, a je tedy pro nízkomolekulární látky nevhodný. V uplynulých letech však bylo navrženo několik typů nekompetitivních imunoanalýz, kterými lze tyto látky detegovat. Mezi používané formáty patří např. otevřená sendvičová imunoanalýza (a), imunoanalýza založená na „anti-metatypových" protilátkách (b), idiometrická metoda využívající anti-idiotypové protilátky (c), Giraudiho metoda (d), přímý nekompetitivní imunosenzor (odd. 2.2.2) (Liu a kol., 2018).

a) ***Otevřená sendvičová imunoanalýza*** (OS-ELISA) byla poprvé navržena v roce 1996 pracovní skupinou Ueda a kol. Využívá jevu, při kterém se v přítomnosti analytu zvyšuje asociace mezi variabilní oblastí těžkého řetězce V_H a variabilní oblastí lehkého řetězce V_L. Fragment V_H nebo V_L je imobilizován do jamek mikrotitrační destičky a poté je přidán V_H nebo V_L značený enzymem. Následuje enzymová reakce a detekce signálu. Stejně jako u tradičních sendvičových imunoanalýz je detekovaný signál úměrný obsahu analytu. Na Obr. 7 je zobrazeno schéma OS-ELISA s proteinem vázajícím maltosu jako imobilizačním ligandem a alkalickou fosfatasou jako značkou (Liu a kol., 2018).

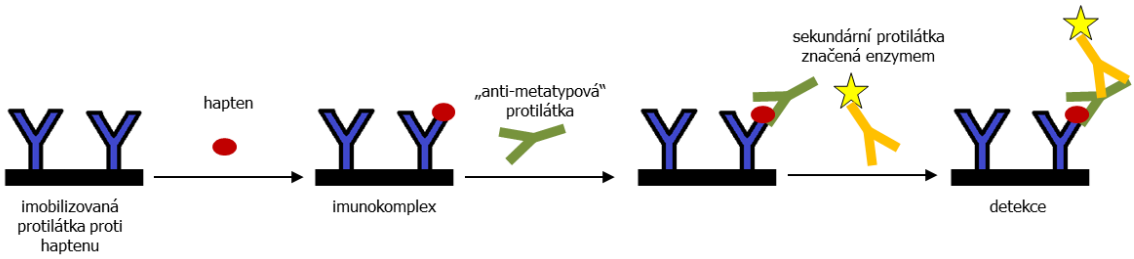


Obr. 7: Schéma otevřené sendvičové imunoanalýzy (OS-ELISA) s proteinem vázajícím maltosu jako imobilizačním ligandem a alkalickou fosfatasou jako značkou (převzato a upraveno Liu a kol., 2018)**.**

V původních experimentech byla síla interakce V_H-V_L po přidání analytu zjišťována až při vlastním měření a tato skutečnost představovala výrazné omezení. Tato nevýhoda byla odstraněna díky navržení metody s použitím V_H (V_L) genů klonovaných z hybridomů (Liu a kol., 2018). Systém je široce využíván, protože zkracuje ve srovnání s tradičním uspořádáním inkubační dobu a usnadňuje detekční proces. Oproti korespondujícímu kompetitivnímu formátu může nekompetitivně detekovat mnoho typů haptenů a dosáhnout nižšího limitu detekce (Liu a kol., 2012). Na základě dosud publikovaných studií lze říci, že OS-ELISA může najít uplatnění v detekci haptenů v potravinách, krmivech, zemědělských

produktech nebo v klinické oblasti (toxin gonyautoxin z měkkýšů (Hara a kol., 2013); estradiol (Liu a kol, 2012); benzaldehyd v broskvových nápojích (Shirasu a kol., 2009); biomarker osteokalcin (Chung a kol., 2017)).

b) Další nekompetitivní metodou je ***imunoanalýza založená na „anti-metatypových" protilátkách***. „Anti-metatypová" protilátka je konformačně specifická protilátka vážící se na komplex antigen-protilátka a nereagující samostatně s antigenem ani protilátkou. Lze ji tedy použít až po vytvoření imunokomplexu analytu s jeho protilátkou. Zjištěný signál je úměrný obsahu analytu ve vzorku (schéma metody na Obr. 8) (Liu a kol., 2018).

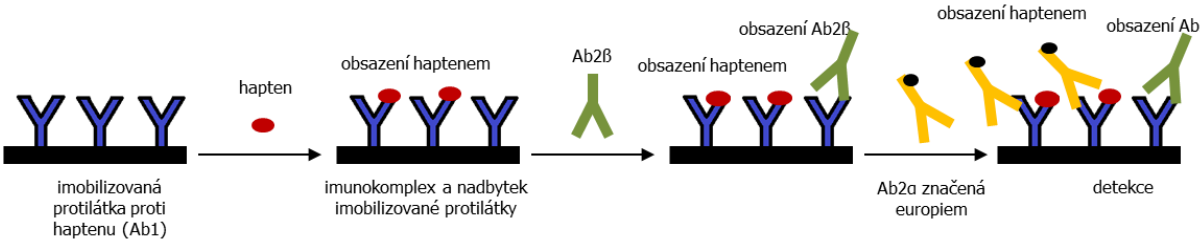


Obr. 8: Schéma nekompetitivní imunoanalýzy založené na „anti-metatypových" protilátkách (převzato a upraveno Liu a kol., 2018)**.**

„Anti-metatypové" protilátky byly nejprve získávány imunizací komplexem protilátka-hapten. Tento způsob přípravy je však velmi obtížný, protože je závislý na stabilitě tohoto komplexu. Během imunizace může dojít k disociaci imunokomplexu nebo molekuly haptenu mohou být téměř úplně zahaleny v paratopech protilátek a tím omezit možnost získat imunoreagencie schopné rozlišit mezi navázanými a nevázanými protilátkami. V současné době se pro přípravu využívají knihovny protilátek. Nicméně zmiňované faktory (disociace komplexu, maskování haptenu) jsou stále limitující pro tuto metodu (Liu a kol., 2018). Do dnešní doby byly vytvořeny „anti-metatypové" protilátky např. pro detekci toxinů (mikrocystin (Nagata a kol., 1999); mykotoxin (Arola a kol., 2016)).

c) ***Idiometrická metoda*** byla vyvinuta na základě teorie, v které je imunitní systém přirovnán k síti interaktivních idiotypů, které se podílejí na regulaci imunitních odpovědí. Teorie vychází z předpokladu, že jedinečná aminokyselinová sekvence variabilních domén těžkého a lehkého řetězce dané protilátky může fungovat nejen jako místo pro vazbu antigenu, ale také jako soubor antigenních determinant. Každá individuální antigenní determinanta variabilní oblasti je označována jako idiotop. V některých případech může být idiotop součástí místa, které váže antigen, a v některých případech idiotop může obsahovat sekvence variabilní oblasti mimo místo vazby s antigenem. Každá protilátka obsahuje více idiotopů; součet jednotlivých idiotopů se nazývá idiotyp protilátky.

Anti-idiotypové protilátky (Ab2) mohou být vytvořeny po imunizaci idiotypovými protilátkami (Ab1). Obecně lze Ab2 klasifikovat do Ab2α, Ab2β, Ab2γ a Ab2ε, mezi kterými se Ab2β váže přímo na idiotop v rámci paratopu Ab1. Ab2α rozpoznává idiotopy, které jsou mimo vazebné místo haptenu, ale kvůli stérické překážce se neváže na komplex Ab2β-Ab1. Preferenčně se tedy váže na Ab1, které jsou obsazeny haptenem. Signál generovaný vázaným Ab2α je tedy úměrný obsahu haptenu. Na Obr. 9 je schéma první publikované metody využívající anti-idiotypové protilátky pro detekci estradiolu v krevním séru (Barnard a Kohen, 1990). Nejprve byly do mikrotitračních jamek imobilizovány monoklonální protilátky proti estradiolu, poté byl jimi zachycen estradiol ze vzorku a neobsazené imobilizované protilátky byly blokovány nadbytkem Ab2ß. Nakonec bylo množství zachyceného estradiolu stanoveno fluorometricky přidáním nadbytku Ab2α značeného europiem. Vzhledem k časově náročné a velmi obtížné přípravě a identifikaci Ab2α a Ab2β není tato metoda příliš rozšířená v oblasti detekce nízkomolekulárních látek (He a kol., 2010, Liu a kol., 2018).



Obr. 9: Schéma idiometrické metody využívající anti-idiotypové protilátky (Ab2α, Ab2β) (převzato a upraveno Liu a kol., 2018)**.**

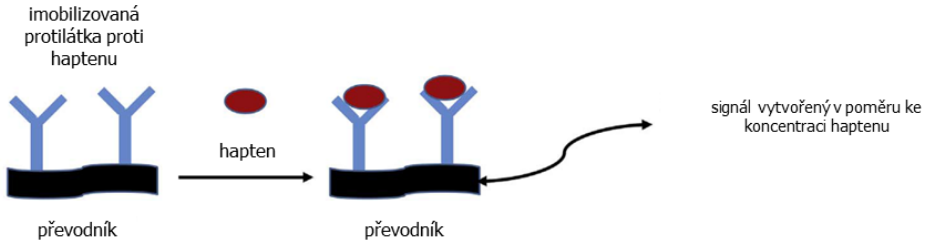
d) ***Giraudiho metoda*** je založena na interakci analytu ze vzorku s imobilizovanou specifickou protilátkou a následném blokování volných míst této protilátky blokujícím činidlem (hapten navázaný na vysokomolekulární látce). V prvním kroku této metody dochází ke vzniku komplexu analytu s imobilizovanou protilátkou, po němž následuje blokování volných vazebných míst protilátky činidlem. Vazba činidla s protilátkou je výrazně silnější než vazba analyt-protilátka. V druhém kroku je promytím pufrem odstraněn pouze analyt z komplexu. Následně přidaný enzymově značený analyt se pak může vázat pouze na místa, které byly dříve obsazené analytem ze vzorku. Měřený signál pak lineárně koreluje s koncentrací analytu. Metoda byla aplikována pro detekci kortizolu ve slinách (Anfossi a kol., 2002) nebo pro detekci celkových aflatoxinů v plodinách (Acharya a Dhar, 2008). Využití této techniky pro detekci jiných kontaminantů s nízkou molekulovou hmotností znesnadňuje navržení vhodného blokujícího činidla. Přístup je však atraktivní, protože nevyžaduje výrobu dalších protilátek, jako jsou např. V_H a V_L nebo protilátky proti imunokomplexu.

2.2.2 Metody nevyužívající značení imunokomplexu

Z metod založených na interakci antigen-protilátka, které nevyužívají značku k detekci vzniklého imunokomplexu, se v oblasti detekce nízkomolekulárních látek používá především ***imunochemický biosenzor***. Jeho výhodou oproti jiným imunochemickým screeningovým metodám je jednoduchost a rychlost analýzy, analýza v reálném čase a možnost automatizace. Cílem je vyrobit malé přenosné systémy použitelné přímo v místě odběru vzorku (McGrath a kol., 2012, Mičková a kol., 2004).

Imunochemický biosenzor patří mezi afinitní biosenzory a kombinuje principy imunoanalýzy na pevné fázi s fyzikálně-chemickým převodníkem. Skládá se z biologického prvku (imobilizovaný antigen nebo imobilizovaná protilátka), převodníku přeměňujícího biochemický nebo biofyzikální děj na elektrický signál a elektronické části. Pro jednoanalytové i multianalytové stanovení haptenů se převážně používají kompetitivní formáty imunosenzoru na bázi optických, elektrochemických a piezoelektrických senzorů (Farre´a kol., 2007b, McGrath a kol., 2012).

Alternativní možností pro detekci haptenů je přímé nekompetitivní uspořádání imunosenzoru. Stejně jako kompetitivní imunosenzor se skládá z imunoreceptoru, převodníku, zařízení pro záznam dat a detektoru měřícího změny v optickém, elektrochemickém nebo jiném signálu (Obr. 10) (Liu a kol., 2018). Své uplatnění našel například v oblasti detekce kontaminantů v potravinách nebo zemědělských produktech (Funari a kol., 2013; Dia a kol., 2017). Přestože dosud vyvinuté nekompetitivní senzory jsou jednoduché, citlivé a využívají přímou detekci analytu, jejich zařízení jsou velmi drahá, imobilizované molekuly jsou nestabilní a často dochází k interferenci s jinými sloučeninami (Liu a kol., 2018).



Obr. 10: Schéma metody založené na přímém nekompetitivním imunosenzoru (převzato a upraveno Liu a kol., 2018)**.**

Mezi metody nevyužívající značku k detekci vzniklého imunokomplexu lze zařadit i ***imunoafinitní chromatografii***. Tato technika využívá vratné nekovalentní vazby mezi protilátkou a antigenem pro selektivní extrakci analytu ze složitých environmentálních matric. Je obzvláště vhodná pro polární organické analyty. Imunoafinitní sorbenty se používají k předkoncentrování strukturně příbuzných sloučenin, které se později eluují, separují

a analyzují konvenčními metodami (HPLC, GC). Chromatografie může být provedena dvěma způsoby podle toho, zda je na nerozpustný nosič kovalentně imobilizován antigen nebo protilátka. Kolona s navázaným antigenem se používá při čištění polyklonálního antiséra pro získání jeho vyšší specifity. Kolonu s imobilizovanou protilátkou lze použít ke specifickému zachycení analytu ze vzorku (Farre´a kol., 2007b, Mičková a kol., 2004).

2.3 Trendy v imunochemických metodách pro detekci nízkomolekulárních látek

Hlavní úsilí ve vývoji nových imunochemických metod se dnes soustřeďuje na snížení doby analýzy, možnost provedení analýzy v polních podmínkách, snížení nákladů na analýzu, miniaturizace, automatizace stanovení a současné stanovení několika analytů (Morozova a kol., 2005, Vashist a Luong, 2018). Podařilo se tak například automatizovat ELISA formát, který slouží jako zlatý standard pro analýzu množství analytů. Vzorky se pak analyzují pomocí robotických pracovních stanic, které mohou využít i vícejamkové mikrotitrační destičky než konvenční 96 jamkové. Výkon ELISA se také výrazně zlepšil díky novým přístupům při imobilizaci imunoreagencií, vývoji ultra senzitivních enzymových substrátů, použití rekombinantních protilátek, použití nekompetitivního uspořádání a použití strategií pro zvýšení signálu na bázi mikromateriálů nebo nanomateriálů. (Jiang a kol., 2012, Li a kol., 2017, Topkas a kol., 2012, Vashist a Luong, 2018, Waller a kol., 2010).

Trendem v současné době je i vývoj multianalytových imunochemických technik, které umožní současné stanovení několika analytů. Kritickým krokem této strategie je stále výběr vhodného imunizačního konjugátu. Tradičně se tento výběr dělá metodou pokus/omyl. Tento postup je časově náročný a v některých případech získané protilátky postrádají požadované vlastnosti pro vývoj multianalytové techniky. Jako dobrá alternativa se v této oblasti jeví počítačové molekulové modelování. Tato metoda se stává užitečným nástrojem pro přípravu imunizačního konjugátu, protože může poskytnout představu o vztahu jeho molekulové struktury a biologické aktivity, kterou je obtížné nebo přímo nemožné jinak získat (Farre´a kol., 2007b; Jiang a kol., 2012; Li a kol., 2017; Vashist a Luong, 2018).

Nejrozšířenějším formátem rychlé imunochemické metody, která je vhodná pro analýzu v polních podmínkách, je imunochromatografický test (LFIA). Jedním z cílů v oblasti vývoje těchto testů je příprava tzv. multiparametrových imunochromatografických testů. Principem je kombinace proužků a čipových technologií (vytváření testovacích zón na proužku jako „mikrodotů" a následný instrumentální záznam výsledků). Technika tak umožní rychle a současně analyzovat velké množství sloučenin.

Většina dosud vyvinutých LFIA je vyhodnocováno vizuálně (kvalitativně). V posledních letech je možné i jejich kvantitativní využití. Velký potenciál v této oblasti má vyhodnocování pomocí inteligentních telefonů (Obr. 11). Vývoj formátů a zařízení založených na inteligentních telefonech je významným trendem v celé oblasti imunochemických technik. Použití inteligentního telefonu zlepšuje možnosti diagnostiky nemocí, umožňuje analýzu ze vzdálených míst nebo zjednodušuje požadavky na personál. Odečtené výsledky analýzy současně s informací o čase, datu a místě analýzy mohou být bezpečně uloženy v telefonu a následně bezdrátově nebo pomocí internetu přeneseny do centrální laboratoře. Výsledky mohou být vyhodnoceny pomocí programu přímo v telefonu nebo po přenosu dat v centrální laboratoři, odkud se mohou informace zpět odeslat uživateli (Dzantiev a kol., 2014, Vashist a Luong, 2018).

Obr. 11: Ukázka mobilních čteček rychlých imunochromatografických testů.
A – převzato z https://angel.co/projects/261153-mobile-diagnostic-rapid-test-reader-mreader, VIII/2018; **B** – převzato z http://ngbiotech.com/about-us/, VIII/2018

3 Přehled řešené problematiky

V této kapitole budou popsány přístupy k přípravě imunizačních a imobilizačních konjugátů haptenu s nosičem a jejich následné využití v naší laboratoři při vývoji jednoanalytových a multianalytových imunochemických metod pro detekci vybraných nízkomolekulárních látek. Řešená problematika je rozdělena do tří částí. První část je zaměřena na problematiku detekce pesticidů v životním prostředí nebo v potravinách. V druhé části je popsán postup přípravy konjugátů nosiče s anabolickým androgenním steroidem. Nový způsob přípravy derivátů nových psychoaktivních látek použitý pro přípravu konjugátů je uveden ve třetí části této kapitoly. Navržené imunoanalytické postupy byly v případě některých analytů dovedeny do fáze přípravy funkčních vzorků analytických souprav (rychlé testy ELISA a LFIA pro detekci anabolických steroidů v potravinových vzorcích; ELISA kit pro detekci vybraného pesticidu ve spolupráci s firmou Immunotech, s.r.o.; rychlé testy LFIA pro detekci drog v biologických matricích ve spolupráci s firmou Dynex).

3.1 Detekce pesticidů a jejich degradačních produktů

Otázka reziduí pesticidů je jedním z nejrozšířenějších problémů týkajících se bezpečnosti potravin a životního prostředí na celém světě. Významným nástrojem pro rychlý screening těchto kontaminantů jsou imunochemické metody (**Příloha 1**). Naše laboratoř se několik let zabývala vývojem jednoanalytových nebo multianalytových imunochemických metod pro detekci organochlorových insekticidů (DDT, endosulfan) a jejich nástupců karbamátových pesticidů (karbaryl, karbofuran, methiokarb). Vzhledem k tomu, že v reálných situacích nedochází ke kontaminaci pouze jednou sloučeninou, bylo naším úkolem vyvinout především multianalytové metody pro detekci vybraných pesticidů.

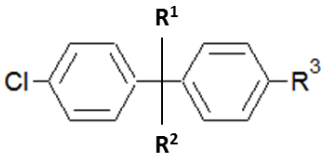
3.1.1 Organochlorové pesticidy

Pesticid *DDT* (1,1,1-trichlor-2,2-bis(4-chlorfenyl)ethan) a jeho analoga představují významnou skupinu organochlorových insekticidů. Jeho rozsáhlé používání vyvolalo díky jeho stabilitě a perzistenci insekticidního účinku vážné ekologické problémy, a proto bylo stejně jako používání jiných chlorovaných uhlovodíků v mnoha zemích silně omezeno, popř. přímo zakázáno. Přestože DDT existuje ve dvou isomerních formách (*p,p'*-DDT a *o,p'*-DDT), pouze prvně jmenovaný isomer má insekticidní vlastnosti. Jeho hlavní metabolity DDE (1,1-dichlor-2,2-bis(4-chlorfenyl)ethen) a DDA (2,2-bis(4-chlorfenyl) octová kyselina) jsou také lipofilní a dokonce se pomaleji degradují než DDT. *Endosulfan* je významným zástupcem cyklodienových pesticidů, mezi které patří i další nebezpečné látky (aldrin, dieldrin, aj.). Všechny zmiňované látky jsou pod dohledem státních institucí i EU a jsou pro ně stanoveny

přísné maximální reziduální limity. Výhodnější by tedy bylo vyvinout metody, které by detekovaly nejen DDT nebo endosulfan, ale i jejich metabolity a příbuzné sloučeniny.

Pro přípravu multianalytových metod byla vybrána strategie přípravy skupinově specifické protilátky s použitím jednoho typu haptenu navázaného na nosiči. Většina imunoreagencií pro vývoj metod byla získána od spolupracující vědecké skupiny z Polytechnické univerzity ve Valencii (Universidad Politéccnica de Valencia, Spain). Tato vědecká skupina publikovala studii o vlivu struktury imunizačních konjugátů na specifitu monoklonálních protilátek v metodě ***ELISA ke stanovení DDT a jeho derivátů*** (Abad a kol., 1997a). V této práci byl haptenům zabudován alifatický řetězec jako spojovací raménko s koncovou karboxylovou skupinou – buď na centrální uhlíkový atom (Obr. 12A – hapten 1) nebo na benzenový kruh struktury DDT (Obr. 12A - hapten 2). Poté byly připojeny metodou aktivovaného esteru k proteinu a vzniklé konjugáty byly použity pro imunizaci.

A



Název sloučeniny	**R^1**	**R^2**	**R^3**
DDT	CCl_3	H	Cl
Hapten 1	OH	$(CH_2)_3COOH$	Cl
Hapten 2 (DDT5)	CCl_3	H	$(CH_2)_3COOH$

B

Obr. 12: ELISA pro stanovení DDT a jeho derivátů.
A – Chemická struktura DDT a haptenů použitých pro přípravu imunizačních konjugátů v práci Abad a kol., 1997a; **B –** Sestavená ELISA souprava (využívající hapten 2) pro kvantitativní stanovení DDT ve spolupráci s firmou Immunotech s.r.o., Praha, ČR.

Připravené monoklonální protilátky byly charakterizovány z hlediska afinity a specifity k *p,p´*-DDT metodou ELISA. Všech sedm protilátek, které byly odvozeny od haptenu 2 (I_{50} v rozmezí 2,1 – 6,5 nM), vykazovalo vyšší afinitu k p,p´-DDT než protilátka odvozená od haptenu 1 (I_{50} = 11,1 nM). Protilátka odvozená od haptenu 1 vykazovala nízké křížové interakce (CR) s DDT a jeho analogy. Čtyři protilátky odvozené od haptenu 2 vykazovaly silné křížové interakce se sloučeninami ze třídy DDT a byly označeny jako vhodné skupinově specifické protilátky pro multianalytové imunochemické stanovení DDT. Zbylé protilátky odvozené od haptenu 2 byly specifické pro DDT, protože rozeznávaly pouze *p,p´*-DDT nebo *o,p´*-DDT, a byly tedy označeny jako vhodné pro jednoalytové imunochemické stanovení DDT. My jsme v naší práci využili hapten 2 (označený DDT5) a jeho konjugáty s proteiny, skupinově

specifickou protilátku (označená LIB-DDT5.25) a DDT-specifickou protilátku (označená LIB-DDT5.52). Jejich významné charakteristiky jsou uvedeny v Tab. 2.

Tab. 2: Přehled významných charakteristik použitých imunoreagencií pro imunochemické stanovení vybraných organochlorových a *N*-methylkarbamátových pesticidů (Holubová, 2005)**.**

Analyt	Reagens	Název	Konjugační metoda	Molární poměr hapten/protein	Reference
DDT	Imunizační konjugát	BSA-DDT5	DCC-NHS (1)	23	Abad a kol., 1997a
	Monoklonální protilátka	LIB-DDT5.25 LIB-DDT5.52	---	---	
	Imobilizační konjugát	OVA-DDT5	CFI-TBA (2)	9	
Endosulfan	Imunizační konjugát	BSA-CCD2	DCC-NHS (1)	21	Příloha 3
	Monoklonální protilátka	LIB-CCD2.2 LIB-CCD2.3	---	---	
	Imobilizační konjugát	OVA-CCD2	DCC-NHS (1)	8	
Karbaryl	Imunizační konjugát	BSA-CNH	CFI-TBA (2)	36	Abad a kol., 1997b
	Monoklonální protilátka	LIB-CNH45	---	---	
	Imobilizační konjugát	OVA-2NAH	CFI-TBA (2)	6	
Karbofuran	Imunizační konjugát	BSA-BFNB	DCC-NHS (1)	17	Abad a kol., 1999
	Monoklonální protilátka	LIB-BFNB67	---	---	
	Imobilizační konjugát	OVA-BFNH	CFI-TBA (2)	5	
Methiokarb	Imunizační konjugát	BSA-MXNB	DCC-NHS (1)	10	Abad a kol., 1998
	Monoklonální protilátka	LIB-MXNB31	---	---	
	Imobilizační konjugát	OVA-DPNH	CFI-TBA (2)	8	

(1) DCC-NHS: metoda aktivovaného esteru
(2) CFI-TBA: smíšená anhydridová metoda

Získané imunoreagencie jsme použili k vývoji nepřímé kompetitivní ELISA se spektrofotometrickou detekcí. Zároveň se podařilo zavést v naší laboratoři i ELISA s chemiluminiscenční detekcí (CL-ELISA). CL-ELISA měla čtyřikrát nižší detekční limit než původní spektrofotometrický formát ELISA a byla úspěšně aplikována v analýze potravinových (ryba) a environmentálních vzorků (voda, půda) (**Příloha 2**). Získané výsledky z analýzy vzorků byly srovnatelné s hodnotami naměřenými GC-ECD jako referenční metodou (Holubová, 2005). V době své publikace byla naše ELISA s chemiluminiscenční detekcí jediná s touto koncovkou a její detekční limit (0,06 ng.ml^{-1} s použitím LIB-DDT5.52; 0,04 ng.ml^{-1} s použitím

LIB-DDT5.25) byl srovnatelný nebo lepší než do té doby prezentované ELISA nebo komerční soupravy pro detekci DDT a příbuzných sloučenin (**Příloha 1**).

Ve spolupráci s firmou Immunotech a.s., Praha byl podle vyvinutého protokolu vytvořen funkční vzorek ELISA soupravy pro kvantitativní stanovení DDT pomocí skupinově specifické monoklonální protilátky LIB-DDT5.25 (Obr. 12B) a úspěšně aplikován při analýze vzorků potravin, vody a půdy (Holubová, 2005).

Vliv struktury imunizačního konjugátu na specifitu monoklonálních protilátek v metodě ***ELISA ke stanovení cyklodienů*** jsme publikovali v článku Manclus a kol., 2004 (**Příloha 3**). Cílem této práce bylo vytvořit multianalytovou ELISA s použitím skupinově specifické monoklonální protilátky. Byly připraveny čtyři různé konjugáty hapten-protein (CCD1-4), které obsahovaly charakteristickou strukturu (hexachlorobicyklická struktura) a lišily se strukturou vazby na nosičový protein. Z myší imunizovaných těmito konjugáty se získalo několik monoklonálních protilátek se schopností vázat testované cyklodieny. Nejvhodnější pro vývoj ELISA byly zvoleny monoklonální protilátky získané imunizací pomocí haptenu CCD2 – protilátky LIB-CCD2.2 a LIB-CCD2.3. Významné charakteristiky použitých imunoreagencií jsou uvedeny v Tab. 2. Obě skupinově specifické protilátky jsme použili k vývoji ELISA se spektrofotometrickou detekcí a úspěšně aplikovali v analýze vzorků potravin, vody a půdy. Získané výsledky ELISA opět korelovaly s hodnotami naměřenými GC-ECD (Holubová, 2005).

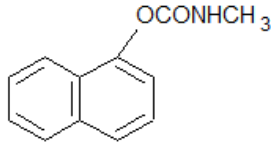
3.1.2 *N*-Methylkarbamátové pesticidy

Vzhledem k vážným ekologickým problémům, na kterých se podílely také organochlorové pesticidy, začal chemický průmysl vyvíjet nové typy insekticidů. Díky své nízké toxicitě pro savce, snadné degradovatelnosti a zachovanému širokému spektru účinku získaly na významu *karbamátové insekticidy*. Přesto ani jejich používání není zcela bezpečné. I pro tyto pesticidy jsou stanoveny velmi přísné maximální reziduální limity. Většina karbamátů se přeměňuje ve velmi krátké době na své metabolity, které jsou často stejně nebo dokonce více škodlivé než původní sloučenina. Výhodné by tedy byly imunochemické metody, které by byly schopné detegovat nejen původní molekulu pesticidu, ale také jeho degradační produkty.

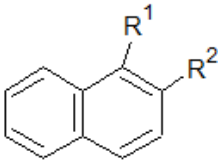
Při vývoji imunoanalýz pro karbamáty bylo stejně jako pro organochlorové pesticidy důležitým krokem navržení a příprava imunizačního a imobilizačního konjugátu. I v tomto případě byly spolupracující vědeckou skupinou z Polytechnické univerzity (Valencie) pro přípravu monoklonálních protilátek použity konjugáty jednoho typu haptenu navázaného na nosiči. Imobilizační konjugáty byly navrženy pro heterologní formát imunochemické metody. Použití tohoto formátu je jedním z přístupů, jak zlepšit citlivost imunochemických testů. Sedm navržených haptenů pro ***imunoanalýzu karbarylu*** (Obr. 13) mělo stejné

rozložení aromatických kruhů jako karbaryl a obsahovaly karboxylovou skupinu pro připojení na bílkovinu. Při výběru nejvhodnějších haptenů pro přípravu monoklonálních protilátek byly vlastnosti připravených imunogenů nejprve testovány při tvorbě polyklonálních protilátek. Nejlepších výsledků bylo dosaženo v ELISA pro hapteny CNH a CNA, a proto byly použity pro přípravu monoklonálních protilátek. Spojovací raménko je k CNH a CNA připojeno přes methylovou skupinu analytu, a tak je zachována charakteristická skupina pro tyto pesticidy (Abad a kol., 1997b). Během testování specificity protilátek bylo zjištěno, že *N*-methylkarbamátová skupina je velmi důležitou antigenní determinantou, protože žádný z testovaných metabolitů ani pesticidů nebyl významně rozpoznán (CR <0,1 %). Pro sestavení imunoanalýzy v naší laboratoři byla vybrána monoklonální protilátka získaná pomocí haptenu CNH a imobilizační konjugát ovalbuminu s navázaným haptenem 2NAH. Hapteny se od sebe liší pouze polohou funkční skupiny na aromatickém kruhu, ke které byl poté připojen protein.

KARBARYL



NAVRŽENÉ HAPTENY

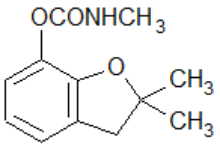


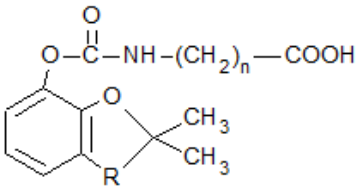
Název sloučeniny	**R^1**	**R^2**
CNH	$OCONH(CH_2)_5COOH$	H
CNA	$OCONH(CH_2)_2COOH$	H
CPNU	$NHCONH(CH_2)_5COOH$	H
1NAH	$CONH(CH_2)_5COOH$	H
2NAH	H	$CONH(CH_2)_5COOH$
1NA	OCH_2COOH	H
2NA	H	OCH_2COOH

Obr. 13: Chemická struktura karbarylu a navržených haptenů použitých v práci Abad a kol., 1997b.

Pro ***imunoanalýzu karbofuranu*** byly navrženy čtyři hapteny s různou délkou alkylového spojovacího raménka (Obr. 14), která jsou připojena přes charakteristickou karbamátovou skupinu pesticidu. Pro přípravu monoklonálních protilátek pak byly použity imunizační konjugáty s hapteny BFNP, BFNB a BFNH. Studie specifity protilátek ukázala, že kromě karbofuranu jsou křížovými reaktanty strukturně podobné a také nebezpečné látky jako bendiokarb, karbosulfan, furathiokarb a benfurakarb; tyto protilátky jsou proto vhodné pro přípravu testu s širokou skupinovou specifitou. Naše laboratoř vyvíjela imunochemické metody s použitím monoklonální protilátky získané pomocí haptenu BFNB a imobilizačního konjugátu ovalbumin-BFNH – tato kombinace byla podle dosažených výsledků nejvhodnější pro vývoj metody (Abad a kol., 1999).

KARBOFURAN **NAVRŽENÉ HAPTENY**





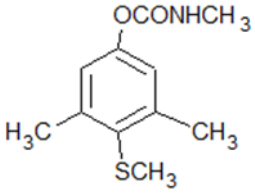
Název sloučeniny	n	R
BFNP	2	CH_2
BFNB	3	CH_2
BFNH	5	CH_2
BDNH	5	O

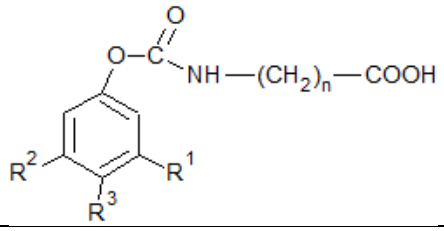
Obr. 14: Chemická struktura karbofuranu a navržených haptenů použitých v práci Abad a kol., 1999.

Imunizační konjugáty pro ***imunoanalýzu methiokarbu*** byly připraveny stejnou strategií jako karbarylové a karbofuranové konjugáty (Obr. 15). Pro imunizaci byly vybrány hapteny MXNP, MXNB a MXNH. Navzdory velké strukturální podobnosti mezi methiokarbem a jeho metabolity byly všechny monoklonální protilátky mimořádně specifické pro methiokarb. Ostatní připravené hapteny, které se lišily od imunizačních haptenů počtem substituentů kruhu, byly použity k přípravě imobilizačních konjugátů. Z těchto imunoreagencií pak byla vybrána vhodná kombinace pro sestavení heterologní metody. Naše laboratoř k vývoji imunochemických metod využila monoklonální protilátku získanou pomocí haptenu MXNB a imobilizační konjugát ovalbumin-DPNH. Významné charakteristiky všech použitých imunoreagencií pro vývoj imunochemických metod karbamátových pesticidů jsou uvedeny v Tab. 2.

Vzhledem ke specifitě použitých monoklonálních protilátek byly v naší laboratoři vyvinuty především jednoanalytové imunochemické metody. S vybranými imunoreagenciemi byly pro karbaryl, karbofuran nebo methiokarb sestaveny heterologní formáty ELISA se spektrofotometrickou koncovkou, které umožňovaly detekovat nižší koncentrace cílových pesticidů, než jsou maximální reziduální limity určené Evropskou legislativou (**Příloha 4**). Detekční limit těchto metod byl poté snížen díky zavedení chemiluminiscenční detekce (**Příloha 5**). Během validace metod byly provedeny korelační studie s HPLC/ESI/MS/MS pro ovocné džusy a dětské přesnídávky (R^2 = 0,991).

METHIOKARB **NAVRŽENÉ HAPTENY**



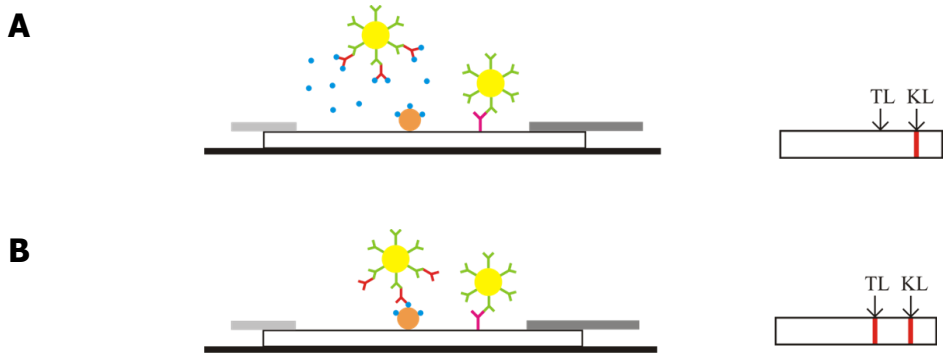


Název sloučeniny	n	R^1	R^2	R^3
MXNP	2	CH_3	CH_3	SCH_3
MXNB	3	CH_3	CH_3	SCH_3
MXNH	5	CH_3	CH_3	SCH_3
MCNP	2	H	CH_3	SCH_3
MCNB	3	H	CH_3	SCH_3
MCNH	5	H	CH_3	SCH_3
DPNP	2	CH_3	CH_3	H
DPNB	3	CH_3	CH_3	H
DPNH	5	CH_3	CH_3	H
MPNP	2	H	H	SCH_3
MPNB	3	H	H	SCH_3
MPNH	5	H	H	SCH_3
PNP	2	H	H	H
PNB	3	H	H	H
PNH	5	H	H	H

Obr. 15: Chemická struktura methiokarbu a navržených haptenů použitých v práci Abad a kol., 1998.

Zároveň byly vyvinuty rychlé imunochromatografické testy (LFIA) v kompetitivním formátu. Na testovací linii byl imobilizován konjugát haptenu s ovalbuminem a na kontrolní linii terciární protilátka. Vzorek byl aplikován na podložku pro vzorek spolu s primární protilátkou a detekční molekulou (nanočástice s navázanými sekundárními protilátkami). Výsledkem pozitivního testu byla pouze jedna barvená linie – kontrolní, na které interagovala terciární protilátka se sekundární protilátkou ukotvenou na nanočásticích (Obr. 16A). V případě nepřítomnosti analytu ve vzorku neměl imobilizovaný konjugát s čím soutěžit a výsledek se projevil vybarvením dvou linií (Obr. 16B).

Jako detekční molekulu jsme použili uhlíkaté nanočástice s navázanými sekundárními protilátkami. Výhodou této detekční molekuly je její univerzálnost. Vzhledem k tomu, že pro její přípravu není potřeba specifická protilátka, lze ji využít ve všech námi připravených testech. Publikován byl vývoj a aplikace testu pro detekci methiokarbu ve vzorcích vody (**Příloha 6**) a karbarylu ve vzorcích ovocných džusů (**Příloha 7**). Ve spolupráci s firmou VIDIA s.r.o. byl sestaven funkční prototyp rychlého imunoanalytického testu pro detekci karbofuranu (Rapid-VIDITEST karbofuran) (Blažková, 2017).



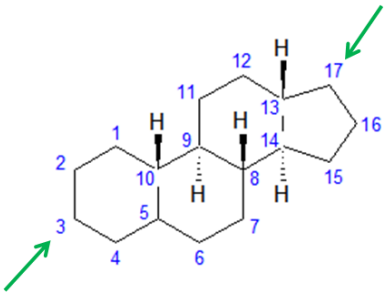
Obr. 16: Schéma imunochromatografického testu pro detekci haptenů (Tajnaiová, 2015).
A – pozitivní vzorek; **B** — negativní vzorek
– imobilizační konjugát hapten-protein, – primární (specifická) protilátka, – nanočástice s navázanými sekundárními protilátkami, – terciární protilátka proti sekundární protilátce, – hapten, – nitrocelulosová membrána, – podložka pro vzorek, – absorpční podložka, TL – testovací linka, KL – kontrolní linka

3.2 Detekce anabolických androgenních steroidů

Další oblastí, které se věnuje naše laboratoř, je vývoj rychlých imunochemických metod pro detekci ***anabolických androgenních steroidů (AAS)*** ve vzorcích potravin. AAS jsou syntetické deriváty testosteronu. V dnešní době jsou využívány nejen v lékařství na podporu léčebných postupů, ale i ve sportu jako nepovolený doping na podporu rychlého nárůstu svalové hmoty a celkového zesílení organismu. Volný prodej těchto látek je zakázaný, přesto se mohou nelegálně dostat do prodeje například ve formě doplňků stravy, aniž je to uvedeno na etiketě obalu. Toto jednání ohrožuje především zákazníky, kteří kupují produkt bez deklarovaného obsahu anabolik, a tudíž tyto látky užívají nevědomky. Z těchto důvodů by bylo výhodné, aby byly k dispozici metody, jež by umožnily AAS v doplňcích stravy spolehlivě odhalit. Vzhledem k vzrůstajícímu trendu mezi uživateli AAS (užívání více druhů AAS v menších množstvích) se jeví jako vhodná multianalytová metoda. Detekce steroidů v různých biologických matricích se běžně provádí pomocí plynové chromatografie (GC) nebo kapalinové chromatografie (LC). V České republice je při kontrole potravin na průkaz anabolických steroidů využívána laboratoří Státní zemědělské a potravinářské inspekce metoda dvojrozměrné plynové chromatografie (**Příloha 8**). Alternativou ke stávajícím metodám by mohla být imunochemická technika, pomocí které by se provedl jednoduchý, rychlý a levný screening AAS v testovaných vzorcích.

Strategií pro přípravu polyklonálních protilátek byla opět příprava imunizačního konjugátu jednoho typu haptenu navázaného na nosiči. Vzhledem k tomu, že všechny AAS jsou odvozeny od testosteronu, mají stejnou charakteristickou strukturu – tetracyklický skelet nazývaný gonan. V rámci projektu byly tedy ve spolupráci s Ústavem přírodních látek připraveny imunizační a imobilizační konjugáty, v kterých byly hapteny na molekulu proteinu

připojeny alifatickým raménkem navázaným v poloze C17 nebo C3 (Obr. 17). Konjugace s nosičem probíhala metodou aktivovaného esteru.



Obr. 17: Charakteristická struktura anabolických androgenních steroidů (gonan) s vyznačenými polohami, přes které byly pomocí alifatického spojovacího můstku připojeny nosiče.

Polyklonální protilátky získané pomocí imunizačních konjugátů a připravené imobilizační konjugáty byly v naší laboratoři použity k vývoji nepřímých kompetitivních ELISA pro screening většího počtu vzorků. Zároveň byly vyvinuty i rychlé imunochromatografické testy pro použití v polních podmínkách (Obr. 16). Specifita vyvinutých metod byla ověřena pomocí křížových interakcí s více než 60 AAS. Jako vhodné pro multianalytové stanovení AAS byly vybrány metody, které používaly protilátky získané pomocí haptenů navázaných na protein přes polohu C17 (**Příloha 9, 10**). Protilátky připravené pomocí haptenů navázaných přes polohu C3 byly vysoce specifické pro cílový AAS (**Příloha 11, 12**). Vyvinuté metody byly použity k analýze vzorků doplňků stravy a získané výsledky vykazovaly vysokou shodu s výsledky z UHPLC-MS/MS. Skupinově specifická protilátka připravená pomocí imunizačního konjugátu odvozeného od stanazololu byla v naší laboratoři použita pro sestavení funkčního vzorku imunoanalytické soupravy (Obr. 18). Vzorky byly předvedeny např. zástupcům vězeňské služby nebo nabídnuty majitelům fitness center. Výsledky z projektu byly využity firmou SEDIUM R&D k sestavení funkčních vzorků analytických souprav na bázi ELISA pro detekci anabolických steroidů v potravinách (Obr. 18).

A **B** **C**

Obr. 18: Soupravy využívající imunochemických metod pro detekci anabolických androgenních steroidů nebo nových psychoaktivních látek.
A – STANAZOLOL IMUNOSTRIP; **B –** ELISA kit pro detekci anabolických androgenních steroidů; **C –** Diagnostická souprava pro rychlé stanovení nových syntetických drog (5-MeO-DMT test na sliny)

3.3 Detekce nových psychoaktivních látek

Nové psychoaktivní látky (NPS, z angl. New Psychoactive Substances) nebo také nové syntetické drogy, „legal highs", „designer drugs", případně „research chemicals" jsou syntetizovány jako strukturní analoga nebo chemické deriváty již zákonem kontrolovaných látek. Drobné strukturní změny v molekulách způsobí, že dostupné imunochemické techniky zaměřené na klasické drogy (kokain, opiáty, benzodiazepiny, THC) je nejsou schopny stanovit. Kromě tradičních instrumentálních metod jako je LC/MS nebo GC/MS bylo vyvinuto i několik uživatelsky nenáročných imunochemických metod (ELISA, LFIA), které jsou schopny detegovat alespoň některé zástupce NPS v různých matricích (sliny, moč, sérum, plazma, vlasy nebo bylinné směsi). Velkým problémem některých z těchto metod je malá skupinová specifita. Výsledky studie zaměřené na testování specifity komerčních ELISA souprav pro detekci NPS (16 typů) odhalily, že valná většina kitů je vysoce specifická pouze na určitý strukturní typ, přestože výrobce uvádí skupinovou specifitu použitých protilátek (**Příloha 13**).

Naše laboratoř se v rámci projektu zaměřila na vývoj imunochemických metod pro detekci NPS v biologických tekutinách. Jedním z cílů bylo sestavit multianalytovou metodu pro detekci *syntetických kanabinoidů*. Vývoj takové metody je velmi aktuální i vzhledem k prvnímu doloženému úmrtí na našem území (září 2018) v důsledku požití syntetického kanabinoidu v kombinaci s alkoholem. Pro imunizační a imobilizační konjugáty byl použit nový způsob přípravy derivátů syntetických kanabinoidů, jejichž součástí je krátký spojovací můstek nesoucí karboxylovou funkční skupinu. Tento způsob byl patentován (**Příloha 14**). Hapten je pak připojen k nosiči metodou aktivovaného esteru. Publikována byla ELISA a imunochromatografický test pro detekci syntetických kanabinoidů ve slinách. Součástí práce byla i korelační studie s UHPLC-MS/MS (R^2_{ELISA} = 0,99, R^2_{LFIA} = 0,99) (**Příloha 15**). Nový způsob přípravy haptenů byl použit i pro imunizační a imobilizační konjugáty, které byly využity pro vývoj metod detekujících další skupinu NPS – *tryptaminů*. I v tomto postupu byly haptenové struktury modifikovány připojením krátkého spojovacího raménka s karboxylovou skupinou a s nosičem spojeny metodou aktivovaného esteru. Skupinová specifita připravené protilátky k syntetickým tryptaminům byla ověřena metodou ELISA (**Příloha 16**). Ve spolupráci s firmou DYNEX byly sestaveny funkční vzorky pro detekci vybraných NPS v moči nebo slinách (Obr. 18).

4 Závěr

Předkládaná práce, zabývající se imunochemickým stanovením nízkomolekulárních látek, byla v první části zaměřena na problematiku výběru vhodného imunizačního konjugátu, který lze použít pro přípravu protilátky s požadovanými vlastnostmi. Na tuto část navázal přehled imunochemických metod používaných k detekci nízkomolekulárních látek především v potravinách a životním prostředí. V přehledu byly uvedeny nejen běžně používané metody (kompetitivní ELISA, LFIA), ale i netradiční metody, které by mohly pozitivně ovlivnit rychlost, specifitu nebo citlivost detekce těchto látek.

V druhé části jsou popsány konkrétní přístupy pro přípravu konjugátů s navázaným haptenem a jejich aplikace ve vývoji různých formátů imunochemických metod. V naší laboratoři se podařilo vyvinout imunochemické metody pro detekci významných kontaminantů životního prostředí (*organochlorové a N-methylkarbamátové pesticidy, anabolické androgenní steroidy a nové psychoaktivní látky*). Podařilo se patentovat nový způsob přípravy derivátů jedné ze skupin drog (syntetické kanabinoidy), který lze využít při přípravě imunizačních konjugátů. Navržené imunoanalytické postupy byly v případě některých analytů dovedeny až do fáze přípravy funkčních vzorků analytických souprav. Otevřela se tak možnost jejich komerčního využití v oblasti detekce pesticidů v životním prostředí, steroidů v potravinách nebo drog v biologických vzorcích.

5 Seznam zkratek

AAS	anabolické androgenní steroidy
CFI-TBA	smíšená anhydridová metoda
CL-ELISA	ELISA s chemiluminiscenční detekcí
CR	křížové interakce
DCC-NHS	metoda aktivovaného esteru
DDT	1,1,1-trichlor-2,2-bis(4-chlorfenyl)ethan
DDE	1,1-dichlor-2,2-bis(4-chlorfenyl)ethen
DDA	2,2-bis(4-chlorfenyl) octová kyselina
ECD	detektor elektronového záchytu
ELISA	enzyme linked immunosorbent assay (enzymová imunoanalýza na pevné fázi)
ESI	elektrosprejová ionizace
GC	plynová chromatografie
HPLC	vysokotlaká kapalinová chromatografie
HRP	křenová peroxidasa
I_{50}	koncentrace analytu, která způsobuje 50% inhibici navázání protilátky s imobilizovaným haptenem při kompetitivní ELISA
Ig	imunoglobulin
KL	kontrolní linka
LC	kapalinová chromatografie
LFIA	lateral flow immunoassay
MDPV	methylenedioxypyrovaleron
MS	hmotnostně spektrometrická detekce
NPS	nové psychoaktivní látky
OS-ELISA	otevřená sendvičová imunoanalýza
THC	tetrahydrokanabinol
TL	testovací linka
TMB	3,3´,5,5´-tetramethylbenzidin
UHPLC	extrémně vysokotlaká kapalinová chromatografie
V_H	variabilní část těžkého řetězce protilátky
V_L	variabilní část lehkého řetězce protilátky

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7 Seznam příloh a přílohy

Tato práce se opírá o původní vědecké i referátové články v impaktovaných časopisech. Tyto práce jsou přiloženy v následujícím pořadí:

Příloha 1:
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MOŽNOSTI IMUNOCHEMICKÉHO STANOVENÍ ORGANOCHLOROVÝCH A KARBAMÁTOVÝCH PESTICIDŮ

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Došlo 10.5.04, přijato 20.7.04.

Klíčová slova: imunoanalýza, organochlorové pesticidy, karbamátové pesticidy

Obsah

1. Úvod

Pesticidy jsou chemické sloučeniny používané pro preventivní zničení, odpuzování nebo zmírňování účinků škodlivých živočichů, plevelů a parazitujících hub, které ohrožují zemědělské, zahradní a lesní rostliny, zásoby potravin a zemědělských produktů, průmyslové materiály, hospodářská zvířata i samotného člověka. Tuto velkou skupinu látek lze rozdělit do různých tříd, např. podle cílového organismu nebo podle způsobu účinku. Základní třídění dělí chemické pesticidy do těchto hlavních skupin: insekticidy (k hubení hmyzu), herbicidy (k hubení plevelů), fungicidy (proti parazitujícím houbám a plísním), akaricidy (proti pavoukům), moluskocidy (proti měkkýšům), rodenticidy (proti hlodavcům) a regulátory růstu rostlin.

Podle chemické struktury lze pesticidy dále členit do skupin na organofosfátové, karbamátové, pyrethroidní a organochlorové pesticidy. V tomto přehledu se zaměříme na stanovení těch zástupců, kteří nejvýznamněji ovlivňují životní prostředí kolem nás, tj. představitele organochlorových insekticidů a zástupce karbamátových pesticidů.

Organochlorové pesticidy jsou velkou a různorodou skupinou, která způsobuje velké problémy v životním prostředí. V době zavedení těchto pesticidů se jako největší výhoda uváděla jejich stabilita, perzistence insekticidního účinku, levnost výroby, malá toxicita pro savce a široké spektrum insekticidní účinnosti[1]. Jejich rozsáhlé používání však vyvolalo díky těmto vlastnostem vážné ekologické problémy, a proto bylo stejně jako používání jiných chlorovaných uhlovodíků v mnoha zemích silně omezeno, popř. přímo zakázáno. Rozdělují se do tří hlavních skupin: DDT a jeho analogy, hexachlorcyklohexan (HCH) a cyklodieny (např. endosulfan, aldrin, dieldrin). Asi nejznámějšími a nejdéle používanými insekticidy jsou DDT a endosulfan.

Vzhledem k vážným ekologickým problémům, které způsobily především organochlorové pesticidy, začal chemický průmysl vyvíjet nové typy insekticidů. Díky své nízké toxicitě pro savce, snadné degradovatelnosti a zachovanému širokému spektru účinku získaly na významu karbamátové insekticidy (např. karbaryl, karbofuran nebo methiokarb). Používají se především k omezování hmyzích škůdců v zemědělství a zahradnictví. Přesto ani jejich používání není zcela bezpečné. Vstupem karbamátových pesticidů do organismu dojde k inhibici acetylcholinesterasy, která zajišťuje konečnou fázi při přenosu signálu mezi synaptickými buňkami – uvolnění mediátoru acetylcholinu z receptoru[2].

Toxicita a perzistence zmiňovaných pesticidů si vyžaduje kontrolu jejich výskytu v životním prostředí a v potravinách. Volba vhodné analytické metody pro stanovení pesticidu ve vzorku závisí na řadě faktorů: účel analýzy (např. kontrola hygienické nezávadnosti, orientační screening), povaha cílového analytu (původní sloučenina, její metabolit nebo degradační produkt) nebo znalost „historie“ vzorku[3]. Díky výrobě nových pesticidů, snižování hodnot hygienických limitů a zdokonalování instrumentace je třeba dále optimalizovat používané metody (především multireziduální metody) a zavádět nové analytické postupy (např. imunochemické metody).

Významným nástrojem při kontrole dopadu používání pesticidů na životní prostředí jsou multireziduální metody, které jsou schopné během jedné analýzy současně stanovit velké množství těchto látek. Identifikace a kvantifikace analytu se velmi často provádí pomocí plynové nebo kapalinové chromatografie spojené s vhodným detektorem.

Hlavní roli v oblasti analýzy organických kontaminantů hraje plynová chromatografie. Protože v analyzovaných vzorcích jsou pouze stopová množství analytů, je nutné pracovat s příslušnými selektivními detektory. Pro látky s vysokým zastoupením elektronegativních skupin nebo s výrazným podílem dvojných vazeb či aromatických kruhů (jako jsou organochlorové pesticidy) slouží detektor elektronového záchytu (ECD, cit.[4–7]). Zvýšení selektivity

detekce nabízí hmotnostní detektor[8,9]. Hmotnostní detektor s iontovou pastí (ITD) deteguje tyto pesticidy na hladinách ppt-ppb (cit.[10–12]).

Pro karbamátové pesticidy, které nelze pro jejich fyzikálně-chemické vlastnosti (polarita, termolabilita) stanovit metodou GC, byly vyvinuty metody kapalinové chromatografie (LC) a její varianty vysoce účinné kapalinové chromatografie (HPLC). Běžně používané ultrafialové detektory (UV) většinou nejsou schopné dostatečně splnit požadavky, které jsou kladeny na detekční limity metody, a proto se častěji používají detektory MS s různými technikami ionizace: chemická ionizace za normálního tlaku (APCI, cit.[13]), elektrosprayová ionizace (ESI, cit.[14,15]), termosprayová ionizace (TSI, cit.[16]) nebo ionizace pomocí částicového zářiče (PBI). Detekční limity pro LC/MS nebo HPLC/MS se pohybují pro potravinové vzorky v rozmezí 5–50 ppb (ng.ml^{-1}), cit.[17].

Zmíněné konvenční chromatografické techniky ve spojení s citlivým detekčním systémem jsou široce používány kontrolními laboratořemi. Tyto metody vyžadují velice nákladné přístrojové zařízení a časově náročné mnohastupňové čištění extraktů vzorků před vlastní analýzou. V posledních dvaceti letech lze pozorovat zvýšené úsilí při snahách o vypracování imunochemických metod ke stanovení pesticidů jako určité alternativy ke stávajícím metodám[18–20]. Imunochemické techniky představují jednoduché, rychlé a levné metody, které se stávají populárním nástrojem pro rychlý screening organických sloučenin v životním prostředí[21].

V tomto článku podáváme přehled dosud vyvinutých imunochemických metod a aplikací ke stanovení organochlorových a karbamátových pesticidů.

2. Princip imunochemických metod

Imunochemické metody využívají k důkazu, stanovení nebo separaci analytu biospecifické interakce s protilátkami. *In vitro* se při nich využívá produkt imunitního systému obratlovců zaměřený původně na specifické rozlišení a zneškodnění cizorodého agens (antigenu) *in vivo*. Tento produkt (specifické protilátky) umožňuje poměrně citlivé a specifické rozpoznání analytu v komplexních směsích biologických vzorků. Podstatou všech imunochemických metod je tedy nekovalentní interakce protilátky s antigenem. Stěžejním analytickým nástrojem je specifická protilátka. Nejjednodušší způsob její přípravy představuje injekční aplikaci antigenu (analytu) do organismu zvoleného zvířete a po určité době izolaci krevního séra.

Pesticidy ale mají vlastnosti tzv. haptenů – jejich molekuly jsou poměrně malé (< 2000 Da), a proto samy o sobě v organismu neprovokují imunitní systém k produkci protilátek. Pro tyto účely se musí hapten nejprve navázat na vysokomolekulární nosič (nejčastěji protein). Přitom by nemělo jít pouze o vytvoření kovalentní vazby mezi dvěma atomy, ale hapten by měl pomocí krátkého spojovacího raménka (3–6 atomů) mírně vyčnívat z molekuly bílkoviny, aby epitopově charakteristické atomy jeho molekuly nebyly stíněny prostorovou členitostí bílkovinné struktury.

Pesticid lze obvykle navázat na bílkovinu alternativně přes více než jeden reaktivní atom v jeho molekule. Přitom výběr místa na molekule pesticidu pro kovalentní vazbu s proteinem velice ovlivní specifitu produkovaných protilátek. Nabízí se možnost připravit řadu různých konjugátů (budou se lišit umístěním kovalentní vazby na molekule haptenu, event. typem a délkou spojovacího raménka) a ze získaných protilátek pak vybrat ty, které nejvíce vyhovují našim potřebám. A nebo se můžeme při volbě přípravy menšího počtu konjugátů řídit následujícími doporučeními, která vyplývají z mnoha dosud provedených experimentů:

a) Místo v molekule haptenu, na kterém se vytváří vazba s proteinem, by mělo být co nejvíce vzdáleno od té části haptenové molekuly, proti které chceme vytvořit specifické protilátky (od charakteristického seskupení atomů v molekule haptenu). Tím se docílí toho, že epitopově významné skupiny atomů nejsou pozměněny ani prostorově stíněny nosičem ani spojovacím raménkem.
b) Pokud je to možné, konjugovat hapten přes atom uhlíku v jeho molekule, a ne přes heteroatom, což by mohlo vést ke změně polarity, a tím i ke změně charakteru epitopu a odezvy imunitního systému.
c) Spojovací raménko vzniklé konjugací haptenu s proteinem by nemělo být výraznějším epitopem než molekula haptenu. Na druhou stranu delší raménko může zvýraznit imunitní odpověď na hapten. Je ale vhodnější, je-li raménko alifatické a bez heteroatomů.

Výběr epitopově významné skupiny atomů v molekule pesticidu, která má být v molekule konjugátu zvýrazněna (viz výše ad a) může výrazně ovlivnit další použitelnost protilátky. Zohledněním výše uvedených zásad lze totiž cíleně připravit buď úzce specifické protilátky, které budou interagovat pouze s jediným vybraným zástupcem ze skupiny strukturně podobných pesticidů, anebo široce specifické protilátky, reagující se všemi členy pesticidové skupiny[22–24].

3. Metody pro imunochemické stanovení pesticidů

Pesticidy jako hapteny spontánně neprecipitují s protilátkami. Proto se jejich stanovení provádí především neprecipitačními imunochemickými metodami, které využívají vhodnou značku k zesílení a zviditelnění primární reakce hapten-protilátka. Označením jednoho z partnerů této interakce se značně zvýší citlivost imunochemického stanovení. Přestože jako značky přicházejí v úvahu radionuklidy, fluorescenční a chemiluminiscenční látky, ferritin, liposomy aj., v praxi se nejvíce rozšířilo značení enzymem.

3.1. Metoda ELISA

Při stanovení pesticidů enzymovou imunoanalýzou se téměř výhradně používá kompetitivní uspořádání heterogenní analýzy, kdy je buď protilátka nebo pesticid zakotven na pevnou fázi (ELISA – Enzyme Linked Immuno-Sorbent Assay).

Tzv. přímá ELISA spočívá v soutěži značeného pesticidu a pesticidu-analytu o vazebná místa omezeného množství molekul protilátek, které jsou navázány na nerozpustný nosič. Po ustavení rovnováhy je odstraněn reakční roztok a kvantifikován enzym (enzymová aktivita), který je zprostředkovaně navázán na pevnou fázi[25].

V nepřímém uspořádání ELISA je na pevnou fázi navázán pesticid. Ten soutěží s rozpustným pesticidem-analytem o vazebná místa omezeného množství molekul protilátek, které jsou také volně v roztoku. Po ustavení rovnováhy je odstraněn reakční roztok s rozpustnými reaktanty a protilátka navázaná na imobilizovaný pesticid je kvantifikována přídavkem protilátky proti protilátce (tzv. druhá protilátka), která byla předem označena enzymem. Po dalším ustavení rovnováhy a odstranění reakčního roztoku je měřena enzymová aktivita zachycená na pevné fázi[25].

Obě popsané varianty ELISA umožňují stanovení pesticidů až do koncentrace 1 ng – 1 $\mu g.ml^{-1}$ v závislosti na kvalitě protilátky. Standardní křivka (hodnoty enzymové aktivity resp. absorbance reakčního roztoku proti logaritmu koncentrace standardu analytu) má v obou případech sigmoidní tvar se strmou lineární oblastí kolem inflexního bodu (bod 50% inhibice, I_{50}).

Technické uspořádání ELISA se liší podle účelu a rozsahu prováděných stanovení.

a) Ve velké sérii malých reakčních prostorů mikrotitrační destičky se taková stanovení provádějí při screeningu většího počtu vzorků v kontrolních a výzkumných laboratořích. Formát destičky je vhodný pro aplikaci automatizačních prvků, a to jak při pipetování reagencií shodných pro všechny reakční prostory, tak pro promývání těchto prostorů po jednotlivých inkubacích, i při finální kvantifikaci absorbance.
b) V jednotlivých zkumavkách se realizují při analýzách několika málo vzorků. Jako nerozpustný nosič pro imobilizaci jednoho z imunoreaktantů mohou v obou případech sloužit stěny plastových zkumavek nebo jamek mikrotitračních destiček, popř. částice s ferromagnetickým jádrem, které lze separovat od roztoku vnějším magnetem. Kvantitativní analýza vyžaduje u obou variant fotometr. Semikvantitativní hodnocení analýz vystačí se subjektivním porovnáním zbarvení reakčních roztoků při enzymové přeměně přidaného substrátu.
c) Nejpohodlnější je semikvantitativní provedení ELISA v podobě tyčinek (dipstiků) nebo kartiček s aktivními zónami. Po aplikaci imunoreaktantů a testovaného vzorku je porovnáván odstín zbarvení aktivní zóny se standardní stupnicí.

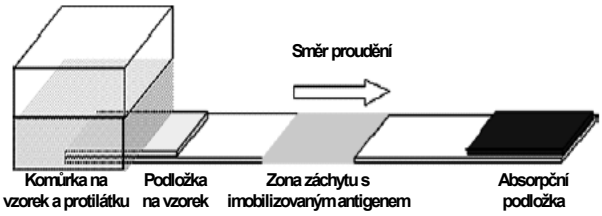
3.2. Imunoafinitní chromatografie

Principy imunochemické reakce jsou použity také v jiných technikách, např. imunoafinitní chromatografie nebo imunosenzor. Imunoafinitní chromatografie může být provedena dvěma způsoby podle toho, zda je na nerozpustný nosič kovalentně imobilizován antigen nebo protilátka. Kolona s navázaným antigenem se používá při čištění polyklonálního antiséra pro získání jeho vyšší specifity. Kolonu s imobilizovanou protilátkou lze použít ke specifickému zachycení analytu ze vzorku. V poslední době se při stanovení pesticidů rozšiřuje zařazení této imunoafinitní techniky před konvenční HPLC nebo GC (cit.[26–30]). Účelem je buď nakoncentrování analytu nebo čištění extraktu od interferujících látek z matrice vzorku, tzv. imunoextrakce.

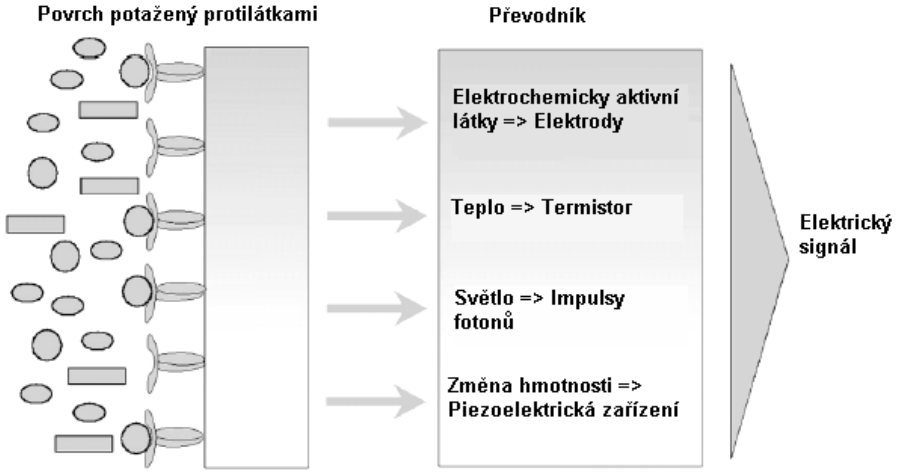
Mezi imunochromatografie řadíme i metodu LFIA (lateral flow immunoassay). Tato metoda je vyvíjena pro stanovení nejrůznějších analytů a jako atraktivní se jeví i v analýze pesticidů[31–34]. LFIA je v podstatě navržena jako semikvantitativní, robustní, snadno proveditelná imunochemická metoda, která je vhodná pro specifickou semikvantitativní detekci analytů. Vyžaduje pouze levné přístrojové vybavení a umožňuje ve srovnání s metodou ELISA na mikrotitračních destičkách podstatné snížení doby analýzy. Specifické protilátky imobilizované na koloidní částice jsou smíchány s analytem (extrakt vzorku) v aplikační komůrce. Všechny složky takového roztoku potom kapilárními silami migrují vrchní porézní vrstvou destičky (viz obr. 1). V tzv. zóně záchytu je na destičku předem imobilizován ekvivalent analytu, na nějž jsou při migraci destičkou poutány volné protilátky, které se nenavázaly na analyt ze vzorku. Čím více je analytu v aplikovaném vzorku, tím méně protilátek je zachyceno na zmíněné zóně. Semikvantitativní vizuální hodnocení záchytu umožňují koloidní částice. Pokud vzorek neobsahuje stanovovaný analyt, v zoně záchytu se v důsledku přítomnosti volných koloidních protilátek objeví výrazný barevný proužek. Pokud vzorek obsahuje sledovaný analyt, v zóně záchytu se objeví slabší nebo žádný barevný proužek[35,36].

3.3. Imunosenzor

Imunochemické biosenzory patří mezi afinitní biosenzory, které využívají specifickou interakci mezi komple-



Obr. 1. **Schéma metody LFIA**[36]



Obr. 2. **Základní princip imunosenzoru**[38]

mentárními oblastmi biomolekul (antigen-protilátka) vedoucí ke vzniku komplexních struktur. Cílem integrace imunochemických stanovení s technologií biosenzorů je snaha zrychlit, zjednodušit jejich použití a vyvinout malé přenosné systémy použitelné přímo v místě odběru vzorku[37].

Imunosenzory se skládají ze tří částí: (*i*) biologický prvek (protilátka, která je imobilizovaná na převodník a interaguje se stanovovaným analytem), (*ii*) převodník, který přeměňuje biochemický nebo biofyzikální děj (vazba analytu) na elektrický signál a (*iii*) elektronická část. Tyto senzory jsou klasifikovány na základě fyzikálněpřevodních mechanismů: elektrochemické, optické, teplotní nebo piezoelektrické (obr. 2, cit.[38]). Pro stanovení pesticidů se převážně používají imunosenzory na bázi optických, elektrochemických a piezoelektrických senzorů.

Optické imunosenzory jsou založeny na spektrofotometrické, spektrofluorimetrické, chemiluminometrické či reflektometrické detekci optického signálu. Mohou detegovat přímo vznik imunokomplexu (bez značení), značenou látku a nebo produkt enzymové reakce (fluorofor či luminofor). Pro detekci haptenů bez značení se používá jejich imobilizace na převodník. Změny způsobené interakcí mezi imobilizovaným haptenem a protilátkou jsou nepřímo úměrné koncentraci volného haptenu v reakčním mediu[39].

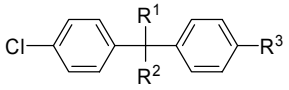
Elektrochemické převodníky jsou založeny na značení protilátky či stanovovaného analytu enzymem, který katalyzuje produkci elektroaktivního produktu[40]. Oproti optickým imunosenzorům jsou citlivější a nevyžadují speciální optické vlastnosti reakční směsi.

Piezoelektrický imunosenzor měří změny hmotnosti způsobené vznikem komplexu antigen-protilátka pomocí piezoelektrického krystalu[41]. K sledování těchto změn se využívá přirozená rezonanční frekvence tohoto senzoru. Její hodnota je nepřímo úměrná hmotnosti piezoelektrického senzoru. Navázání haptenu na imobilizovanou protilátku však nezpůsobuje dostatečně velkou změnu hmotnosti. Proto při použití tohoto imunosenzoru je využíván konjugát haptenu s proteinem a kompetitivní schéma stanovení.

4. Imunochemické stanovení vybraných organochlorových pesticidů

Klíčovým krokem ve vývoji imunoanalýzy pro haptény je navržení a příprava imunogenu. Např. pro DDT, který se vyskytuje ve dvou izomerních formách *p*,*p*'-DDT a *o*,*p*'-DDT, a příbuzné sloučeniny bylo provedeno mnoho pokusů s různými typy imunogenů pro získání specifických protilátek. První práce používající jako imunogen konjugát protein-DDA (DDA - 2,2-bis(4-chlorfenyl)octová kyselina) poskytovaly nízkoafinitní antiséra, která hlavně rozpoznávala DDA, hlavní metabolit DDT vyskytující se v moči[42,43]. Další práce (Burgisser a spol.[44]) použila jako imunogen pro přípravu monoklonálních protilátek v kompetitivní radioimunoanalýze dicofol (2,2,2-trichlor-1,1-bis(4-chlorfenyl)ethanol), který je strukturně podobný DDT. Tato metoda nenašla uplatnění v praxi, protože je málo citlivá (I_{50}=100 nM) a používá radioaktivní značku. Stejně tak jako radioimunoanalýzy pro endosulfan a jiné cyklodieny (aldrin, endrin, dieldrin aj.) byla tato metoda pro větší bezpečnost a snazší manipulaci nahrazena enzymovými imunoanalýzami.

Vliv struktury haptenů pro přípravu imunogenu na specifitu monoklonálních protilátek při metodě ELISA ke stanovení DDT a jeho derivátů byl zhodnocen v publikované práci Abada a spol.[45]. Hapteny byly připraveny zabudováním alifatického řetězce jako spojovacího raménka s koncovou karboxylovou skupinou – buď na centrální uhlíkový atom (hapten 1, obr. 3) nebo na



Název sloučeniny	R^1	R^2	R^3
DDT	CCl_3	H	Cl
DDA	COOH	H	Cl
Hapten 1	OH	$(CH_2)_3COOH$	Cl
Hapten 2	CCl_3	H	$(CH_2)_3COOH$

Obr. 3. **Chemická struktura DDT a haptenů použitých v práci Abad a spol.[45]**

benzenový kruh struktury DDT (hapten 2, obr. 3). Připravené monoklonální protilátky byly charakterizovány z hlediska afinity a specifity k *p*,*p*'-DDT metodou ELISA. Všech sedm protilátek, které byly odvozeny od haptenu 2 (I_{50} v rozmezí 2,1–6,5 nM), vykazovalo vyšší afinitu k *p*,*p*'-DDT než protilátka odvozená od haptenu 1 (I_{50} = 11,1 nM). Specifita protilátek, schopnost interagovat i s analogy DDT, byla prověřena pomocí hodnot křížových interakcí. Protilátky byly na jejich základě rozděleny do tří skupin. První skupina zahrnuje čtyři protilátky odvozené od haptenu 2, které vykazovaly silné křížové interakce se sloučeninami ze třídy DDT. Tyto protilátky jsou tedy skupinově specifické. Druhá skupina protilátek, kam byly zařazeny ostatní protilátky odvozené od haptenu 2, jsou specifické pro DDT, protože rozeznává pouze *p*,*p*'-DDT nebo *o*,*p*'-DDT. Protilátka odvozená od haptenu 1 vykazovala rozdílné křížové interakce s analogy DDT, a proto byla zařazena do třetí skupiny. Vzhledem k těmto výsledkům byly pro imunochemická stanovení DDT doporučeny protilátky odvozené od haptenu 2.

Do dnešní doby byly publikovány enzymové imunoanalýzy pro stanovení chlorovaných bifenylů[46,47], DDT, endosulfanu, hexachlorcyklohexanu[48], dieldrinu[49], pentachlorfenolu, pikloramu nebo kaptanu[50]. Dostupné publikované metody ELISA s fotometrickou nebo chemiluminiscenční detekcí pro DDT, endosulfan a jejich degradační produkty jsou shrnuty s vybranými charakteristikami v tabulce I a II.

Pro detekci a kvantifikaci cyklodienů (dieldrin, aldrin, heptachlor, chlorden, endrin a endosulfan) byl vyvinut optický imunosenzor využívající spektrofluorometrickou detekci optického signálu. Na převodník byly imobilizovány polyklonální protilátky proti chlorované kapronové kyselině. Imunosenzor detegoval cyklodieny na hladině ppb a vykazoval nízkou křížovou interakci s hexachlorcyklohexanem[51].

5. Imunochemické stanovení vybraných karbamátových pesticidů

Mezi nejznámější karbamátové pesticidy patří karbaryl, karbofuran, aldikarb, methonyl, oxamyl nebo methiokarb. Většina těchto látek se přeměňuje ve velmi krátké době na své metabolity, které jsou často stejně nebo dokonce více aktivní než původní sloučenina. Např. aldikarbsulfát je silnějším inhibitorem cholinesterasy než aldikarb[70]. Imunochemické metody jsou vyvíjeny tak, aby protilátka rozpoznávala nejen původní molekulu pesticidu, ale také jeho degradační produkty.

Při vývoji imunoanalýzy pro karbamáty je stejně jako pro organochlorové pesticidy důležitým krokem navržení a příprava imunogenu. Hodnocení vlivu haptenové struktury na afinitu protilátek byla publikována Abadem a spol. pro *N*-methylkarbamátový pesticid karbaryl[71]. Navržené hapteny (obr. 4) měly stejné rozložení aromatických kruhů jako karbaryl a obsahovaly karboxylovou skupinu pro

Tabulka I
Přehled publikovaných metod ELISA pro stanovení DDT a jeho degradačních produktů

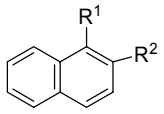
Uspořádání metody[a]	PL	Detekční limit [ng.ml^{-1}]	I_{50} [ng.ml^{-1}]	Matrice vzorku	Lit.
ELISA II	mPL	N	0,3–2,8	pufr	52
ELISA II	pPL	0,12	2,5	odpadní voda	53,54
ELISA I	pPL	N	13	rajčatový protlak, zemina, voda, rozinky, mléko	55–59
ELISA II	pPL	N	120	pufr	60,61
ELISA II	mPL	0,006 / 0,04	0,6 / 0,2	voda, zemina, hlávkový salát, jahody	45,62,63
ELISA VII	mPL	0,4 / 0,4	21 / 8	voda, zemina, hlávkový salát, jahody	45,62,63

[a]N – neuvedeno; pPL – polyklonální protilátka; mPL – monoklonální protilátka; ELISA I – přímá kompetitivní ELISA, na mikrotitrační destičky vázána protilátka; ELISA II – nepřímá kompetitivní ELISA, na mikrotitrační destičky vázán antigen; ELISA VII – průtoková ELISA

Tabulka II
Přehled publikovaných metod ELISA pro stanovení endosulfanu a jeho degradačních produktů

Uspořádání metody[a]	PL	Detekční limit [ng.ml^{-1}]	I_{50} [ng.ml^{-1}]	Matrice vzorku	Lit.
ELISA I / ELISA V	pPL	0,2/0,2	25–50/N	voda, zemina, zrnka vína, zelí, květák, špenát, rajčata, rýže	56, 64–66
ELISA I / ELISA II	pPL	3/5	N	pufr	67
ELISA I	pPL	3	N	pufr	68
ELISA (komerční souprava)	pPL	10–30	6–100	jablko, rajčata, hlávkový salát	69

[a]N – neuvedeno; pPL – polyklonální protilátka; mPL– monoklonální protilátka; ELISA I – přímá kompetitivní ELISA, na mikrotitrační destičky vázána protilátka; ELISA II – nepřímá kompetitivní ELISA, na mikrotitrační destičky vázán antigen; ELISA V – přímá kompetitivní ELISA, na stěnách zkumavky vázána protilátka



Sloučenina	R^1	R^2
Karbaryl	$OCONHCH_3$	H
Hapten A	$OCONH(CH_2)_5COOH$	H
Hapten B	$OCONH(CH_2)_2COOH$	H
Hapten C	$NHCONH(CH_2)_5COOH$	H
Hapten D	$CONH(CH_2)_5COOH$	H
Hapten E	H	$CONH(CH_2)_5COOH$

Obr. 4. **Chemická struktura karbarylu a haptenů použitých v práci Abad a spol.[71]**

připojení na bílkovinu. Teoreticky jsou za nejlepší hapteny považovány hapteny A a B. V jejich molekulách je spojovací raménko připojeno přes methylovou skupinu analytu, a tak je zachována charakteristická skupina pro tyto pesticidy. V haptenech C a D je raménko připojeno stejným způsobem, ale chybí karbamátová skupina. Hapten E se od haptenu D liší pouze polohou funkční skupiny na aromatickém kruhu. Při výběru nejvhodnějších haptenů pro přípravu monoklonálních protilátek byly připravené imunogeny použity nejprve pro imunizaci králíků. Získané polyklonální protilátky byly charakterizovány metodou ELISA. Podle očekávání se jako nejlepší potvrdily hapteny A a B, které poskytly výrazně nižší I_{50} než ostatní hapteny. Proto byly použity pro přípravu monoklonálních protilátek.

Pro analýzu *N*-methylkarbamátů je k dispozici několik komerčních souprav ELISA používajících mikrotitrační destičky nebo magnetické částice s fotometrickou detekcí. Takováto souprava byla použita např. ke stanovení karbofuranu a aldikarbsulfátu v mase, játrech, krvi nebo moči[72]. ELISA založená na využití magnetických částic byla vyvinuta pro rychlé stanovení karbarylu ve vodných vzorcích[73] nebo pro karbofuran ve vodě a v půdě[74]. Publikována byla jednostupňová kompetitivní ELISA používající monoklonální protilátky proti pesticidu propoxur (I_{50} v rozmezí 6,5–17,9 nM) bez křížových interakcí s významnými *N*-methylkarbamátovými pesticidy[75]. Dostupné publikované metody ELISA pro karbaryl, karbofuran a methiokarb jsou shrnuty s vybranými charakteristikami v tabulce III.

Protilátky, které byly připraveny Abadem a spol.[71], byly použity při vývoji imunosenzoru pro detekci karbarylu[76,77]. Senzor je založen na principu heterogenní kompetitivní enzymové imunoanalýzy, která používá protilátky v roztoku (nepřímé provedení) nebo imobilizované protilátky (přímé provedení). V obou případech byla použita enzymová značka (křenová peroxidasa) a fluorimetrická detekce. Jedna imunoanalýza pro přímé provedení trvá asi 11 min s detekčním limitem 26 ng.ml^{-1}, pro nepřímý asi 17 min s detekčním limitem 284 ng.ml^{-1}. Metoda byla aplikována na analýzu vzorků pitné vody a džusu a získané výsledky byly porovnány s ELISA jako referenční metodou. Vyvinutý imunosenzor byl použit také pro analýzu vzorků zeleniny a získané výsledky byly porovnány s HPLC jako referenční metodou[78]. Pro vzorky vody byl sestaven imunosenzor pro současné stanovení více pesticidů (karbaryl, atrazin a irgarol 1051). Detekční limit byl pro všechny pesticidy nižší než 0,1 μg.ml^{-1}, cit.[79]. Stejný typ imunosenzoru jako pro karbaryl byl použit i pro jeho hlavní degradační metabolit 1-naftol s detekčním limitem ve vodném prostředí 16,2 μg.l^{-1}, cit.[80].

Tabulka III
Přehled publikovaných metod ELISA pro *N*-methylkarbamátové pesticidy karbaryl, karbofuran a methiokarb

Uspořádání metody[a]	PL	Detekční limit [ng.ml^{-1}]	I_{50} [ng.ml^{-1}]	Matrice vzorku	Lit.
Karbaryl					
ELISA IV	pPL	0,25	N	voda	73
ELISA III	pPL	200	92,8	zelenina	81
ELISA I	pPL	1000	N	obilí	82
ELISA II	pPL	N	1,48	pomeranče, broskve, banány, mrkev, fazole, brambory	83
ELISA I / ELISA II	pPL	50 / 200	400 / 2000–5000	voda	84,85
ELISA I / ELISA II	mPL	N / 0,01	0,13 / 0,058	jablka, rajčata, jablkový a grepový džus, voda, okurky, jahody, brambory, pomeranče, papriky, dětská výživa	15,71, 86-90
Methiokarb					
ELISA I	mPL	N	0,044	dětská výživa, okurky, jahody	15, 89, 91
ELISA II / ELISA VI	mPL	N / N	0,12 / 0,16	dětská výživa, okurky, jahody	15, 89, 91
Karbofuran					
ELISA I / ELISA II	mPL	N / 0,2	0,7 / 1,8	jablka, rajčata, jablkový a grepový džus, voda, okurky, jahody, brambory, pomeranče, papriky, dětská výživa	89,92, 93
ELISA IV komerční souprava	pPL	0,056 / 5,6	N	voda / zemina	74
ELISA IV komerční souprava	pPL	N	N	hovězí játra	94
ELISA V komerční souprava	pPL	N	N	mleté maso a játra	72

[a]N – neuvedeno; pPL – polyklonální protilátka; mPL – monoklonální protilátka; ELISA I – přímá kompetitivní ELISA, na mikrotitrační destičky vázána protilátka; ELISA II – nepřímá kompetitivní ELISA, na mikrotitračnídestičky vázán antigen; ELISA III – dipstik; ELISA IV – přímá kompetitivní ELISA, na magnetických částicích navázána protilátka; ELISA V – přímá kompetitivní ELISA, na stěnách zkumavky vázána protilátka; ELISA VI – přímá kompetitivní ELISA, na mikrotitrační destičky vázán antigen

Tabulka IV
Přehled hlavních charakteristik komerčních souprav ELISA pro analýzu organochlorových a karbamátových pesticidů uvedených v různých dokumentech firem Strategic Diagnostics, Inc., Abraxis a EnviroLogix

Pesticid	Formát[a]	Rozmezí kvantifikace [$\mu g.ml^{-1}$]	Matrice vzorku
Aldikarb	I	1–100	vzorky vody, půdy, potravin
Kaptan	I	0,08–3	vzorky vody
Karbaryl	I	0,4–5	vzorky vody, půdy, potravin
Karbofuran	I	0,1–5	vzorky vody, půdy, potravin
Cyklodieny	I	0,6–26,6	vzorky vody
Aldikarb	II	5–100	vzorky vody
Chlordan	II	20–600	vzorky půdy
Cyklodieny	II	10–100	vzorky vody
DDT	II	0,2–10	vzorky půdy
Lindan	II	0,4–40	vzorky půdy
Aldikarb	III	1–20	vzorky vody
Cyklodieny	III	1–100	vzorky vody
	III	5–100	vzorky vody
DDT	III	1,25–75	vzorky vody, půdy, sediment, rybí plazma
	III	0,025–0,875	vzorky půdy
Endosulfan	III	0,25–25	vzorky vody, půdy, sediment, rybí plazma
	III	0,075–1	vzorky vody

[a]Formát: I – protilátky navázané na magnetické částice, II – protilátky navázané na stěny zkumavek, III – protilátky navázané na mikrotitračních destičkách s 96 jamkami

6. Validace imunoanalýz

Na desítkách pesticidů bylo ukázáno, že imunoanalýzy jsou dobrou alternativou ke konvenčním metodám pro svou vysokou specifitu a citlivost a pro nižší náklady na stanovení. Validace imunoanalýzy by měla být integrální součástí vývoje metody. Při analýzách určitých typů vzorků bylo dosaženo vysoké korelace mezi výsledky imunoanalýzy a konvenční metody[95–97]. Řada státních i profesních organizací (především v USA a v Evropě) z oblasti kontroly potravin a životního prostředí a z oblasti analytické chemie (EPA, AOAC, FSIS, UK ESCA, GISG) dává závazná písemná doporučení pro evaluaci imunoanalýz a kontroluje validační protokoly u komerčních souprav. Na seznamu validovaných oficiálních metod podle EPA a AOAC pro stanovení určitých pesticidů v určitém typu vzorku je již imunoanalýza uvedena ve více než dvaceti případech (včetně DDT, pentachlorfenolu, chlordanu).

Imunoanalýzu analytu je nutno ověřit pro každý typ vzorku zvlášť, aby se předešlo nežádoucím nespecifickým interakcím složek matrice vzorku s protilátkami, které by mohly ovlivňovat získané výsledky stanovení (např. způsobovat falešně pozitivní či falešně negativní hodnocení kontaminace sledované komodity)[56,98]. Předpokládat bez ověření, že selektivita protilátek je natolik vysoká, že nedovolí zmíněný projev matrice, je nekorektní. Ostatně takový vliv matrice je nutné eliminovat i u konvenčních metod[99].

7. Budoucnost imunoanalýzy pesticidů

Popularita imunochemických metod při stanovení pesticidů se zvyšuje, jak se tyto metody stále více přeměňují z výzkumného nástroje v rutinní techniku, a jak jsou komerčními výrobci nabízeny v širší škále kompletní analytické soupravy. (Přehled souprav ke stanovení organochlorových a karbamátových pesticidů je uveden v tabulce IV).

Zatímco mezi současnými aplikacemi imunoanalýz při stanovení pesticidů naprosto převažuje ELISA, lze v budoucnu očekávat četnější aplikace imunoafinitní chro-

matografie, ať již v podobě LFIA nebo kolonek pro imunoextrakci při kombinovaném použití konvenčních analytických postupů a imunosorbentu.

Vzhledem k značnému úsilí vynaloženém na vývoj imunosenzorů roste i počet publikací o multianalytových imunosenzorech[79], ať již v provedení tyčinek[82], protilátkový mikroarray[100,101], svazku kapilár[102] nebo dokonce komerčního přístroje[103,104]. Zavedení takových zařízení do analytické praxe by bylo z hlediska efektivity výkonu laboratoře velice prospěšné.

Vedle již naprosto běžně používaných monoklonálních protilátek určitě při imunoanalýze pesticidů naleznou širší uplatnění rekombinantní protilátky[105,106]. Při jejich přípravě lze genovými manipulacemi snáze ovlivňovat specifitu protilátek než změnou struktury imunogenů při přípravě polyklonálních protilátek či selekcí hybridomů u monoklonálních protilátek.

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Seznam zkratek

DDA	2,2-bis(4-chlorfenyl)octová kyselina
DDT	1,1,1-trichlor-2,2-bis(4-chlorfenyl)ethan
ELISA	enzyme linked immunosorbent assay
LFIA	lateral flow immunoassay
I_{50}	bod 50% inhibice

B. Mičková, P. Rauch, and L. Fukal (*Department od Biochemistry and Microbiology, Institute of Chemical Technology, Prague*): **Possibilities of Immunochemical Determination of Organochlorine and Carbamate Pesticides**

Immunoassay is recognized as a promising method for screening environmental contaminants. The article deals with principles and practical applications of immunochemical methods in pesticide analysis. An overview is given of organochlorine and carbamate pesticides for which immunoassays, in particular the ELISA test, were developed, including commercially available kits. In addition, a survey is given on further developments of existing or new immunoassays and on the application of immunochemistry in other fields (immunoafinity chromatography, immunosensors).

Příloha 2:

Botchkareva A.E., Eremin S.A., Montoya A., Manclus J.J., Mickova B., Rauch P., Fini F., Girotti S.: Developmnet of chemiluminescent ELISAs to DDT and its metabolites in food and environmental samples. *J. Immunol.Methods* 283(1-2): 45-57, 2003.

ELSEVIER

Journal of Immunological Methods 283 (2003) 45–57

JIM
Journal of Immunological Methods

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Development of chemiluminescent ELISAs to DDT and its metabolites in food and environmental samples

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Received 24 January 2003; received in revised form 29 May 2003; accepted 5 August 2003

Abstract

Two enzyme-linked immunosorbent assays (ELISA) with chemiluminescent (CL) detection for the insecticide DDT and the group of DDT-related compounds have been optimized and characterized. Both conjugate-coated ELISAs are based on monoclonal antibodies (MAbs) of different specificity and homologous protein conjugates. Effects of several physicochemical factors (ionic strength, pH, Tween-20 and Bovine serum albumin (BSA) concentrations) and solvents (methanol, ethanol, acetone and *N*,*N*′-dimethylformamide) on the performance of the assays were studied and optimized. For the DDT-selective assay, the sensitivity, estimated as the I_{50} value, was 0.6 μg/l, with a linear working range between 0.1 and 2 μg/l and a limit of detection of 0.06 μg/l. For the DDT group-selective assay, the sensitivity was 0.2 μg/l, with a linear working range between 0.07 and 1 μg/l and a limit of detection of 0.04 μg/l. CL-ELISAs were four times more sensitive compared to colorimetric ELISAs. Finally, both immunoassays were applied to the detection of DDT and group of DDT-related compounds in spiked real water, soil and food samples.

Keywords: DDT; Monoclonal ELISA; Enhanced chemiluminescence reaction; Environmental samples; Food

Abbreviations: ARPA, Regional Agency for Environmental Protection; BSA, bovine serum albumin; CL, chemiluminescent; CR, cross-reactivity; CV, coefficient of variation; ECD, electron-capture detectors; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; EU, European Union; GPC, gel permeation chromatography; HRP, horseradish peroxidase; I_{50}, concentration giving 50% inhibition of maximum response; LOD, limit of detection; MAbs, monoclonal antibodies; OVA, ovalbumin; PBS, 10 mM phosphate buffer; 150 mM NaCl, pH 7.4; PBST, 10 mM phosphate buffer; 150 mM NaCl, 0.05% Tween-20, pH 7.4; RLU, relative light units.

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doi:10.1016/j.jim.2003.08.016

1. Introduction

Immunochemical techniques have gained an increasing importance for screening and quantification of pesticides due to their sensitivity, speed, simplicity and low cost (Hennion and Barcelo, 1998; Knopp, 1995; Dankwardt, 2001). Since the European Union (EU) has limited the maximal level for a single pesticide to 0.1 μg/l and for the total of all pesticides to 0.5 μg/l in drinking water (EC Drinking water quality directive, 1980, 1998), a need exists for methods with very high sensitivity.

The sensitivity of an immunoassay strongly depends on the affinity of specific antibodies and on the sensitivity of the detection method. The enhanced chemiluminescence (ECL) reaction offers the possibility of improving the sensitivity of immunoassays to at least 2–3 orders of magnitude compared to conventional colorimetric detection (Dzgoev et al., 1999; Rubtsova and Wittman, 1997). The light intensity of ECL reaches a maximum 1–2 min after the start of the reaction, thus providing a rapid detection of the analytical signal (Arefyev et al., 1990). These advantages of chemiluminescent (CL) techniques make them useful detection systems for ecotoxicological analysis (Danielsson et al., 2001; Navaz and Jimenez, 1996).

Among the several kinds of pesticides employed in agriculture and vector control applications, DDT displays a recognized toxicity (ATSDR, 2000) and a long persistence in the environment. Although its use was banned in developed nations after 1970, there is still an important presence in the food chain. In addition, DDE and DDD, the two major metabolites of DDT, and other DDT-related compounds are usually present together with the parent compound in DDT-contaminated environmental matrices. Several groups have developed immunoassays for DDT and related compounds (Banerjee, 1987; Banerjee et al., 1996; Beasley et al., 1998; Burgisser et al., 1990). Commercial enzyme-linked immunosorbent assay (ELISA) kits for detecting residues of DDT, available from Strategic Diagnostics (Newark, DE, USA) and EnviroLogix (Westbrook, MA, USA), are based on the use of polyclonal antibodies.

Recently, we reported on the development of flow immunoassays for DDT and related compounds with ECL as a detection system (Botchkareva et al., 2002). These chemiluminescent assays were based on monoclonal antibodies (MAbs) against DDT with different patterns of cross-reactivity to DDT-related compounds (DDT-selective and class-selective) (Abad et al., 1997). Although the resulting flow assays had LODs of 1.1 and 0.4 μg/l with DDT-selective and class-selective MAbs, respectively, their application for the quantification of DDT in real samples was limited by a short life-time of flow immunosupport. In the present study, we adapted the ECL detection to the development and optimization of sensitive ELISAs based on the same monoclonal antibodies (MAbs). We also describe the application of the developed CL-ELISAs to the analysis of residues in water, soil and food samples.

2. Materials and methods

2.1. Reagents and instruments

Pesticide standards (Pestanal grade) were purchased from Riedel-de-Haen (Seelze, Germany). Stock solutions were prepared in methanol (1 g/l) and stored in the dark at 4 °C. Working standard solutions (0.001–200 μg/l) were prepared daily in double-distilled water from medium stock solution in methanol (1 mg/l) in borosilicate glass tubes and used within 1 h to avoid pesticide loss through adhesion to glass surfaces.

The preparation of the hapten–protein conjugate (OVA-DDT5, hapten-to-protein molar ratio 11:1) and the production of LIB-DDT5-25 and LIB-DDT5-52 monoclonal antibodies against DDT were previously described (Abad et al., 1997). The chemical structure of hapten DDT5 is shown in Fig. 1. Coating conjugate solutions were kept frozen in PBS at −20 °C, and concentrated MAb solutions (1 g/l) were kept precipitated with 1 volume of saturated ammonium sulphate

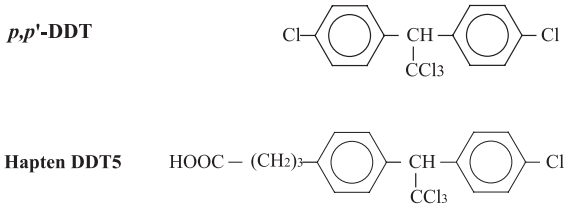

Fig. 1. Structure of *p,p'*-DDT and hapten DDT5.

solution at 4 °C, from which intermediate solutions in PBS were prepared and kept at 4 °C.

Peroxidase-labeled rabbit anti-mouse immunoglobulins were from Dako (Glostrup, Denmark). Bovine serum albumin (BSA), Tween-20, luminol, and *p*-iodophenol were from Sigma (USA). All other chemicals and organic solvents were of reagent grade. All solutions were prepared with water purified with a Milli-Q system (Millipore, USA). Opaque high binding plates for chemiluminescent measurements were from Costar (Cambridge, USA). Pyrex borosilicate glass tubes were from Corning (Corning, USA). Plates were washed in a WellWash 4 from Labsystems (Sweden) and chemiluminescent intensity was measured with a Victor reader (Wallac, Finland).

2.2. ELISA

Two ELISAs with antibodies LIB-DDT5-25 and LIB-DDT5-52 were chosen for optimization. Both of them were based on the conjugate-coated format with OVA-DDT5 as coating conjugate. Optimum concentrations were determined by checkerboard titration before the optimization of the physicochemical assay parameters. The optimum reagent concentrations were defined as those which gave the maximum intensity of chemiluminescence with minimum reagent expense. A final volume of 100 μl/well was used in all steps; all incubations were performed at room temperature. Plates were washed four times between steps with PBST [PBS (10 mM phosphate, 150 mM NaCl, pH 7.4) containing 0.05% Tween-20].

2.3. Procedure

Plates were coated overnight with 0.5 mg/l of the OVA-DDT5 conjugate in coating buffer (10 mM sodium carbonate/bicarbonate, pH 9.6). The plates were washed as described above, and 50 μl/well of standard in double-distilled water or sample solution, followed by 50 μl/well of LIB-DDT5-25 (assay 1) or LIB-DDT5-52 (assay 2) MAbs at 60 μg/l, were added. MAb solutions were prepared in different buffers depending upon the experiments. Inhibition standard curves were prepared by serial dilutions from 200 to 0.001 μg/l with a dilution factor of 5. The competitive reaction was allowed to take place for 2 h. After washing, a 1/2000 dilution of peroxidase-labeled rabbit anti-mouse immunoglobulins in PBST was added, and plates were incubated for 1 h. Plates were then washed and finally peroxidase activity was revealed by adding 100 μl/well of a freshly prepared substrate mixture (1 mM luminol, 0.5 mM *p*-iodophenol, 1 mM H_2O_2 in 0.2 M borate buffer, pH 8.5). Chemiluminescence emission was measured immediately after the addition of the substrate (1 s/well). Each microplate was read five times.

2.4. Data analysis

Standards and samples were run in quadruplicate wells, and mean chemiluminescence intensity values were processed. Standard curves were obtained by plotting chemiluminescence intensity against the logarithm of analyte concentration and fitted to a four-parameter logistic equation using either Origin (Microcal, USA) or Sigmaplot (Jandel Scientific, Germany) software packages

$$y = \{(A - D)/[1 + (x/C)^B]\} + D$$

where A is the asymptotic maximum (chemiluminescence intensity in the absence of analyte, RLU_{max}), B is the curve slope at the inflection point, C is the x value at the inflection point (corresponding to the analyte concentration that reduces RLU_{max} to 50%), and D is the asymptotic minimum (background signal).

2.5. Cross-reactivity studies

Inhibition curves with a set of pesticides or related compounds were executed in both optimized ELISAs and their I_{50} values were compared to those from a standard curve for DDT run in the same plate. Cross-reactivity (CR) was calculated as follows:

$$CR = 100 \times I_{50}(p,p' - DDT)/I_{50}(cross - reactant)$$

2.6. Physicochemical parameter influence and optimization

Several physicochemical factors influencing immunoassay performance were studied in both ELISAs. Modifications of RLU_{max} and I_{50} parameters of the standard curves were evaluated under different conditions.

2.6.1. Tween-20 and BSA concentrations

Competitive assays were performed using different concentrations of Tween-20 and BSA separately. Briefly, standard analyte curves were prepared in double-distilled water while MAbs were added to serial dilutions of BSA (from 0.8% to 0%, w/v) or Tween-20 (from 0.1% to 0%, v/v) in PBS. Thereafter, assays were executed as described above.

2.6.2. Buffer ionic strength and pH

Competitive curves were performed with buffers of different ionic strength but at constant pH (7.4). Standard analyte curves were prepared in double-distilled water, and a constant concentration of MAbs was added to serial dilutions (from 10 × PBS to 0) of a concentrated buffer in double-distilled water. Thereafter, assays were executed as described above. To evaluate the influence of pH, competitive analyte curves were prepared in double-distilled water and a constant concentration of MAbs in 1 × PBS at different pH values covering the range 4.7–10.5 was added.

2.6.3. Studies of solvent effects

The assays tolerance to methanol, ethanol, acetone, and DMF was evaluated between 0% and 60% solvent concentration (v/v). Standards curves of DDT were prepared in double-distilled water, and MAbs were dissolved in PBS containing different amounts of solvent.

2.6.4. Spiked water sample analysis

Water samples were fortified with DDT to evaluate potential matrix effects on ELISA. Mean chemiluminescence intensity values obtained from quadruplicate wells were interpolated in a standard curve run in the same plate. The water samples tested were the Bologna tap water, a commercial bottled water, and wastewater collected in Emilia–Romagna region, Italy. Turbid water samples were filtered prior to use. Samples were spiked to 0, 0.1, 0.5 and 1.0 μg/l from 1 mg/ml DDT stock solution.

2.6.5. Spiked soil and food analysis

Samples kindly provided by ARPA (Regional Agency for Environment and Prevention, Bologna, Italy) were used: food extracts in methanol and lyophilized samples of fish (commercially available baby's food) and soil. Samples were fortified in ARPA with different amounts of *p*,*p*′-DDT, *o*,*p*′-DDT, *p*,*p*′-DDE, *p*,*p*′-DDD and other organochlorine pesticides (α- and β-endosulfan, endosulfan ether, endosulfan sulphate, heptachlor, aldrin, dieldrin, endrin, lindane, hexachlorane, α- and β-hexachlorocyclohexane). Five hundred grams of vegetables samples (strawberry, salad, beet root) were homogenized with a mixer (5 min) and added with 5 ml of acetone containing the listed pesticides to obtain concentrations ranging from 0.03 to 2.53 mg/kg. Samples were left with agitation for 30 min and finally lyophilized and stored at 4 °C. Ten grams of soil and fish lyophilized samples were added with 50 ml of acetone and subjected to agitation for 30 min while adding acetone containing the listed pesticides (0.03–2.53 mg/kg). Samples were then lyophilized and kept at 4 °C.

Lyophilized samples were extracted with methanol according to the following procedure. Fortified samples (0.1 g) were suspended in 1 ml of methanol and shaken for 30 min. The extracts were left to settle for 16 h. The organic layer was isolated by centrifugation for 5 min at 2056 × *g*. Finally, extracts were dissolved with double-distilled water (1:100, v/v) prior to use in the immunoassays.

2.6.6. Fish meat analysis

Barbel fish (*Barbus barbus*) from the Elbe river (Czech Republic) was analyzed by both immunoassay and chromatographic methods. Thirty five grams of homogenized tissue (anhydrous sodium sulphate added for desiccation) were extracted for 8 h in a Soxhlet apparatus using 340 ml of a hexane–dichloromethane mixture (1:1, v/v). After solvent evaporation, approx. 300 mg of isolated lipids were dissolved in 4 ml of chloroform. A 2-ml aliquot was then purified by gel permeation chromatography (GPC) employing a Bio Bead SX-3 column (500 × 8 mm) and chloroform as a mobile phase (flow rate 1 ml/min). Fractions corresponding to the elution volume 13–33 ml were collected. Chloroform was evaporated under vacuum and the residue was dissolved in methanol prior to dilution (1:4 with dilution buffer). Purified extracts were then analyzed.

2.6.6.1. Analysis of fish meat samples by gas chromatography (GC-ECD). Using the procedure of Hajslova et al. (1993), a HP 5890 series II gas

chromatograph (Agilent, Waldbronn, Germany) equipped with two parallel capillary columns (DB-5 and DB-17, 5% and 50% fenyl-methylpolysilicon, respectively, both 60-m column length, 0.25 mm I.D., J&W Scientific, USA) and 63 Ni-electron-capture detectors (ECD) (Hewlett-Packard, USA) was used for the analysis of chloroform extracts obtained as above. Samples (1 μl) were injected splitless (splitless time 2.5 min). Helium was used as carrier gas (30 cm/s). The following temperature program was applied: 60 °C (hold time 2 min), then at 30 °C/min to 220 °C, at 0.5 °C/min to 240 °C, and at 2.5 °C/min to the final temperature 280 °C (hold time 10 min). The detector was operated at 300 °C with nitrogen (50 ml/min) as make-up gas.

For results comparison, the values obtained by GC-ECD were recalculated on the basis of CR values of DDT isomers and metabolites as follows:

$$\begin{aligned}\text{GC}-\text{ECD recalculated (mg/kg)} &= M(p,p-\text{DDT})\times \text{CR}(p,p-\text{DDT}) \\ &+ M(o,p-\text{DDT})\times \text{CR}(o,p-\text{DDT}) \\ &+ M(p,p-\text{DDE})\times \text{CR}(p,p-\text{DDE}) \\ &+ M(o,p-\text{DDE})\times \text{CR}(o,p-\text{DDE}) \\ &+ M(p,p-\text{DDD})\times \text{CR}(p,p-\text{DDD}) \\ &+ M(o,p-\text{DDD})\times \text{CR}(o,p-\text{DDD})\end{aligned}$$

where M (analyte) is the value obtained by GC-ECD (mg/kg) and CR (analyte) is relevant cross-reactivity (in %/100).

3. Results and discussion

In a previous paper (Abad et al., 1997), the synthesis of haptens and the production of high-affinity monoclonal antibodies for DDT and related compounds were described. In order to improve the sensitivity of the colorimetric ELISAs based on these reagents, the enhanced luminescence reaction (ECL) was adapted as an end-point detection system.

The introduction of chemiluminescent detection in ELISA leads to the utilization of nontransparent plates. Since the adsorption capacities of different plates may be different, the reagent concentrations should be readjusted to achieve the best immunoassay performance. Using a checkerboard titration, the optimum concentrations of the immobilized conjugate and monoclonal antibodies that gave the highest sensitivity (lower I_{50} value) toward *p,p′*-DDT were found. The ability of the chemiluminescent technique to detect lower concentrations of HRP allowed the optimal antibody (30 μg/l for both LIB-DDT5-25 and LIB-DDT5-52) and conjugate (0.5 mg/l) concentrations to be decreased, compared to colorimetric ELISA for DDT (1 mg/l of OVA-DDT5; 100 and 200 μg/l for LIB-DDT5-25 and LIB-DDT5-52, respectively) (Abad et al., 1997). These concentrations were used in subsequent experiments.

3.1. Physicochemical parameter optimization

The application of ELISA to environmental samples requires consideration to be given to several experimental factors such as salt concentration, pH, and the presence of surfactants and solvents affecting the performance of the immunoassay (Manclús and Montoya, 1996). The $\text{RLU}_{\text{max}}/I_{50}$ ratio has been shown to be a useful parameter with which to estimate the effect of a certain factor on the ELISA performance, the highest ratio indicating the highest sensitivity (Mercader and Montoya, 1999).

3.1.1. Effect of Tween-20 and BSA concentrations

Because inert proteins (such as BSA) and surfactants (such as Tween-20) are commonly used in ELISA to reduce nonspecific interactions, their influence on assay performance (I_{50}, RLU_{max}, and $\text{RLU}_{\text{max}}/I_{50}$) was examined in both CL-ELISAs. Tween-20 concentrations higher than 0.05% reduced RLU_{max}, whereas I_{50} increased with lower Tween-20 concentrations (0.005%). RLU_{max} was considerably reduced with low concentrations of BSA. Under these conditions, I_{50} values did not change significantly. Fig. 2A shows the variation of the $\text{RLU}_{\text{max}}/I_{50}$ ratio as a function of the concentration of additive. It was observed that Tween-20 and BSA had similar effects on both ELISAs: the $\text{RLU}_{\text{max}}/I_{50}$ ratio values were higher at the lower concentrations of additive. Therefore, the use of Tween-20 as well as BSA should be limited to a minimum in these ELISAs.

3.1.2. Effect of buffer ionic strength and pH

Both ion concentration and pH may affect ELISA performance. Because antigen-antibody binding is

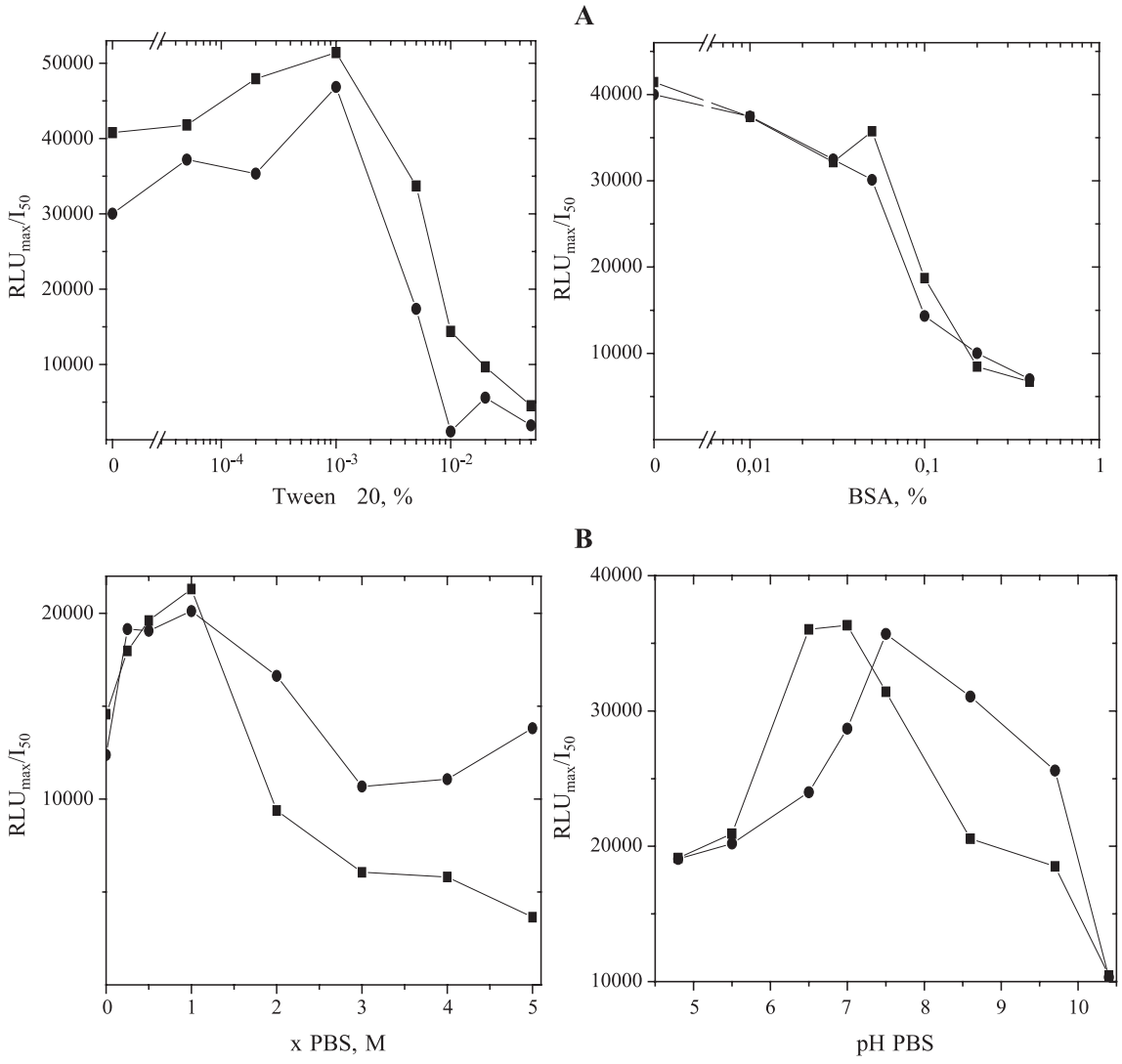

Fig. 2. Representation of the influence of (A) Tween-20 and BSA and (B) salt concentration and pH on the RLU_{max}/I_{50} ratio for each ELISA: (■) LIB-DDT5-25 MAb; (●) LIB-DDT5-52 MAb. Data were obtained from standard curves performed in triplicate.

characterized by weak intermolecular bonds, a change in either ionic strength or pH could affect this interaction (Schneider et al., 1995), especially in the case of monoclonal antibodies with isoelectric points (p*I*) between 5 and 7 (Selby, 1999). It was observed that salt concentration had a similar effect on both ELISAs. By increasing the ionic strength, the recognition of the conjugated hapten (RLU_{max}) diminished, while the recognition of *p*,*p*′-DDT (I_{50}) was improved. As shown in Fig. 2B, the RLU_{max}/I_{50} ratio increased with salt concentrations up to 1 × PBS. This salt concentration was selected for the buffer of the competition step in both ELISAs. The effect of ionic strength on both ELISAs can be related to the high hydrophobicity of *p*,*p*′-DDT. Similar effects have been observed for other hydrophobic pesticides (Mercader and Montoya, 1999; Abad et al., 1999).

Changes in pH had different effects on the I_{50} values and RLU_{max} of the ELISAs. The I_{50} value of the DDT-selective assay (with LIB-DDT5 52 MAb)

did not change markedly up to pH 9.0, whereas an increase of I_{50} was found in the class-selective ELISA (LIB-DDT5-25 MAb). RLU_{max} values also showed a maximum over different pH ranges: 7.5–9.5 and 6.5–7.5 for LIB-DDT5-52 and LIB-DDT5-25, respectively. Fig. 2B shows the dependence of the RLU_{max}/I_{50} ratio on pH. This ratio reached a maximum at pH 6.5–7.5 for LIB-DDT5-25 and 7.5 for LIB-DDT5-52. Finally, for both ELISAs, a pH of 7.5 was chosen as optimum. Differences in the p*I* values of the MAbs could explain the observed behavior.

3.2. Organic solvent tolerance

The preparation of environmental samples for pesticide analysis often includes an extraction step with organic solvents. Four organic solvents were included in competitive assays of both optimized ELISAs to evaluate their tolerance from 5% to 60% solvent contents. RLU_{max} and I_{50} changes were investigated, and the results are shown in Fig. 3A and B. DMF up to 10% was the best tolerated solvent in both cases, although it caused some decrease of the RLU_{max}. In contrast, RLU_{max} was not markedly affected by methanol over the range 5–20%, but the loss of sensitivity was significant, especially in the case of LIB-DDT5-25 MAb (Fig. 3A). Ethanol and acetone were the worst tolerated solvents in both cases. In general, the DDT-selective assay (LIB-DDT5-52 MAb; Fig. 3B) was more tolerant to the presence of solvents than the class-selective assay (LIB-DDT5-25 MAb).

3.3. Cross-reactivity studies

The specificity of the ELISAs was evaluated using a wide range of compounds structurally related to DDT and other organochlorine agrochemicals (Table 1). As previously shown using a colorimetric ELISA (Abad et al., 1997), antibody LIB-DDT5-25 exhibited high cross-reactivity values for almost all DDT-related compounds, showing preferential recognition of *p*,*p*′-isomers. Therefore, it can be considered to be a class-selective antibody. On the other hand, LIB-DDT5-52 cross-reacted only with *p*,*p*′- and *o*,*p*′-DDT and methoxychlor, all containing the $-CCl_3$ moiety and is therefore a DDT-selective antibody. Dicofol and DDA, sharing polar groups, were not detected by any of the antibodies, thus suggesting that the chemical nature of substituents at the central carbon plays an important role in influencing antibody specificity. The *p*-chloro groups appeared to be non-important in analyte recognition in both ELISAs, because methoxychlor, which is an analogue of *p*,*p*′-DDT with *o*-methyl groups in place of the chlorine substituents on the aromatic rings, was detected in each assay with even greater sensitivity than *p*,*p*′-DDT itself. Perthane, which is identical to *p*,*p*′-DDD except for two *p*-ethyl groups, was detected by the class-selective assay (LIB-DDT5-25) but not by the DDT-selective assay (LIB-DDT5-52), indicating that the retention of trichlorethane structure of *p*,*p*′-DDT is critical for the binding with LIB-DDT5-52 MAb. None of the assays detected 2,4-D, hexachlorocyclohexane, cyclodiene insecticides and chlorosubstituted phenylureas (CR < 0.1%).

Results were also compared to those reported with polyclonal antibodies (Beasley et al., 1998). Rabbit polyclonal antibodies described in this work were obtained against hapten 3-[4-{1-(4-chlorphenyl)-2,2,2-trichlorethyl} benzoylamino] propanoic acid (hapten VI), which is similar but not identical to DDT5. Similar to our results and to those reported by Abad et al. (1997), these antibodies did not recognize haptens that were substituted through the central carbon atom and did not cross-react with dicofol and *p*,*p*′-DDA. However, in contrast to our findings, methoxychlor was not detected by these antibodies, and the authors concluded that the *p*-chloro group appeared to be very important in analyte recognition. The fact that the selectivity data did not agree at all was accounted for by the use of different antibody production methods.

3.4. Analytical parameters of the optimized DDT immunoassays

Final assay conditions are summarized in Table 2. Sensitivities, estimated as I_{50} values for *p*,*p*′-DDT, were 0.2 and 0.6 μg/l with curve slopes 1.0 and 1.1 for class-selective and DDT-selective ELISAs, respectively. Typical competitive curves for *p*,*p*′-DDT in both ELISAs are shown in Fig. 4. The linear working ranges determined as the concentration causing 20–80% inhibition of the maximal chemiluminescence intensity were 0.07–1.1 and 0.1–2.2 μg/l for class-

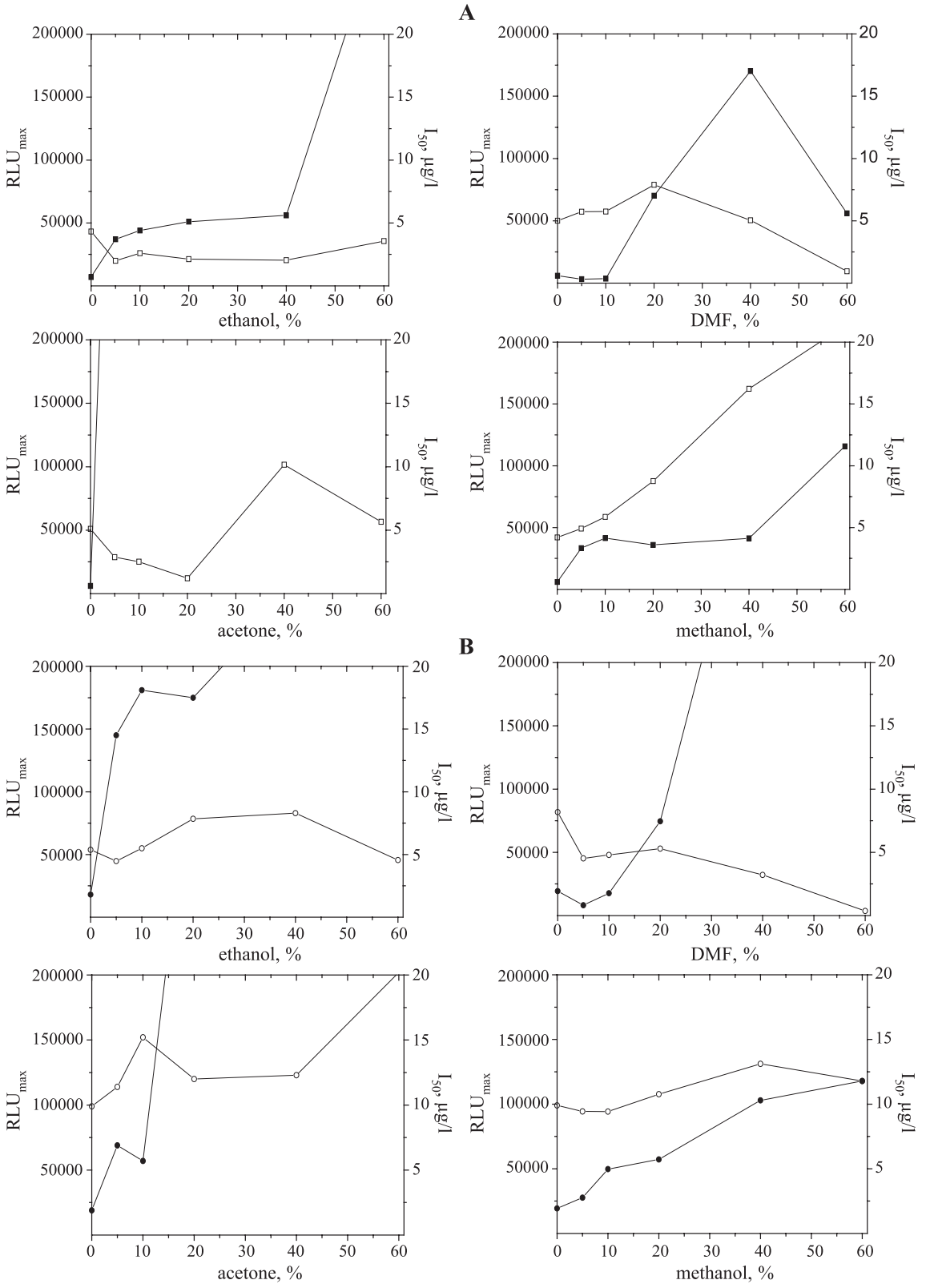

Fig. 3. Effect of the organic solvent concentration on the RLU_{max} (open symbols) and I_{50} (solid symbols) of *p,p′*-DDT standard curves for each ELISA: (A, ■) LIB-DDT5-25 MAb; (B, ●) LIB-DDT5-52 MAb. Data were obtained from standard curves performed in triplicate.

Table 1
Cross-reactivity (CR) of isomers and metabolites of DDT and related compounds in class-selective and DDT-selective ELISAs[a]

Compound	Class-selective ELISA (LIB-DDT5-25 MAb)		DDT-selective ELISA (LIB-DDT5-52 MAb)	
	I_{50}, μg/l	CR, %	I_{50}, μg/l	CR, %
p,p′-DDT	0.20	100	0.60	100
p,p′-DDE	0.22	92	20	3
p,p′-DDD	0.09	224	12	5
o,p′-DDT	0.33	61	0.48	124
o,p′-DDE	0.74	27	30	2
o,p′-DDD	0.25	81	15	4
p,p′-DDA	400	<0.1	600	<0.1
Dicofol	550	<0.1	780	<0.1
Methoxychlor	0.09	213	0.21	280
Perthane	0.12	166	550	<0.1

[a] Cross-reactivity values for 2,4-D, Endosulfan, α- and β-HCCH, and chlorosubstituted phenylureas were <0.1.

and DDT-selective ELISAs, respectively. Assay precision was evaluated by spiking double-distilled water with different concentrations of *p,p′*-DDT in the 10–90% inhibition range and determining the recovery in both assays on the same day (within assay) and on different days (between assay). Within- and between-assay coefficients of variation varied from 4.6% to 17.0%. The highest CV values were observed at the lowest concentrations of analyte.

Limits of detection (LODs) were experimentally determined as the *p,p′*-DDT concentration giving a 10% inhibition of the maximal chemiluminescence intensity. LODs for *p,p′*-DDT were established at 0.04

Table 2
Analytical characteristics of the ELISA procedures for DDT and related compounds

Characteristic	Class-selective ELISA (LIB-DDT5-25 MAb)	DDT-selective ELISA (LIB-DDT5-52 MAb)
Immunoreagent concentrations		
OVA-DDT5, μg/l	500	
MAb, μg/l	30	
Buffer conditions		
%Tween-20	0.001	
Salt concentration	5 mM phosphate, 150 mM NaCl	
pH	7.4	
Best tolerated solvent, %	*N,N′*-dimethylformamide (up to 10%)	
Analytical parameters of standard curve		
RLU_{max}	49 400	38 400
RLU_{min}	500	1200
I_{50}, μg/l	0.2	0.6
Slope	1.0	1.1
LODs		
CV, %	6.7–17.0	4.6–11.5
Concentrations giving		
20–80% inhibition, μg/l	0.07–1.1	0.1–2.2
10 % inhibition, μg/l	0.04	0.06

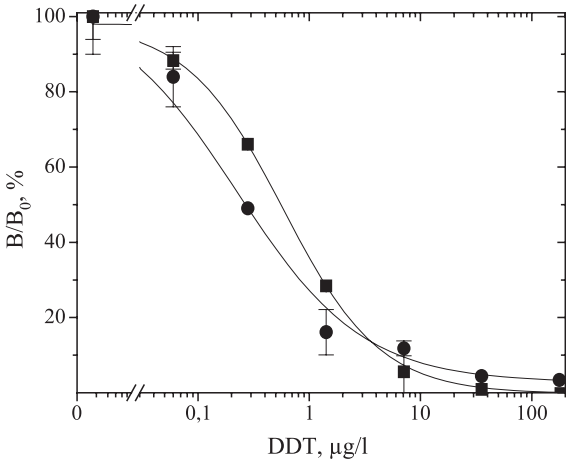

Fig. 4. Normalized standard curves for *p,p′*-DDT obtained under the optimized conditions with class-selective (■) and DDT-selective (●) ELISA. Each plot represents the mean ± S.D. of 10 independent curves run in triplicate wells. Competitive curves were fitted to experimental points by the four-parameter logistic equation.

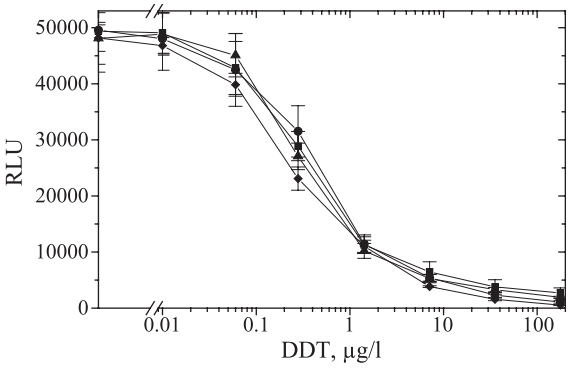

Fig. 5. Comparison of class-selective ELISA standard curves for *p,p′*-DDT obtained with spiked water samples: double-distilled water (■), tap water (□), bottled water (●), and wastewater (▲). Each plot represents the mean ± S.D. of two independent curves run in triplicate wells.

Table 3
Recovery of DDT from Spiked Water Samples (class-selective ELISA)

Water sample	DDT added, μg/l	DDT recovered, μg/l[a]	Recovery, %	CV, %
Bottled	0	–	–	–
	0.1	0.07	73	17
	0.5	0.38	75	9
	1	0.89	89	10
Tap	0	–	–	–
	0.1	0.12	115	8
	0.5	0.7	139	5
	1	1.11	111	13
Wastewater	0	–	–	–
	0.1	0.1	100	15
	0.5	0.4	70	11
	1	0.9	88	13

[a] Data listed are the mean of the six independent determinations.

and 0.06 μg/l for class-selective and DDT-selective assays, respectively. The sensitivity (I_{50}) reached by both immunoassays was higher by a factor of 4 compared to the values obtained for colorimetric ELISAs (Abad et al., 1997). The decrease of the I_{50} value for *p*,*p*′-DDT was probably a direct consequence of the decrease in the immunoreagent concentrations and of the accurate optimization of assay parameters.

3.5. Analysis of spiked water, soil, and food samples

The analytical performance of ELISAs is commonly assessed by spiking matrix samples with the target analyte. To study possible matrix interferences from different water types (tap, mineral and wastewater), standard curves were prepared in water samples and in double-distilled water as a control. As shown in Fig. 5 for the class-selective CL-ELISA, parallel calibration curves were obtained irrespective of the nature of the water sample. Results of the analysis, expressed as a percentage of recovery, are shown in Table 3. Added *p*,*p*′-DDT was accurately recovered (73–89%) in bottled water. For the wastewater sample, positive results were obtained when a blank was assayed probably indicating the presence of some interfering compounds in the sample. Probably for the same reason, spiked tap water samples gave rather high recovery values (111–139%). All CVs were acceptable, never being higher than 17%.

Both assays were next applied to the analysis of soil and several food samples. Two kinds of samples were used: fortified soil and food extracts in methanol and pesticide-free and fortified lyophilized samples of soil and fish. Lyophilized samples were extracted with methanol prior to use as described in Materials and methods. Initial examination of sample matrix effects was performed with pesticide-free extracts of lyophilized samples. *p*,*p*′-DDT standard curves produced in several dilutions of each sample extract (typically, 1/20, 1/50, 1/100, 1/200 and 1/400 prepared in 0.5 × PBS) showed that extracts diluted 1/100 and higher did not affect the chemiluminescence intensity and the sensitivity of the assays. Therefore, methanol extracts of both kind of fortified samples were diluted 1/100

Table 4
Recovery of DDT and related compounds from spiked food and soil samples

Sample	Class-selective ELISA (LIB-DDT5-25 MAb)			DDT-selective ELISA (LIB-DDT5-52 MAb)		
	DDT added, mg/kg	DDT recovered, mg/kg[a]	Recovery, % (CV, %)	DDT added, mg/kg	DDT recovered, mg/kg[a]	Recovery, % (CV, %)
Soil	0.59	0.81	136 (14)	0.88	1.00	114 (15)
Soil1[b]	0.03	0.02	81 (10)	0.04	0.04	119 (25)
Soil2[b,c]	0.98	0.85	87 (18)	–	–	–
Strawberry	1.76	1.99	108 (14)	2.34	2.24	96 (18)
Salad1	0.09	0.11	125 (26)	0.11	0.16	144 (12)
Salad2	0.08	0.08	88 (14)	0.15	0.13	90 (16)
Beet root	1.52	1.52	100 (23)	2.53	2.68	106 (17)
Fish[b]	0.12	0.07	54 (14)	0.18	0.12	70 (17)

[a] Data listed are the mean of the six independent determinations.
[b] Extract of lyophilized samples.
[c] Only *p*,*p*′-DDE.

before analysis. Using class-selective and DDT-selective chemiluminescent ELISAs, recoveries between 54% and 136% and between 70% and 144%, respectively, were obtained for samples spiked with mixtures of DDT-related compounds and other organochlorine pesticides (Table 4). These high recoveries for both types of samples indicated that the extent of pesticide removal did not depend upon the manners of fortification. In both cases, the higher spiked concentrations were closer to 100% recovery, with the lowest spiked concentrations being overestimated as well as underestimated. It should be noted that the lowest recoveries were observed with fish sample due to its fat content and high fat solubility of DDT-related compounds.

Samples of barbel fish (*B. barbus*) from the Elbe river were finally analyzed by both immunoassay and chromatographic methods (Hajslova et al., 1993) (see Table 5). The GC-ECD method allowed us to obtain individual values for each of the DDT isomers and metabolites present, while with immunoassay, only summa DDT was measured. In order to compare the results obtained, the values obtained by GC-ECD were recalculated on the basis of CR values of DDT isomers and metabolites. In this way, the results obtained by both methods were found to be in good agreement, showing that the chemiluminescent ELISA can detect, both qualitatively and quantitatively, the presence of these pesticides in fish.

Table 5
Analysis of barbel fish samples by immunoassay and gas chromatography-ECD methods

Sample	GC-ECD recalculated[a], mg/kg	Value obtained by ELISA[b]		ELISA/GC-ECD[c], %
		Average value, mg/kg	CV, %	
1	2.46	2.54	14	103
2	2.46	2.89	15	118
3	2.46	2.58	26	105
4	2.46	4.24	33	173
5	2.45	2.19	9	89
6	2.45	2.29	4	94
7	2.45	2.58	18	105
8	2.45	2.99	56	122

[a] Values obtained by GC-ECD (summa DDT) were recalculated on the basis of CR values of DDT isomers and metabolites.
[b] n (number of analysis) = 5.
[c] Value obtained by ELISA/GC-ECD recalculated.

4. Conclusions

A number of antibodies have been developed for the analysis of p,p'-DDT and its derivatives and metabolites (Abad et al., 1997; Banerjee et al., 1996; Beasley et al., 1998; Giraudi et al., 1998). These have been applied in different immunoassay techniques. Competitive immunoassays were mainly developed with colorimetric detection, and 0.12 ng/ml was the lowest detection limit obtained (Giraudi et al., 1998). More recently, two fluorescent polarization immunoassays were developed by Eremin et al. (2002), obtaining limits of detection of 12 and 3 ng/ml for the DDT specific and the DDT-class specific assay, respectively.

In European countries, very low residue limits are set for pesticides in foods, from 50 to 10 μg/kg in baby food (EC Pesticides MRLs in and on cereals, foodstuffs of animal origin and certain products of plant origin, including fruit and vegestables, 1976, 1986, 1990, 2000), and in water (EC Drinking water quality directive, 1980, 1998). Therefore, sensitive methods are needed. Chemiluminescent detection appears to be an effective analytical technique for use in ecotoxicological monitoring due to its high sensitivity and ease of handling. No chemiluminescent ELISAs for DDT have been published till now. In the current study, the sensitivity of the ELISAs was improved and reagent concentrations were lowered with respect to colorimetric detection (Abad et al., 1997) by introducing an enhanced chemiluminescence (ECL) reaction as the end-point detection system. We have already reported on the development of ECL immunoassays for DDT and related compounds, in which a flow system was employed (Botchkareva et al., 2002). Although the resulting flow assays had LODs of 1.1 and 0.4 ng/ml with DDT-selective and class-selective MAbs, respectively, its application for the quantification of DDT in real samples was limited by the short lifespan of the flow immunosupport. In the present study, we adapted the ECL detection to the development and optimization of sensitive ELISAs based on the same monoclonal antibodies (MAbs). For the DDT-selective assay, the sensitivity, estimated as the I_{50} value, was 0.6 ng/ml, with a linear working range between 0.1 and 2 ng/ml and a limit of detection of 0.06 ng/ml. For the DDT group-selective assay, the sensitivity was 0.2 ng/ml, with a linear working range between 0.07 and 1 ng/ml and a limit of detection of

0.04 ng/ml. CL-ELISAs were about four times more sensitive compared to colorimetric ELISAs (Abad et al., 1997).

Optimized CL-ELISAs were applied to different sample matrices. DDT-related compound residues in tap, mineral and wastewater could be quantitatively analyzed directly without the need for sample clean-up. Soil and food extracts proved to be difficult matrices for analysis. However, a simple method involving the dilution of sample extracts with the assay buffer allowed soil and food samples to be analyzed using standard curves prepared in double-distilled water.

Luminescent detection proved to be quicker and to possess lower detection limits than the colorimetric one. Also, the consumption of immunoreagents was lower, but black microplates are more expensive than colorimetric ones. Therefore, the chemiluminescent class-selective and DDT-selective ELISAs presented here are very sensitive compared to the methods found in the literature and they could be employed for the analysis of DDT and related compounds in environmental and food samples.

Acknowledgements

This work was supported by EC INCO-COPERNICUS Grant ERBIC-15CT961001. The authors acknowledge CIRB (Centro Interdipartimentale per le Ricerche Biotecnologiche), the University of Bologna, and MIUR (Ministero Istruzione, Università e Ricerca Scientifica, projects of National Interest). S.A. Eremin thanks for the Fellowship of the Italian Ministry of Foreign Affairs organized by the Landau Network-Centro Volta.

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Příloha 3:

Manclus J.J., Abad A., Lebedev M.Y., Mojarrad F., Mičková B., Mercader J.V., Primo J., Miranda M.A., Montoya A.: Development of a monoclonal immunoassay selective for chlorinated cyclodiene insecticides. *J. Agric. Food Chem.* 52(2): 2776-2784, 2004.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Development of a Monoclonal Immunoassay Selective for Chlorinated Cyclodiene Insecticides

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Organochlorine pesticides still generate public health concerns because of their unresolved health impact and their persistence in living beings, which is demanding appropriate analytical techniques for their monitoring. In this study, an enzyme-linked immunosorbent assay based on monoclonal antibodies (MAbs) for the detection of an important group of organochlorine pesticides, the cyclodiene group, has been developed. With this aim, several hapten−protein conjugates, characterized by exposure of the common hexachlorinated bicyclic (norbornene) moiety and differing in the linking structure to the carrier protein, were prepared. From mice immunized with these conjugates, several MAbs with the ability to sensitively bind the majority of cyclodienes were obtained. Among them CCD2.2 MAb displaying the broadest recognition to cyclodiene compounds (endosulfan, dieldrin, endrin, chlordane, heptachlor, aldrin, toxaphene: I_{50} values in the 6−25 nM range) was selected for the assay. Interestingly, this MAb showed certain stereospecificity toward other polychlorinated cycloalkanes because the γ-isomer of hexachlorocyclohexane (lindane) was also very well recognized (I_{50} value of 22 nM). This immunoassay is potentially a very valuable analytical tool for the rapid and sensitive determination of cyclodiene insecticides and related compounds, which in turn may contribute to the understanding of the biological activities and of the overall environmental impact of these persistent organic pollutants.

KEYWORDS: Organochlorine pesticides; cyclodiene insecticides; endosulfan; lindane; monoclonal antibodies; immunoassay; ELISA

INTRODUCTION

Beginning with their irruption in the 1940s, organochlorine pesticides (OCPs) were widely used in agriculture and malarial control programs with dramatic beneficial effects, but they have fallen into disuse because of their persistence in the environment. Chlorinated cyclodiene (CCD) insecticides are a group of OCPs that includes compounds such as endosulfan, heptachlor, chlordane, aldrin, endrin, and dieldrin. Their mode of action, toxicology, metabolism, and distribution have been extensively reviewed (*1*, *2*). Although to a different extent for each compound, CCD residues and their metabolites are prone to bioaccumulation and biomagnification (*3*−*6*). Moreover, some of these insecticides have been shown to have estrogenic activity (*7*, *8*), whereas synergistic estrogenic effects among them and with other environmentally relevant OCPs have also been observed (*9*). In other studies, the estrogenic activity of CCDs has not been demonstrated (*10*), nor can a direct relationship be established between environmental pesticide pollution, especially OCPs, and cancer (*11*). Due to their environmental impact, the use of CCDs on agriculture has been almost banned since the 1970s, but their residues are continuously detected in food and in the environment (*12*−*15*). Endosulfan is one of the few organochlorine insecticides remaining in widespread use, and its residues have been found throughout the world environment (*16*) and with a high frequency of occurrence in foods (*15*, *17*). The ubiquitous presence of CCD residues in the environment and the controversy related to their health impact on humans and wildlife pointed out the need for an adequate methodology for their environmental monitoring, as a tool to complement the great effort in epidemiologic and biological studies currently carried out.

Traditional methods for determining OCPs involve solvent extraction, liquid−liquid partitioning, and cleanup of the extract prior to identification and quantitative determination by chro-

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10.1021/jf035382h CCC: $27.50
Published on Web 04/20/2004

matography (*18*, *19*). Consequently, these methods are time-consuming, labor-intensive, and costly in terms of solvent use and disposal and require sophisticated equipment available only in well-equipped centralized laboratories. Therefore, chromatographic techniques are not suitable for the analysis of the large number of samples required for comprehensive monitoring studies. Immunochemical techniques have lately gained a position as alternative and/or complementary methods for the analysis of agrochemicals because of their simplicity, cost-effectiveness, and high sample throughput. Moreover, immunoassays (IAs) are field-portable and do not require sophisticated instrumentation. All of these features make IAs very valuable methods for large monitoring programs (*20*−*23*).

Immunoassay development requires the production of antibodies to the analytes and their incorporation into adequate assay formats, usually enzyme-linked immunosorbent assays (ELISAs). The successful generation of specific antibodies and sensitive assays to a small molecule is greatly dependent upon a proper design of immunizing and assay haptens. In this respect, it is still unpredictable how haptens are presented to the immune system, making it advisable to examine several haptenic structures (*24*, *25*). Particularly, detection of a group of compounds of similar structure can be often accomplished by judicious synthesis of hapten−protein immunogens to expose common features to all of the members of the group to the maximum while minimizing the presentation of structural differences to the immune system (*26*, *27*). Once analyte immunogens are prepared, the debate arises whether polyclonal or monoclonal antibodies are obtained. If an unlimited supply of a single and homogeneous type of antibody is required, the choice is monoclonal technology. Additionally, standardized immunoreagents may facilitate acceptance of immunoassays in the analytical laboratory by ensuring a long-term supply of kits with a defined performance (*21*, *28*).

Since the pioneering work of Langone and Van Vunakis (*29*), who designed a radio-immunoassay for dieldrin and aldrin, several works have been published reporting on the immunochemical detection of cyclodienes. Thus, using the aldrin derivative hapten described by these authors, an ELISA based on rabbit polyclonal antiserum was developed for detecting dieldrin/aldrin in dairy products (*30*). Polyclonal antibodies were also obtained from a haptenic structure of the endosulfan metabolite endosulfan diol and used in an immunoassay with a limit of detection for endosulfan of 3 ppb in environmental samples (*31*). The first monoclonal antibodies (MAbs) recognizing cyclodienes with different specificities were produced using an ether derivative of aldrin (*32*) and were used in pharmacological studies (*33*). Stanker et al. (*34*) obtained two MAbs derived from a hydroxyl−chlordene hapten, which bound all CCDs tested. Later, enzyme immunoassays for endosulfan and its metabolites based on rabbit polyclonal antibodies was reported (*35*). These authors explored several haptens to obtain appropriate antibodies and to develop immunoassays. Recently, a fiber optic immunosensor, which detects cyclodiene insecticides at the parts per billion level, has been described using rabbit polyclonal antibodies raised from a chlorendic caproic acid hapten (*36*). Several applications to food and environmental samples of commercial ELISA kits for cyclodiene insecticides have been reported (*37*−*39*).

In previous work carried out in our laboratory, IAs based on MAbs for DDT and related compounds were developed (*40*). In the present study, the development of IAs to another important persistent organochlorine pollutant group, CCD insecticides, was undertaken. As mentioned above, MAbs recognizing several of these insecticides have already been obtained, although they showed moderate affinity to the target compounds. Therefore, our main goal was to produce MAbs showing high affinity and selectivity to CCD insecticides. This work comprised the synthesis of several haptens sharing common structures of cyclodiene insecticides and differing in the linker moiety to carrier proteins, the production and characterization of MAbs, and the evaluation and optimization of different assay formats and conditions.

MATERIALS AND METHODS

Reagents and Instruments. Pesticide and metabolite standards were from Riedel-de Haën (Seelze, Germany) and Dr. Ehrenstorfer GmbH (Augsburg, Germany). Stock solutions were prepared in *N*,*N*-dimethylformamide (DMF, dried) and stored at −20 °C. Starting products for hapten synthesis and hapten−protein coupling reagents were from Fluka-Aldrich Química (Madrid, Spain). Analytical grade solvents and $CDCl_3$ were from Scharlau (Barcelona, Spain). Preparative layer chromatography was performed on 2 mm precoated silica gel 60 F_{254} from Merck (Darmstadt, Germany). Ovalbumin (OVA), Freund's adjuvants, Sephadex G-25, and *o*-phenylenediamine (OPD) were obtained from Sigma Química (Madrid, Spain). Bovine serum albumin (BSA) fraction V, enzyme-immunoassay grade horseradish peroxidase (HRP), Hybridoma Fusion and Cloning Supplement (HFCS), and poly(ethylene glycol) (PEG) 1500 were from Roche Applied Science (Heidelberg, Germany). Peroxidase-labeled rabbit anti-mouse immunoglobulins and goat anti-mouse immunoglobulins were obtained from Dako (Glostrup, Denmark). Culture media (high-glucose Dulbecco's modified Eagle's medium with Glutamax I and sodium pyruvate, DMEM), fetal calf serum (Myoclone Super Plus), and supplements were from Gibco (Paisley, Scotland). Culture plasticware was from Bibby Sterilin Ltd. (Stone, U.K.). Flat-bottom polystyrene ELISA plates (high-binding plates, catalog no. 3590) were from Costar (Cambridge, MA).

^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained with a Varian Gemini 330 spectrometer (Sunnyvale, CA), operating at 300 and 75 MHz, respectively. Chemical shifts are given relative to tetramethylsilane. Ultraviolet−visible spectra were recorded on a UV-160A Shimadzu spectrophotometer (Kyoto, Japan). ELISA plates were washed with a 96PW microplate washer from SLT Labinstruments GmbH (Salzburg, Austria), and absorbances were read in dual-wavelength mode (490−650 nm) with an Emax microplate reader from Molecular Devices (Sunnyvale, CA).

Hapten Synthesis. Most of the compounds used in this study present only minor safety concerns. However, it is advisable to work in a well-ventilated fume hood during synthesis work. CCD haptens synthesized for this study are depicted in **Figure 1**. The first reaction of hapten synthesis was, in all of the cases, a Diels−Alder addition of hexachlorocyclopentadiene and a particular alkene for each hapten. For haptens **CCD1** and **CCD4**, the alkene already contained a terminal carboxylic group used for protein coupling, whereas for haptens **CCD2** and **CCD3** the carboxylic group was introduced by succinylation of intermediate hydroxyl compounds.

*4,5,6,7,8,8-Hexachloro-3a,4,7,7a-tetrahydro-4,7-methanoindan-1-yl Acetic Acid (**CCD1**).* Hexachlorocyclopentadiene (2.72 g, 0.01 mol) and 2-cyclopentene-1-acetic acid (1.2 g, 0.01 mol) were placed in a round-bottom flask, heated to 130 °C, and kept at this temperature for 5 h. The crude product was left to reach room temperature, whereby white crystals appeared. They were filtered and recrystallized from dichloromethane: mp 165 °C; ^1H NMR ($CDCl_3$) δ 3.32 (q, 1H), 3.0−2.88 (dd, 1H), 2.67 (dd, 1H), 2.34 (dd, 1H), 2.26−2.14 (m, 1H), 2.04−1.88 (m, 2H), 1.50−1.34 (m, 2H); ^{13}C NMR δ 178.2, 131.7, 131.4, 105.1, 81.5, 81.3, 58.3, 53.9, 39.2, 37.0, 34.2, 25.3; elemental analysis for $C_{12}H_{10}C_{16}O_2$: C, 36.13; H, 2.52; Cl, 53.32; found: C, 36.07; H, 2.56; Cl, 53.30.

*4-(4,5,6,7,8,8-Hexachloro-3a,4,7,7a-tetrahydro-4,7-methano-1H-indenyl-1-oxy) 4-Oxobutanoic Acid (**CCD2**).* This hapten was synthesized as described by Stanker et al. (*34*). Briefly, the adduct obtained after Diels−Alder addition of hexachlorocyclopentadiene and cyclopentadiene was oxidized with SeO_2 to render 1-hydroxychlordene, which was converted into the hemisuccinate **CCD2** by reaction with succinic anhydride.

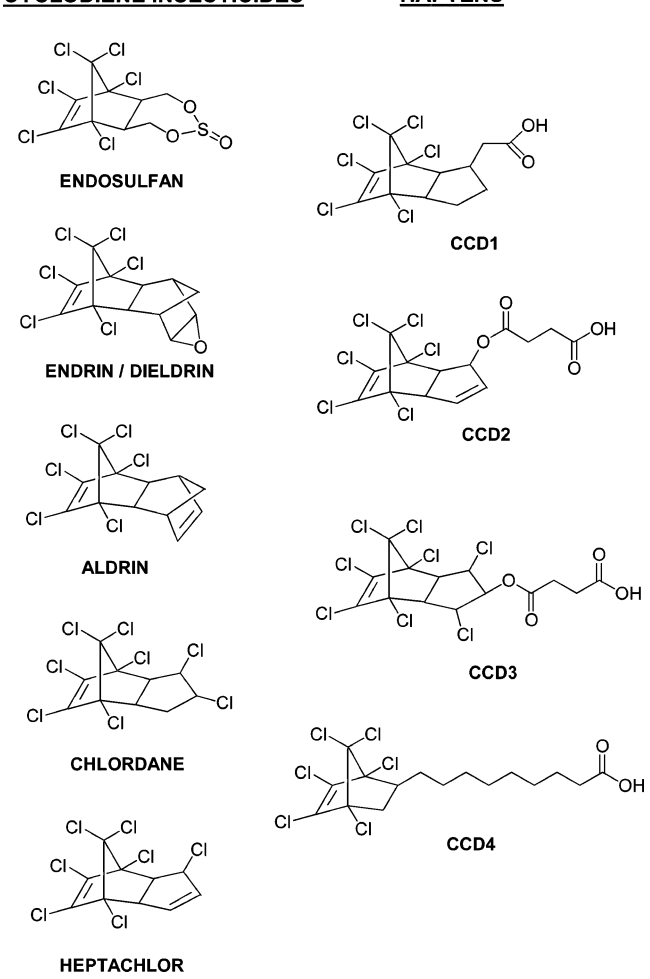

Figure 1. Structures of the chlorinated cyclodiene insecticides and of the haptens synthesized.

*4-(1,3,4,5,6,7,8,8-Octachloro-3a,4,7,7a-tetrahydro-4,7-methanoindanyl-2-oxy) 4-Oxobutanoic Acid (**CCD3**).* The synthetic procedure reported by Lee et al. (*35*) was followed. Briefly, the adduct 1-chlorochlordene was converted into the chlorohydrin of 1-chlorochlordane, which was succinylated.

*1,2,3,4,7,7-Hexachloronorbornen-5-yl Nonanoic Acid (**CCD4**).* Hexachlorocyclopentadiene (2.72 g, 0.01 mol) and 10-undecylenic acid (1.84 g, 0.01 mols) were placed in a round-bottom flask, heated to 130−140 °C, and kept at this range of temperature for 5 h. The crude was purified on silica gel preparative chromatography plates, using dichloromethane as eluant. The adduct was obtained as an oil: ^{1}H NMR ($CDCl_3$) δ 11.51 (bs, 1H), 2.78−2.70 (m, 1H), 2.62 (dd, 1H), 2.34 (t, 2H), 1.78−1.58 (m, 4H), 1.28 (bs, 10H), 0.94 (bs, 1H); ^{13}C NMR δ 180.5, 131.3, 130.4, 102.5, 82.6, 78.7, 47.4, 40.7, 34.0, 30.4, 29.3, 29.1, 29.0, 28.9, 27.3, 24.5; elemental analysis for $C_{16}H_{20}Cl_6O_2$: C, 42.04; H, 4.40; Cl, 46.54; found: C, 42.24; H, 4.41; Cl, 47.0.

Preparation of Protein−Hapten Conjugates. All haptens used in this study contained a free carboxylic group suitable to react with amine groups of proteins. Hapten−protein conjugations were carried out by the *N*-hydroxysuccinimide (NHS)-active ester method of Langone and Van Vunakis (*29*), with slight modifications.

Immunogenic and Coating Conjugates. Typically, haptens (~25 μmol in the appropriate volume of DMF to bring the final concentration of hapten to 100−200 mM) were activated during 2 h at room temperature with a 50% molar excess (molar ratio 1:1.5) of NHS and *N*,*N*′-dicyclohexylcarbodiimide. Next, the mixture was centrifuged and the supernatant collected. To a solution of 10 mg/mL protein (BSA for immunogens, OVA for coating conjugates) in 0.2 M borate buffer, pH 9.0, was added, over 10 min and with vigorous stirring, the activated ester mixture diluted in the volume of DMF necessary to bring the solution to 20% DMF. The initial hapten to protein molar ratios in the mixture were 50:1 for immunogens and 20:1 for coating conjugates. The mixture was stirred at room temperature for 2 h. Finally, conjugates were separated from uncoupled haptens by gel filtration on Sephadex G-25, using PBS (10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.4) as eluant. The degree of hapten conjugation to proteins, that is, the number of amino groups substituted with haptens in carrier molecules, was estimated by the determination of the number of free amino groups before and after conjugation, using trinitrobenzenesulfonic acid as the titration reagent (*41*). Apparent molar ratios in the range of 14−21 and 5−8, for BSA and OVA conjugates, respectively, were estimated.

Enzyme Conjugates. HRP was used to prepare the enzyme tracers. Following the same procedure as before, haptens were first activated and then conjugated to HRP (5 mg/mL) using a 20-molar excess of activated hapten. Enzyme tracers were purified by gel filtration and stored at 4 °C in a 1:1 mixture of saturated ammonium sulfate and PBS containing 0.1% BSA. HRP conjugate concentrations were estimated spectrophotometrically.

Production of Monoclonal Antibodies. *Immunization.* BALB/c female mice (8−10 weeks old) were immunized with BSA−**CCD1**, −**CCD2**, −**CCD3**, and −**CCD4** conjugates. The first dose consisted of 100 μg of conjugate intraperitoneally injected as an emulsion of PBS and complete Freund's adjuvant. At 2 and 4 weeks after the initial dose, mice received booster injections with the same amount of immunogen emulsified in incomplete Freund's adjuvant. One week after the last injection, mice were tail-bled and sera tested for anti-hapten antibody titer by indirect ELISA and for analyte recognition properties by competitive indirect ELISA. After a resting period of at least 3 weeks from the last injection in adjuvant, mice selected to be spleen donors for hybridoma production received a final soluble intraperitoneal injection of 100 μg of conjugate in PBS, 4 days prior to cell fusion.

Cell Fusion. P3-X63/Ag 8.653 murine myeloma cells (ATCC, Rockville, MD) were cultured in high-glucose DMEM supplemented with 2 mM L-glutamine, 1 mM nonessential amino acids, 25 μg/mL gentamicin, and 15% fetal bovine serum (referred to as s-DMEM). Cell fusion procedures were carried out essentially as described by Nowinski et al. (*42*). Mouse spleen lymphocytes were fused with myeloma cells at a 5:1 ratio using PEG 1500 as the fusing agent. The fused cells were distributed in 96-well culture plates at an approximate density of 2×10^5 cells/100 μL of s-DMEM per well. Twenty-four hours after plating, 100 μL of HAT selection medium (s-DMEM supplemented with 100 μM hypoxanthine, 0.4 μM aminopterine, and 16 μM thymidine) containing 2% HFCS (v/v) was added to each well. Half the medium of the wells was replaced by fresh HAT medium on days 4 and 7 postfusion. Cells were grown in HAT medium for 2 weeks, and then HAT was substituted by HT medium (HAT medium without aminopterine).

Hybridoma Selection and Cloning. Eight to 10 days after cell fusion, culture supernatants were screened for the presence of antibodies that recognized the analyte. The screening consisted of the simultaneous performance of a noncompetitive and a competitive indirect ELISA, to test the ability of antibodies to bind the OVA conjugate of the immunizing hapten and to recognize the analyte, respectively. Optimum conditions were pursued for the screenings. Thus, the coating conjugate concentrations were those selected when the analyte recognition by mouse sera was evaluated, and culture supernatants were appropriately diluted to obtain ELISA absorbance below 2.0. Selected hybridomas were cloned by limiting dilution using HT medium supplemented with 2% HFCS (v/v). Stable antibody-producing clones were expanded and cryopreserved in liquid nitrogen.

Purification of Monoclonal Antibodies. MAbs, all being of IgG class, were purified on a small scale directly from late stationary phase culture supernatants by saline precipitation with saturated ammonium sulfate followed by affinity chromatography on protein G−Sepharose 4 Fast Flow (Amersham Biosciences, Barcelona, Spain). Purified MAbs were stored at 4 °C as ammonium sulfate precipitates.

Enzyme-Linked Immunosorbent Assays. ELISA plates were coated overnight with conjugate or antibody solutions in 50 mM carbonate buffer, pH 9.6. A volume of 100 μL per well was used throughout all assay steps, and all incubations were carried out at room

temperature. After each incubation, plates were washed four times with washing solution (0.15 M NaCl containing 0.05% Tween 20). Two basic formats were used depending on the assay component immobilized into the ELISA plates. In the conjugate-coated format, an indirect ELISA was used to estimate mouse serum antibody titers and for the screening of culture supernatants, and a competitive indirect ELISA was used for the study of antibody sensitivity and specificity to analytes. In the antibody-coated format, the specific antibody was coated directly or by using a capture auxiliary antibody, and competitive ELISAs were followed to evaluate the assay properties using different enzyme tracers. For competition assays, the concentrations of antibodies, hapten conjugates, or enzyme tracers were optimized by checkerboard titration. Competitive curves were obtained by plotting absorbance against the logarithm of analyte concentration. Sigmoidal curves were fitted to a four-parameter logistic equation (*43*), using Molecular Devices (Sunnyvale, CA) and Sigmaplot (Jandel Scientific, Weinheim, Germany) software packages.

Preparation of Standards. From a 100 mM stock in DMF, daily serial dilutions (factor 5, 10 dilutions) from 2.5 mM in organic solvent were made. From eight of these dilutions, eight standards were prepared by diluting 1/200 in PBS (6250, 250, 50, 10, 2, 0.4, 0.08, and 0.0032 nM in assay), using borosilicate glass tubes. Initially, 1,4-dioxane was the organic solvent used, but after assay conditions were optimized, DMF was finally selected.

Conjugate-Coated Format. Plates were coated with the selected concentrations of OVA−hapten conjugates. Then, serum, culture supernatant, or antibody dilutions in PBS were added and incubated for 1 h. Next, plates were incubated for 1 h with peroxidase-labeled rabbit anti-mouse immunoglobulins diluted 1/2000 in PBST (PBS containing 0.05% Tween 20). Finally, peroxidase activity bound to the wells was determined by adding the substrate solution (2 mg/mL OPD and 0.012% H_2O_2 in 25 mM citrate and 62 mM sodium phosphate, pH 5.35). After 10 min, the reaction was stopped with 2.5 M sulfuric acid, and the absorbance at 490 nm was read and recorded. For competitive assays, the procedure was the same except that after coating a competition step was introduced by adding 50 μL of the competitor followed by 50 μL of the appropriate concentration of antibody (serum, culture supernatant, or purified MAb).

Antibody-Coated Format. In this format, plates were coated with antibodies at the selected concentrations. Next, the competition was established for 1 h between analyte standards and the selected dilutions of enzyme tracers (hapten−HRP conjugates). Peroxidase activity was measured as above.

Indirect Antibody-Coated Format. The difference with the previous format was that plates were first coated with goat anti-mouse immunoglobulins at 2 μg/mL in carbonate buffer, followed by an incubation for 2 h with the specific antibodies at appropriate concentrations in PBST.

RESULTS AND DISCUSSION

Synthesis of CCD Haptens. It is well-known that antibodies elicited to haptenic conjugates show a preferential recognition to the part of the molecule that is furthest from the attachment site of the hapten to the carrier protein (*24*, *25*, *44*). As the final goal of this study was the production of MAbs capable of recognizing the majority of the cyclodiene insecticides, a hapten design addressed to expose a structure common to these compounds was followed. In this sense, all of these organochlorine insecticides share a characteristic hexachlorobicyclic (norbornene) moiety, which prompted us to synthesize a series of haptens bearing this bicyclic structure and differing in the spacer arm, containing suitable reactive groups for covalent linking to proteins. As can be seen in **Figure 1**, the linker moiety introduced for haptens **CCD1**, **CCD2**, and **CCD3** was a cyclopentane ring with a carboxymethyl substituent (hapten **CCD1**), two chlorines and a hemisuccinate spacer arm (hapten **CCD2**), or a double bond and a hemisuccinate group (hapten **CCD3**). The design of a simple hapten corresponding to a common substructure of the compounds may constitute a valuable approach to class-specific antibody generation (*27*). Thus, a distinctive possibility was provided by hapten **CCD4**, which contains a more flexible, simple spacer arm consisting of a long hydrocarbonate chain ($[-CH_2-]_{10}$). Theoretically, protein conjugates of these haptens should present the characteristic norbornene structure to the immune system.

Table 1. Properties of the Sera of Mice Immunized with Cyclodiene Haptens

	immunizing hapten[a]			
	CCD1	**CCD2**	**CCD3**	**CCD4**
serum titer[b]	1×10^5	5×10^4	1×10^4	
endosulfan I_{50}[c]	ni[d]	0.7 μM	ni	

[a] Representative sera obtained 1 week after the third booster injection of the respective BSA–hapten conjugate. [b] Serum dilution giving 3 times background absorbance in ELISA using homologous haptens. [c] Data obtained from competitive ELISAs performed with optimum concentrations of homologous OVA–hapten conjugates and serum dilutions giving absorbances around 1.0. [d] No inhibition up to 10 μM endosulfan.

Production of Antibodies to CCD Insecticides. *Mouse Polyclonal Response.* To examine the suitability of the synthesized haptens to raise anti-CCD antibodies, mice were immunized with BSA conjugates of haptens **CCD1**, **-2**, **-3**, and **-4**. After the third injection, mouse sera were characterized for the presence of antibodies recognizing the conjugated immunizing haptens (serum titer) and for their ability to bind endosulfan (a representative of the cyclodiene insecticide family), by estimating their I_{50} value. Results of the characterization are summarized in **Table 1**. Serum titers (serum dilution giving 3 times the background absorbance) were estimated by indirect ELISA using the homologous OVA−hapten conjugates. Mouse sera obtained from BSA−**CCD1**, **-2**, and **-3** showed high levels of polyclonal antibodies recognizing each respective homologous hapten conjugate, with titers ranging from $1/10^4$ to $1/10^5$. Surprisingly, the immunization with BSA−**CCD4** did not raise an anti-hapten response. Next, the ability to recognize endosulfan was evaluated by competitive indirect ELISA. Only sera from mice immunized with BSA−**CCD2** bound competitively endosulfan, with an I_{50} of 0.7 μM, but curves showed shallow slopes as well as no complete inhibition of antibody binding (data not shown). No inhibition was found for the rest of the sera with endosulfan up to 10 μM.

Previous works to raise antibodies to members of the cyclodiene family have been carried out using haptens contemplated in this study. Lee et al. (*35*) obtained a high-titer polyclonal response in rabbits, by immunizing with protein conjugates of haptens **CCD2** and **CCD3**. These antibodies were capable of recognizing endosulfan with high affinity. Not surprisingly, considerable differences in the polyclonal response to the same immunogen between rabbits and mice has been previously found (*45*, *46*). In terms of affinity, rabbit polyclonal antibodies are undoubtedly superior to those of mice, but the reason for immunizing mice is mostly for applying the monoclonal antibody technology, as in this work. On the other hand, despite the fact that hapten **CCD4** appeared to be an ideal candidate to obtain antibodies recognizing the cyclodiene compounds because it contains just the common norbornene structure and a simple spacer arm, its protein conjugate was not able to raise anti-hapten antibodies. As suggested by Fasciglione et al. (*47*), this haptenic structure may be not exposed but rather hidden in the three-dimensional structure of the carrier protein.

Table 2. Summary of the Results of Cell Fusions and Hybridoma Selection

immunizing hapten	fusion	no. of wells seeded	positive[a] (hapten)	competitive[b] (analyte)	no. of cloned hybridomas[c]
CCD1	1	192	5	1	
	2	192	20	2	
	3	672	8	1	
	4	672	24	7	3
CCD2	5	864	78	63	4
	6	960	25	1	
	7	768	307	9	3
CCD3	8	768	4		
	9	768	18		
	10	768	15		

[a] Wells with antibodies that recognized the OVA–hapten conjugates (homologous assays) by indirect ELISA (absorbance > 0.5). [b] Wells with antibodies that recognized free endosulfan (inhibition > 50% by 1 μM endosulfan for fusions 1–5 and by 100 nM endosulfan for fusions 6–10). Homologous competitive ELISAs were carried out with the OVA–hapten concentrations previously selected for evaluating mouse sera. Culture supernatants giving absorbances out of range were diluted until absorbance < 2.0. [c] Hybridomas secreting antibodies with the lowest I_{50} for endosulfan were stabilized and cloned.

Production of MAbs. Fusions were undertaken from all mice that responded with an adequate level of anti-hapten antibodies, that is, mice immunized with BSA−**CCD1**, **-2**, and **-3**. Screenings of fusion cultures were performed using optimum coating concentrations (homologous conjugate-coated ELISA format) in simultaneous noncompetitive and competitive assays. Endosulfan was selected as competitor because it is still in use, and therefore it has a particular environmental and food relevance. Results of hybridoma selection are summarized in **Table 2**. As shown, all of the fusions rendered wells with antibodies recognizing the corresponding homologous conjugated haptens (positive wells), although with a wide range of yields, and a few of them were found to be competitive (inhibition > 50% by 1 μM endosulfan). Thus, four fusions were needed to clone and stabilize competitive hybridomas from hapten **CCD1**. The first fusion from mice immunized with hapten **CCD2** rendered a high yield of competitive wells, from which four were selected on the basis of their highest recognition of endosulfan. Thereafter, the endosulfan concentration cutoff used to screen culture supernatants was lowered to 0.1 μM to look for higher affinity monoclonal antibodies. Finally, after two additional fusions, three new cell lines were obtained that fulfilled the latter condition. With regard to hapten **CCD3**, no competitive well (cutoff, 0.1 μM endosulfan) was found after three fusions.

With the hybridoma production and selection procedure performed from haptens **CCD1**, **-2**, and **-3**, best results were obtained with hapten **CCD2**, which is in accordance with its best polyclonal response. Using this hapten, one MAb with moderate affinity was obtained by Stanker et al. (*34*) after five independent fusions.

Characterization of the MAbs. MAbs produced by each of the 10 selected hybridomas were small-scale purified from culture supernatants and were subsequently characterized for affinity and selectivity to the members of CCDs using homologous and heterologous haptens in different ELISA formats.

Affinity. The ability to recognize endosulfan was first estimated using homologous conjugate-coated ELISA format. After adequate assay concentrations were selected, I_{50} values for endosulfan in the 72−250 and 8−22 nM range were obtained for MAbs derived from haptens **CCD1** and **CCD2**, respectively (**Table 3**). All of the MAbs derived from hapten **CCD2** displayed a higher affinity to endosulfan than those from **CCD1**. Next, antibodies were assayed in heterologous assays using all of the haptens synthesized in this study. Haptens **CCD1** and **CCD2** were appropriately recognized, although heterologous competitive assays gave similar I_{50} values rather than homologous ones. In contrast, hapten **CCD3** was poorly recognized, and competitive curves were obtained only with half the MAbs derived from hapten **CCD2**. In any case, I_{50} values of heterologous assays did not get any affinity improvement. As for **CCD4**, there was no antibody recognition at all, confirming the folding-back of this haptenic structure in the carrier protein.

Table 3. I_{50} Values (Nanomolar) for Endosulfan Obtained with Different Haptens and Formats[a]

MAb	conjugate-coated ELISA (OVA–hapten) CCD1	CCD2	CCD3	antibody-coated ELISA (HRP–hapten) CCD1	CCD2	CCD3
CCD1.1	78	pr[b]	nr[c]	84	81	nr
CCD1.2	250	244	pr	241	650	341
CCD1.3	72	122	pr	300	168	nr
CCD2.1	16	18	pr	pr	45	nr
CCD2.2	9	10	nr	nr	nr	nr
CCD2.3	11	9	12	nr	nr	nr
CCD2.4	11	13	14	pr	54	nr
CCD2.5	10	8	ni[d]	13	29	nr
CCD2.6	17	18	nr	nr	nr	nr
CCD2.8	20	22	nr	133	46	pr

[a] Competitive ELISAs were performed in optimum conditions, i.e., limiting concentrations of immunoreagents giving maximum absorbance around 1.0. **CCD4** hapten conjugates were not recognized. [b] Poor recognition. [c] No recognition. [d] No inhibition up to 0.4 μM endosulfan.

The competitive behavior of MAbs in the direct and indirect antibody-coated formats was also evaluated. As shown in **Table 3**, the recognition pattern of immobilized antibodies (antibody-coated format) is clearly different from that of antibodies in solution (conjugate-coated format) and is characterized by a much lower degree of HRP−hapten recognition. Indeed, HRP tracers of haptens **CCD1** and **CCD2** were recognized by all of the CCD1 MAbs and by approximately half of those derived from **CCD2**, whereas HRP−**CCD3** and −**CCD4** were almost unrecognized. In all of the cases, competitive curves obtained with immobilized antibodies provided higher I_{50} values (less sensitive assays) than those in the conjugate-coated format. Moreover, MAbs indirectly immobilized using goat anti-mouse IgG precoated plates did not significantly modify either the recognition pattern or the assay sensitivity (data not shown).

Selectivity. MAbs showing the highest affinity for endosulfan (I_{50} around 10 nM, MAbs CCD2.2, -2.3, -2.4, and -2.5) were further characterized by performing competitive curves using other members of the cyclodiene insecticide family and other organochlorine compounds as competitors. Relative cross-reactivity (CR) data for each compound are shown in **Table 4**. With regard to endosulfan derivatives, all of the MAbs showed stereospecificity toward β-endosulfan, and endosulfan sulfate and endosulfan diol were predominantly better and less recognized than endosulfan, respectively. As expected, all of the CCDs assayed were recognized, although to different degrees for each MAb. Thus, whereas endosulfan, endrin, and dieldrin were recognized to a similar extent by MAbs CCD2.2 and CCD2.4, the recognition of chlordane, heptachlor, and aldrin was lower for all MAbs. Likewise, toxaphene, a complex mixture of compounds including many chlorinated norbornanes, showed a high CR (>100%). In contrast, the caged structure of the hexachloropentadiene dimer, mirex, was not recognized

Table 4. Selectivity of the MAbs to CCD Insecticides

compound	cross-reactivity[a] (%) CCD2.2 MAb	CCD2.3 MAb	CCD2.4 MAb	CCD2.5 MAb
endosulfan (mix)	100	100	100	100
α-endosulfan	82	55	84	18
β-endosulfan	114	168	114	211
endosulfan sulfate	219	181	63	222
endosulfan diol	54	9	104	18
dieldrin	109	34	109	72
endrin	113	59	133	19
chlordane	28	22	34	20
heptachlor	54	24	53	15
aldrin	47	21	39	14
toxaphene	112	101	120	110
mirex	<0.01	<0.01	<0.01	<0.01
γ-HCH (lindane)	30	2	12	10
α-HCH	14	3	4	3
pentachlorophenol	0.19	0.30	0.06	0.04
2,4,5-trichlorophenol	0.03	0.02	<0.01	<0.01
p,*p*-DDT	<0.01	<0.01	<0.01	<0.01
4,4′-PCB	<0.01	<0.01	<0.01	<0.01

[a] Percentage of cross-reactivity = (IC_{50} of endosulfan/IC_{50} of other compound) × 100. Values correspond to the average of three estimations. Competitive ELISAs in the conjugated-coated format were performed in the following conditions: plates were coated with OVA–**CCD1** at 1 μg/mL for MAb CCD2.2 and at 0.5 μg/mL for the rest; MAb concentrations were CCD2.2 (100 ng/mL), CCD2.3 (320 ng/mL), CCD2.4 (140 ng/mL), and CCD2.5 (350 ng/mL).

(CR < 0.01%). The selectivity pattern exhibited by these antibodies partially resembles that previously reported for antibodies raised from the same haptenic structure (*34*, *35*). Concerning the cross-reactivity toward other organochlorine compounds, the most remarkable finding is the notorious recognition of γ-hexachlorocyclohexane (γ-HCH, lindane) shown especially by MAb CCD2.2 (I_{50} = 25 nM for lindane, 30% CR). Again, the recognition of HCH compounds showed certain stereospecificity because the α-isomer CR was lower than that of the γ-isomer for MAbs CCD2.2, -2.4, and -2.5. On the other hand, the recognition of chlorinated compounds bearing aromatic rings diminished considerably. Thus, CR for polychlorinated phenols was <0.3%, whereas it was negligible for important organochlorine pollutants such as *p*,*p*-DDT and 4,4′-PCB (<0.01%). Considering all of this, it seems that MAbs recognition is directed to polychlorinated cyclic, not aromatic, hydrocarbons. Among them the CCD insecticides that contain the original hexachlorobicyclic immunizing structure are the best recognized compounds, but interestingly the γ-isomer of hexachlorocyclohexane is also fairly well recognized. Similar behavior in terms of polychlorocycloalkane functionality as minimum requirement has been found for MAbs derived from an aldrin derivative hapten, which also exposed the norbornene structure (*33*), but in this study I_{50} values were at least 10-fold higher.

Selection of Immunoassay Conditions. *Immunoreagents and Format.* From the characterization study, MAbs derived from hapten **CCD2** showed the highest sensitivity to endosulfan, and among them MAbs CCD2.2, -2.3, -2.4, and -2.5 gave I_{50} values around 10 nM endosulfan in both homologous and heterologous assays (**Table 3**). Hapten **CCD1** was selected as assay hapten because, giving similar behavior, it is easier to synthesize. On the basis of the selectivity data shown in **Table 4**, MAbs CCD2.2 and CCD2.4 recognized the highest number of members of the chlorinated cyclodiene insecticide family, and between them MAb CCD2.2, giving slightly higher sensitive assays, was selected. Moreover, this MAb had the added value

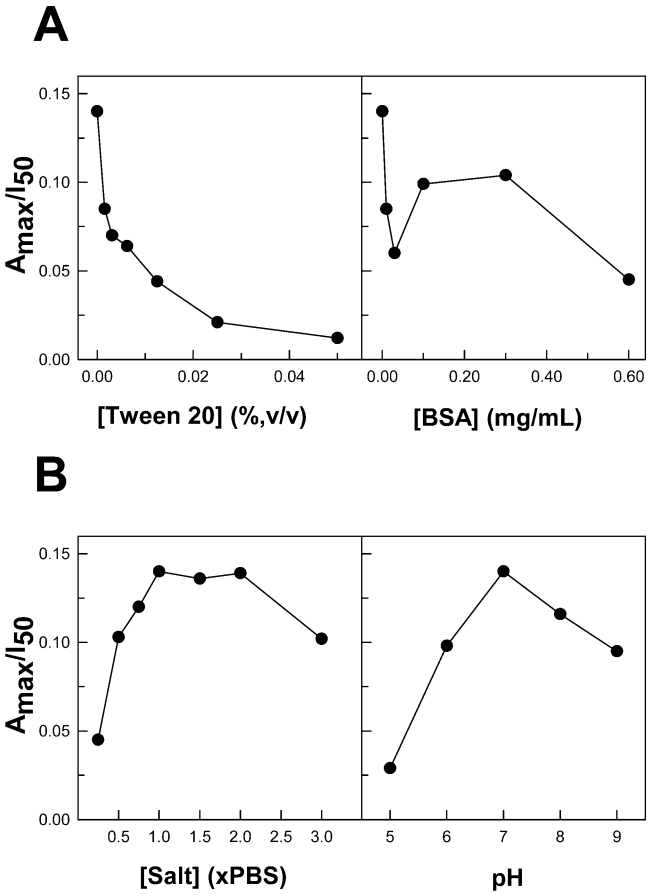

Figure 2. Influence of the assay buffer composition on immunoassay parameters: (**A**) buffer additives, BSA and Tween 20; (**B**) physicochemical conditions, salt concentration, and pH.

of recognizing sensitively lindane, a pollutant for which an efficient analytical methodology in the environment had been long demanded. Referring to the assay format, as MAbs immobilized did not provide useful immunoassays, the conjugate-coated format was hereinafter followed.

Assay Buffer Composition. Once the specific components of the immunoassay were selected (MAb CCD2.2; OVA−**CCD1** as assay conjugate), the influence of several physicochemical properties of the medium on assay characteristics was investigated, to optimize the buffer components. First, the nonionic surfactant Tween 20 and BSA are two additives commonly used in ELISA to reduce nonspecific interactions, but IA characteristics can greatly change as a function of their concentration (*35*, *48*, *49*). To study their influence, competitive curves were obtained in the presence of different additive concentrations. Then, curve parameters were plotted as the A_{max}/I_{50} ratio, which is a convenient estimate of the effect studied on ELISA sensitivity, the higher ratio indicating the higher sensitivity (*48*). **Figure 2A** shows the variation of this ratio as a function of additive concentration. The addition of Tween 20 to the assay buffer of the competitive step affected notoriously the curve parameters—the lower its concentration, the higher the assay sensitivity. A similar tendency was observed for BSA addition. Therefore, optimum assay sensitivity required not to add any of the additives studied.

Next, the influence of buffer composition, ionic strength, and pH on ELISA characteristics was examined. Thus, competitive curves were obtained using several dilutions of 10-fold-concentrated PBS as assay buffer. Likewise, the representation of the A_{max}/I_{50} ratio against the salt concentration was helpful

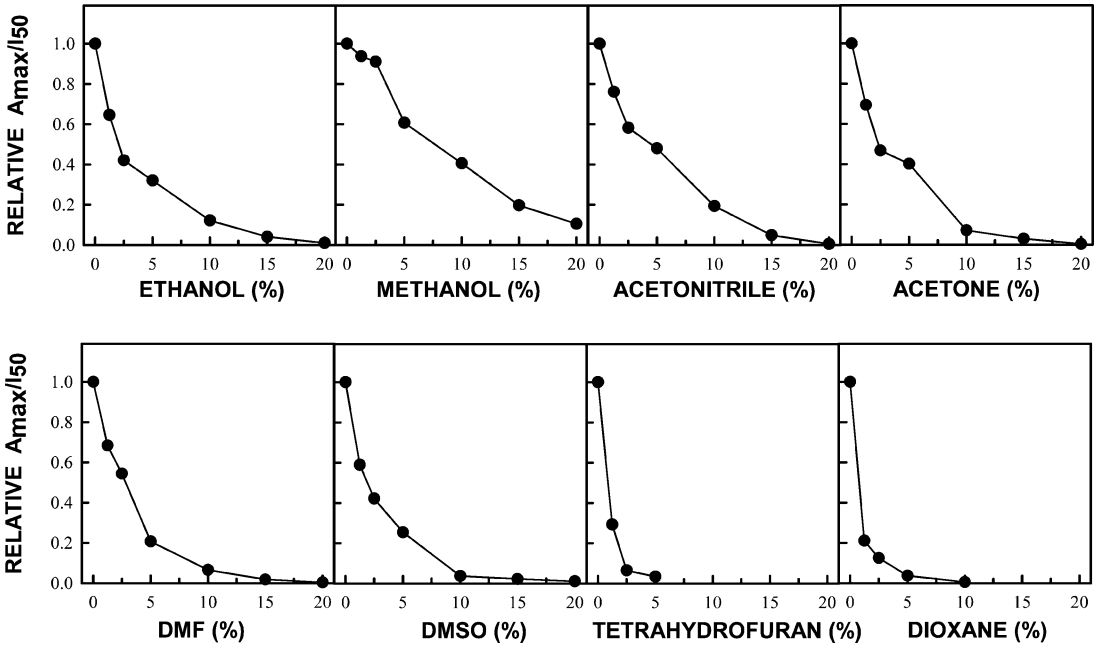

Figure 3. Effect of organic solvent concentrations in immunoassay parameters. Data were obtained from standard curves performed in triplicate.

in the selection of the optimum buffer. Immunoassay did not work in distilled water. As shown in **Figure 2B**, the ratio increased markedly as the salt concentration increased, reaching a plateau between 1 and 2 × PBS, and from here it decreased smoothly. A similar behavior has been found in our laboratory for ELISAs to other nonpolar analytes (*48*−*50*). Among the buffer conditions tested, the lowest salt concentration affording the highest A_{max}/I_{50} ratio was selected (1 × PBS). Finally, the variation of ELISA parameters within a range of assay buffer pH is also depicted in **Figure 2B**. The highest assay sensitivity was achieved at neutral pH, which is the pH routinely used for PBS.

Solvent Tolerance. Solvents are often used to extract analytes from samples, and then extract dilution is a simple, common practice to detect target analytes by ELISA. Moreover, in this work, with the aim of reducing the handling of aqueous solutions of nonpolar analytes to a minimum, intermediate standard dilutions were made in water miscible organic solvents. Therefore, it was imperative to examine how the immunoassay performed in the presence of solvents, to select the most appropriate ones to be used in extraction and in standard preparation. To carry out the experiments, competitive curves were performed by adding different solvent proportions to the assay buffer. Solvents evaluated were those that are water miscible, namely, methanol, ethanol, acetonitrile, acetone, DMF, dimethyl sulfoxide (DMSO), tetrahydrofuran, and 1,4-dioxane. In general, this immunoassay can be considered of low tolerance to the presence of solvents, because assay sensitivity, depicted in **Figure 3** as relative A_{max}/I_{50} ratio, dropped significantly even with solvent proportions as low as 1.25%. As often reported for pesticide immunoassays (*49*, *50*), methanol was the best-tolerated solvent, which might be the choice for analyte extraction from samples when required. For standard preparation, a solvent with a boiling point above 100 °C (DMF, DMSO, dioxane), thereby easier to pipet, was preferred. Initially, dioxane was used for this purpose, but as revealed in this study, this solvent was unfortunately the less tolerated one. Consequently, DMF and DMSO were evaluated as alternatives to dioxane. Standard curves obtained using DMF and DMSO yield assays slightly more sensitive (I_{50} = 7 nM to endosulfan), mainly because assay signal (A_{max}) increased in the presence of 0.25% of these solvents, thus allowing the reduction of MAb concentration necessary to give appropriate signals (absorbance ∼1). Finally, DMF was selected because it required a smaller amount of MAb in the assay (60 ng/mL).

A typical standard curve performed in optimum conditions is shown in **Figure 4**. Endosulfan can be determined in the competitive assay from 2 to 50 nM (20−80% inhibition), with an I_{50} value of 7 nM and a limit of detection (10% inhibition) of 1 nM. Taking advantage of the immunoassay selectivity, chlorinated cyclodiene insecticides can be analyzed with I_{50} values ranging from 6 to 25 nM and, very interestingly, lindane has an I_{50} value of 22 nM.

Conclusions. The goal of this work was the production of class-specific, highly sensitive MAbs and the development of IAs to chlorinated cyclodiene insecticides. This was accomplished by immunizing mice with BSA conjugates of several haptens characterized by presenting the hexachlorinated bicyclic moiety common to all of the cyclodienes and differing in the linking structure to the carrier protein.

After application of the hybridoma technology, four MAbs were selected and characterized in terms of affinity to endosulfan using several assay haptens and formats. In the conjugate-coated format, homologous haptens provided assays with I_{50} values of ∼10 nM, whereas the heterologous haptens used did not afford significant sensitivity improvement. The antibody-coated format was useless because most of the HRP−haptens were unrecognized. With regard to specificity, MAbs showed a broad recognition pattern of CCD insecticides. Among them, CCD2.2 MAb, displaying the highest recognition of these compounds, was selected for the assay. Furthermore, the selectivity of this MAb could be widened to hexachlorinated cyclic hydrocarbons because the γ-isomer of HCH was also very well recognized. This is particularly important because lindane, which is one of the few OCPs still used for animal husbandry and agricultural treatments, is one of the most frequently detected OCPs in total diet studies (*15*, *51*), and an efficient means of environmental monitoring for this pollutant has long been demanded.

Once assay immunoreagents were selected and characterized, the optimum buffer composition for the assay competition step

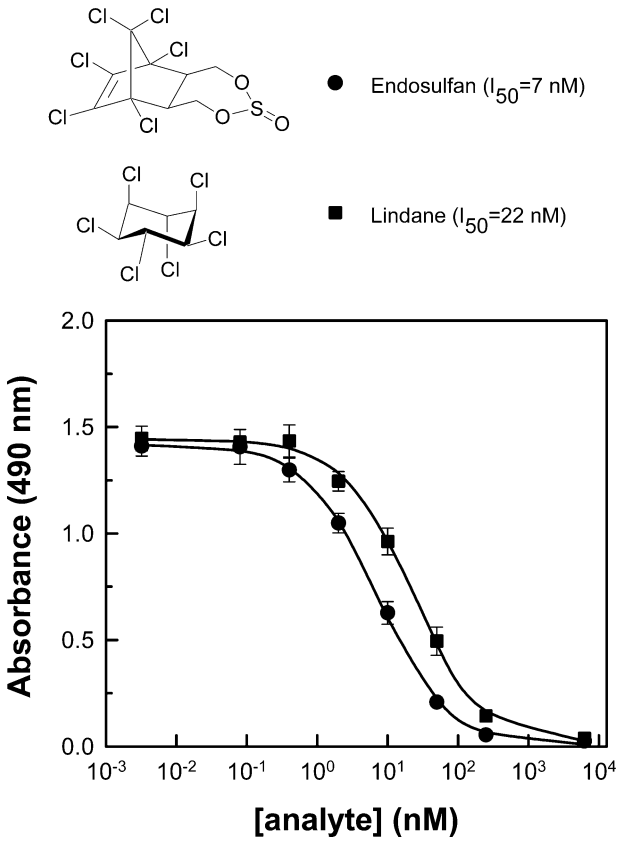

Figure 4. Representative standard curve for endosulfan and lindane obtained under optimized conditions: assay hapten, OVA–**CCD1** (1 μg/mL); CCD2.2 MAb (60 ng/mL); assay buffer, PBS containing 0.25% DMF. Each point represents the mean ± SD of three replicates.

was determined. The optimized immunoassay allows the sensitive detection of not only chlorinated cyclodiene insecticides (I_{50} values in the 6−25 nM range) but also lindane (I_{50} value of 22 nM). To the best of our knowledge, this is the immunochemical detection method of lindane with the highest sensitivity so far reported. In fact, lindane was not recognized in the highly selective polyclonal antibody-based immunoassay described by Lee et al. (*35*), and it was only weakly recognized (I_{50} value around 1 μM) in the monoclonal ELISA reported by Stanker et al. (*34*).

Organochlorine pesticides continue to be a constant source of concern because of their unresolved health impact and their persistence in living beings. Recently, debate has heightened concerning the link of these compounds to certain types of cancers or to endocrine-disrupting activity. As a complement of previous work in this laboratory with IAs to the DDT group, the selective and highly sensitive IAs to cyclodiene insecticides herein described will enable a more comprehensive monitoring of OCPs, which may be very helpful for understanding the biological activities and the overall environmental impact of these persistent organic pollutants.

ABBREVIATIONS USED

A_{max}, maximum absorbance; BSA, bovine serum albumin; CCDs, chlorinated cyclodienes; CR, cross-reactivity; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; HCH, hexachlorohexane; HFCS, hybridoma fusion and cloning supplement; HRP, horseradish peroxidase; IAs, immunoassays; MAb, monoclonal antibody; I_{50}, concentration giving 50% inhibition of maximum response; NHS, *N*-hydroxysuccinimide; NMR, nuclear magnetic resonance; OCPs, organochlorine pesticides; OPD, *o*-phenylenediamine; OVA,ovalbumin; PBS, phosphate-buffered saline; PBST, PBS containing 0.05% Tween 20; PEG, poly(ethylene glycol).

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Received for review November 24, 2003. Revised manuscript received March 10, 2004. Accepted March 10, 2004. Part of this work was supported by IMPIVA (Generalitat Valenciana, Spain), Project 89.93/1035.

JF035382H

Příloha 4:

Mičková B., Zrostlíková J., Hajšlová J., Rauch P., Moreno M.J., Abad A., Montoya A.: Correlation study of enzyme-linked immunosorbent assay and high-performance liquid chromatography/tandem mass spectrometry method for the determination of *N*-methylcarbamate insecticides in baby food. *Anal.Chim.Acta* 495(1-2): 123-132, 2003.

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Correlation study of enzyme-linked immunosorbent assay and high-performance liquid chromatography/tandem mass spectrometry for the determination of *N*-methylcarbamate insecticides in baby food

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Received 21 January 2003; received in revised form 22 July 2003; accepted 11 August 2003

Abstract

In this work, a correlation study of monoclonal antibody-based enzyme-linked immunosorbent assays (ELISAs) and a liquid chromatography–electrospray mass spectrometric (HPLC/ESI/MS/MS) method for the determination of *N*-methylcarbamate insecticides carbofuran, carbaryl and methiocarb in fruit baby food is presented. The comparison of performance characteristics of the two methods was carried out by simultaneous analysis of apple–strawberry baby food (GPC purified) extracts spiked with *N*-methylcarbamates at six different concentration levels. Results obtained by ELISA correlated well with those obtained by LC/MS/MS, both in terms of trueness and precision. Recoveries, i.e. the ratio of the determined concentration to the known spiked concentration, were in the 60–100% range for ELISA and in the 73–104% range by LC/MS/MS with the RSDs from seven replicate analyses 3.6–23.3 and 1.7–8.2%, respectively. The influence of sample pre-treatment on the analytical performance of immunoassay method was also assessed. Using ELISA recoveries close to 90% were obtained even in crude non-purified baby food extracts. The limits of detection (LODs) by ELISA were 0.3, 0.04 and 0.02 $\mu g/kg^{-1}$ for carbofuran, carbaryl and methiocarb, respectively, whereas using LC/MS/MS 1 μg/kg was the detection limit for all three insecticides. The results clearly indicate that the developed ELISA is suitable for the fast, quantitative and reliable determination of carbaryl, carbofuran and methiocarb in baby food even for the analysis of crude non-purified extracts.

Keywords: Correlation study; *N*-methylcarbamate insecticides; Pesticides residues; Baby food; ELISA; HPLC/MS/MS

1. Introduction

N-methylcarbamate insecticides such as carbaryl, carbofuran and methiocarb were introduced worldwide as substituents of persistent organochlorine compounds, due to their broad spectrum of activity and

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doi:10.1016/j.aca.2003.08.022

their low bioaccumulation potential. However, being inhibitors of acetylcholinesterase, they are regarded as toxic for the environment and for humans. As regards the potential risk arising from the use of pesticides, a vulnerable consumer's group are children. As shown by human exposure studies [1], dietary ingestion may be an important pathway to become exposed to pesticides, which is reflected in very low maximum residue limits set for baby food by the EU ($10\,\mu g\,kg^{-1}$).

The thermal instability of most *N*-methylcarbamates makes their sensitive and accurate determination by traditional gas chromatographic methods difficult. Their decomposition to degradation products—corresponding phenols—can occur during the GC separation or in the injection port of a gas chromatograph. Therefore, the preferred analytical technique for these pesticides is high-performance liquid chromatography employing ultraviolet (UV), diode array (DAD), fluorescence (FLD) or mass spectrometric (MS) detection [2–5]. The last mentioned method provides very low detection limits and superior specificity of detection, unless derivatisation of the analytes is needed.

Nowadays, with the increasing development of immunoassay methods for pesticide screening, it is also possible to quantify *N*-methylcarbamates at sub-ppb levels in food and environmental samples by these techniques [6,7]. Enzyme-linked immunosorbent assays (ELISA) have been found to be a simple and cost-effective alternative to instrumental analysis especially when large series of samples are to be analysed. Recently, immunoassays based on polyclonal antibodies have been developed and applied for the analysis of carbofuran and carbaryl in water and soil [8–10], in fruits and vegetables [11,12], in animal feed grains [13] and in meat and liver [14]. For carbofuran the analyses of non-fatty and fatty baby food have been reported. This study included the off-line coupling of supercritical fluid extraction (SFE) with ELISA [1,15,16].

Recently, indirect competitive ELISA format based on monoclonal antibodies for the determination of carbofuran, carbaryl and methiocarb has been developed by Montoya and coworkers [17–20] and the applications to the analysis of various fruits, vegetables [21], fruit juices [22] and water [23] have been reported.

In the study presented here, this monoclonal antibody-based ELISA format was applied for the determination of above pesticides in non-fatty baby food. As already mentioned, relatively strict regulation limits are set for pesticides in baby food commodities. Therefore, availability of rapid, sensitive and cheap analytical methods is highly desirable in this case. In the paper presented here, the performance of ELISA method is compared with liquid chromatography–electrospray mass spectrometric (HPLC/ESI/MS/MS) method. Apple–strawberry baby food was selected as a target matrix since both apples and strawberries represent important sources of baby food contamination by *N*-methylcarbamate insecticides.

2. Experimental

2.1. Chemicals and immunoreagents

For immunoassays, monoclonal antibodies (MAbs) specific for carbaryl, carbofuran and methiocarb, as well as the corresponding OVA–hapten conjugates, were prepared in the Laboratorio Integrado de Bioingeniería, Universidad Politécnica de Valencia, Spain. LIB–CNH45 MAb and OVA–2NAH conjugate were used for carbaryl analysis [23], LIB–BFNB67 MAb and OVA–BFNH conjugate were used for carbofuran analysis [19], and LIB–MXNB31 MAb and OVA–DPNH conjugate were used for methiocarb analysis [20]. Bovine serum albumin (BSA), Tween 20 and *o*-phenylenediamine (OPD) for ELISA colour development were obtained from Sigma Chemical Company (St. Louis, USA). Horseradish peroxidase (HRP)-labelled swine anti-mouse IgG (SwAM, HRP/SwIgG = 1.81, concentration $8.9\,mg\,ml^{-1}$) was obtained from Seva Pharma, Czech Republic.

Certified standards of carbaryl, carbofuran and methiocarb (purity 95–99%) were obtained from Dr. Ehrenstoffer, Germany. Individual stock standard solutions (concentration range $3.00–3.50\,mg\,ml^{-1}$) were prepared by dissolving 30–35 mg of neat pesticide standard in 10 ml of methanol. The mixed standard stock solution of all analytes (ca. $50\,\mu g\,ml^{-1}$ in methanol) was made by diluting these individual pesticide solutions. Stock solutions were stored at $+5\,^{\circ}C$ and used every day to prepare fresh mixed working standard solutions for ELISA and HPLC determinations. Pesticide residue grade solvents were

obtained from Scharlau, Italy (ethyl acetate) and from Merck, Germany (cyclohexane, methanol). Deionised water for mixing of a mobile phase was produced in Milli-Q apparatus (Millipore, Germany). Anhydrous sodium sulphate (Penta Chrudim, Czech Republic) was activated for 5 h at 450 °C.

Apple–strawberry baby food from a retail market was used for the preparation of samples.

2.2. Apparatus and instrumentation

Extractions of target analytes were performed using tissumiser Turrax (IKA Werke, Germany). All solvent reductions were performed on a Büchi rotary evaporator (Büchi, Switzerland). An automated high-performance gel permeation chromatography (HPGPC) system (Gilson, France) equipped with a PL gel (600 mm × 7.5 mm, 0.005 μm) column (Polymer Labs, UK) was used for clean-up of extracts.

Ninety-six-well ELISA polystyrene microplates Costar (catalog no. 9018) were from Corning, USA. ELISA plates were washed with Labsystem Multiwash, USA, and absorbances were read in Labsystem Multiscan MCC/340, Finland. Data were processed using Microsoft Excel software (Microsoft, USA).

HPLC separation was carried out using a HP1100 liquid chromatograph (Hewlett-Packard, USA). HPLC column Discovery C_{18} (15 cm × 3 mm, 5 μm) (Supelco, USA) with mobile phase methanol–water was used and the following linear gradient conditions: 0 min, 50% methanol; 7 min, 80% methanol; 7.2–13 min, 100% methanol. Analysis time was 20 min including the 7 min post-run, when column was conditioned at starting mobile phase composition. Flow rate was 0.5 ml min^{-1}, column temperature was 25 °C and the injection volume was 20 μl.

MS/MS analysis was performed by LCQ Deca ion trap instrument from Finnigan, USA. Electrospray ionisation (ESI) was applied in all experiments. The following experimental conditions were used: capillary temperature, 230 °C; flow rates of sheath gas and auxiliary gas, 1.5 and 3 l min^{-1}, respectively; spray voltage, 6 kV; capillary voltage, 11 V for carbofuran and carbaryl, 18 V for methiocarb. For MS/MS analysis time segments were set up and one analyte was scanned in each segment. All three *N*-methylcarbamates were monitored in positive ion mode with following parent → daughter masses used: carbofuran 222 → 165; carbaryl 202 → 145; methiocarb 226 → 169. Acquired data were processed by XCalibur software (Finnigan, USA).

2.3. Preparation of samples

2.3.1. Blank purified extract

The blank apple–strawberry baby food (25 g), in which the absence of *N*-methylcarbamate residues had been verified by LC/MS analysis, was mixed with 100 ml ethyl acetate and 75 g sodium sulfate and homogenised for 2 min by a Turrax tissumiser. The suspension was filtered under vacuum; the volume of filtrate was reduced by evaporation to 12.5 ml and made-up with cyclohexane in 25 ml volumetric flask. The crude extract was purified by high-performance gel permeation chromatography (HPGPC) under the following conditions—mobile phase: cyclohexane–ethyl acetate (1:1, v/v); flow: 1 ml min^{-1}; injection volume: 2 ml. The eluate within the range 14.5–30 ml was collected. After solvent evaporation in a rotary evaporator and removal of its traces under a mild stream of nitrogen, the residue was redissolved in 2 ml of a methanol–water (1:4, v/v) mixture and passed through a Millipore membrane filter. A volume of 14 ml of purified blank extract was obtained by pooling of seven redissolved fractions after HPGPC. This step was performed to ensure that the matrix composition was exactly the same for all the samples and matrix-matched standards for LC/MS/MS calibration.

2.3.2. Samples for correlation study

Baby food extracts spiked with *N*-methylcarbamate at 1, 5, 15, 50, 250 and 500 ng ml^{-1} were prepared as unknown samples (for details, see Section 2.5.2). An amount of 150 μl of the pesticide standard at the appropriate concentration was added to 2.85 ml of purified blank extract.

2.3.3. Non-purified baby food samples

Non-purified baby food samples were prepared by evaporation of aliquot part of crude extract of blank and redissolving the residue in methanol–water (1:4, v/v). Spiked samples at 10 and 250 ng ml^{-1} were prepared by adding 150 μl of the pesticide standard at the appropriate concentration level to 2.85 ml of this non-purified blank sample.

2.3.4. Matrix-matched standards for HPLC/MS/MS calibration

Matrix-matched standards for HPLC/MS/MS calibration were prepared from the same purified blank extract as the test samples (see above). Calibration solutions at concentrations of 1, 4, 10, 20, 100, 200 and 500 ng ml^{-1} were prepared by adding 100 μl of the appropriate standard solution to 1.9 ml purified sample extract.

2.4. ELISA determinations

Immunochemical determinations of *N*-methylcarbamates in samples were carried out by using three specific monoclonal immunoassays performed in the indirect competitive ELISA format on polystyrene microplates, with photometric detection at 492 nm. Samples were quantitatively analysed for a single pesticide in each plate, irrespective of the presence of the two other analytes. The ELISA conditions were exactly the same for all immunoassays and in fact they were performed simultaneously. Each ELISA plate included its own six-point calibration curve to determine *N*-methylcarbamate concentrations. The central section of the curve with a nearly linear response was accepted as the assay working range. Specific details about immunoreagent concentrations and sample dilutions applied in ELISA are summarised in Table 1, while the used assay working ranges are shown in Table 4. Working standard solutions for each immunoassay were prepared from the standard stock solution by serial dilution in phosphate-buffered saline (PBS, 0.14 M NaCl, 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4, 2.7 mM KCl, pH 7.4).

The immunoassays were accomplished as follows: polystyrene microplates were coated with the ovalbumin–pesticide conjugate solution in 0.05 M carbonate–hydrogen carbonate buffer, pH 9.6 (100 μl per well) and left to incubate overnight at laboratory temperature. Microplates were washed four times with PBS containing Tween 20 (0.05%, v/v). Then the aliquots (50 μl per well) of pesticide standards or sample extracts, diluted in PBS, and the aliquots (50 μl per well) of specific monoclonal antibodies, diluted in PBS containing 0.1% BSA (bovine serum albumin), were pipetted to the microplate wells coated with the conjugates. Microplates were incubated for 2 h at laboratory temperature and washed as described above. Subsequently, the second antibody conjugated with peroxidase (SwAM) was added (100 μl per well), left to interact for 1 h at laboratory temperature, and washed as described above. Then, the peroxidase substrate in reaction buffer (4.6 mM OPD in 0.1 M phosphate—citrate buffer, pH 5.0, containing 0.1% (v/v) of 30% H_2O_2) was added to each well (100 μl per well). After 10 min incubation at laboratory temperature, the enzyme reaction was stopped by adding 50 μl per well of 2.5 M sulphuric acid, and the absorbance at 492 nm was measured directly in the wells with the microplate reader. Absorbance values from standards were mathematically fitted to a four-parameter logistic equation. The analyte concentration in samples was determined by interpolation of their mean absorbance values on the resulting standard curve.

Table 1
Concentrations of immunoreagents and sample dilutions applied in the ELISAs for carbaryl, carbofuran and methiocarb

	Carbaryl	Carbofuran	Methiocarb
Concentration of antibody (μg ml^{-1})	LIB–CNH45: 0.06	LIB–BFNB67: 0.03	LIB–MXNB31: 0.06
Concentration of conjugate (μg ml^{-1})	OVA–2NAH: 0.25	OVA–BFNH: 0.06	OVA–DPNH: 0.5
Sample dilution (ng ml^{-1})			
1	1/20	1	1/5
5	1/25	2/5	1/25
15	1/30	1/15	1/30
50	1/100	1/50	1/100
250	1/500	1/250	1/500
500	1/1000	1/500	1/1000

2.5. Validation study

2.5.1. Determination of overall recovery

Baby food sample was spiked with a mixture of carbaryl, carbofuran and methiocarb at 50 $\mu g\,kg^{-1}$ of each pesticide (1 ml of the standard mixture at 1.25 $\mu g\,ml^{-1}$ was added into 25 g of sample). The extraction and clean-up of spiked samples were carried out according to the method described above. The recovery was determined in five replicates and the repeatability of recovery determination was calculated. HPLC/MS/MS method using matrix-matched standards was applied for quantitation.

2.5.2. Correlation study

To compare the analytical performance of the implemented immunoassay and the HPLC/MS/MS method, purified baby food extracts spiked at 1, 5, 15, 50, 250 and 500 $ng\,ml^{-1}$ with a mixture of carbaryl, carbofuran and methiocarb (see Section 2.3) were analysed by both methods. These testing samples were treated as if they were unknown samples, i.e. the con-

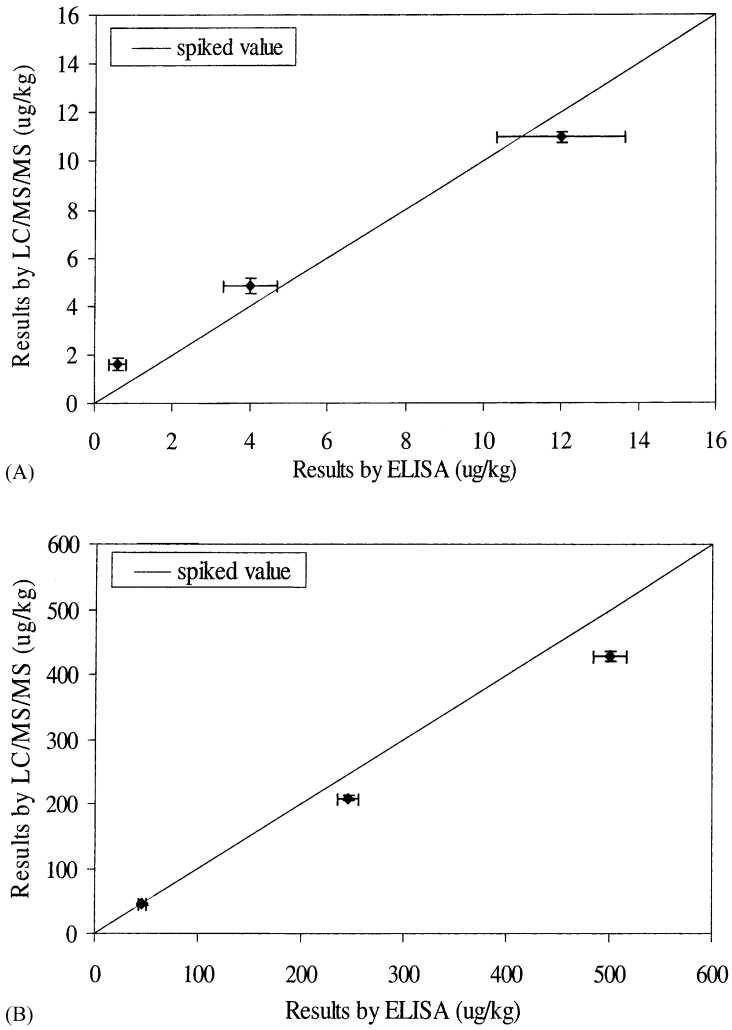

Fig. 1. Correlation between HPLC/MS/MS and ELISA values obtained for carbofuran ($n = 7$, $y = 0.85x + 1.83$, $r^2 = 0.991$) at (A) 1, 5 and 15 $ng\,ml^{-1}$, and (B) 50, 250, 500 $ng\,ml^{-1}$.

tent of *N*-methylcarbamates was quantified by using appropriate calibration procedure by both ELISA and LC/MS method. Whereas for ELISA standards in pure solvent could be used, for LC/MS matrix-matched standards were employed to compensate the so-called matrix effects occurring during LC/MS analysis [24,25]. Several different performance characteristics of both methods were evaluated and compared based on these results (see Section 3.2.).

When using ELISA, previous adjustment of the sample concentration to fit into the linear part of the calibration curve had to be carried out. It should be noted that further dilution of samples for immunoassay was necessary for another reason, i.e. the methanol content in the non-diluted target samples (methanol–water, 1:4, v/v) was too high for antibody–pesticide interactions. Therefore, in our experiments each primary test sample was subjected to seven independent dilutions and subsequently analysed by ELISA. The dilution factors applied for *N*-methylcarbamate analysis are shown in Table 1. Under this experimental setup, the precision parameter for ELISA comprised the uncertainty of both the sample dilution and the quantitative step. For LC/MS/MS, thanks to its much wider dynamic range, no sample dilution was necessary and therefore only the uncertainty of the quantitative step contributes to the results. This experimental approach aimed to reflect real-life situation in the analysis of baby food samples.

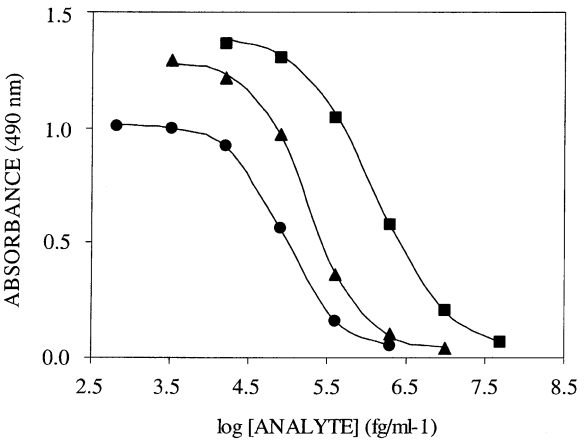

Fig. 2. Representative ELISA standard curves for carbofuran (■), carbaryl (▲) and methiocarb (●). Immunoassay conditions are described in Section 2.4.

The limit of detection (LOD) for ELISA was calculated as the analyte concentration that reduced absorbance to 90% of the maximum. For LC/MS/MS method the limits of detection were determined as a concentration corresponding to a signal to noise ratio of 3:1.

The confidence intervals in Figs. 1 and 2 were determined from the following equation:

$$\mu = \text{S.D.} \times \frac{t_{\text{crit}}(95)}{\sqrt{\text{number of analyses}}}.$$

3. Results and discussion

3.1. Overall recovery of the method

The suitability of the proposed extraction and clean-up procedures was evaluated on the basis of the overall recovery of the target analytes in samples spiked at 50 ng kg^{-1} (i.e. 50 μg ml^{-1}). Employing LC/MS/MS technique as the determination step, the recoveries of carbofuran, carbaryl and methiocarb were 76, 88 and 72%, with relative standard deviations (RSDs) of 13, 10 and 8%, respectively.

3.2. Correlation study

The experimental comparison of the analytical performance of ELISA and LC/MS/MS methods was carried out by simultaneous analysis of apple–strawberry baby food extracts spiked with *N*-methylcarbamates at six different concentrations: 1, 5, 15, 50, 250 and 500 ng ml^{-1} (i.e. 1, 5, 15, 50, 250 and 500 μg kg^{-1}). The lower part of this concentration range was chosen with respect to the maximum residue limit (MRL) for pesticides in baby food (10 μg kg^{-1}). The higher concentrations approximately correspond to the MRLs established for fruit and vegetables. In our experiments, the same analytical conditions as for routine unknown samples were applied.

For the purpose of methods comparison the following characteristics were assessed:

(1) Trueness—as the agreement of the measured concentrations in “unknown” samples with the spiked concentrations.

Table 2
Overview of the results obtained by analysis with ELISA and LC/MS/MS of strawberry baby food extracts spiked with carbaryl, carbofuran and methiocarb at 1, 5, 15, 50, 250 and 500 ng ml^{-1} ($n = 7$ replicates)

Analyte	Spiked level (ng ml^{-1})	ELISA			LC/MS/MS		
		Mean ± S.D. (ng ml^{-1})	Recovery (%)	RSD (%)	Mean ± S.D. (ng ml^{-1})	Recovery (%)	RSD (%)
Carbofuran	1	0.6 ± 0.2	60.0	40.0	1.6 ± 0.3[a]	160.0[a]	17.6[a]
	5	4.3 ± 0.8	86.0	17.6	4.8 ± 0.3	96.0	7.1
	15	12.3 ± 1.8	82.0	14.6	11.0 ± 0.2	73.3	5.8
	50	46.3 ± 3.9	92.6	8.4	45.0 ± 1.5	90.0	3.4
	250	246.0 ± 11.1	98.4	4.5	208.5 ± 3.8	83.4	1.8
	500	500.3 ± 18.1	99.9	3.6	428.7 ± 8.6	85.7	2.0
Carbaryl	1	1.0 ± 0.2	100.0	20.8	1.4 ± 0.3[a]	140.0[a]	18.7[a]
	5	5.0 ± 1.0	100.0	20.0	5.2 ± 0.4	104.0	8.2
	15	14.3 ± 1.0	95.3	6.7	13.6 ± 0.8	90.7	5.8
	50	46.9 ± 5.1	93.8	11.0	46.6 ± 1.6	93.2	3.4
	250	249.1 ± 12.3	99.6	4.9	243.8 ± 4.4	97.5	1.8
	500	484.4 ± 17.6	96.9	3.6	473.2 ± 8.2	94.6	1.7
Methiocarb	1	0.8 ± 0.2	80.0	27.6	1.2 ± 0.1[a]	120.0[a]	8.4[a]
	5	3.9 ± 0.9	78.0	23.3	4.9 ± 0.2	98.0	4.4
	15	13.9 ± 1.2	92.7	8.8	14.1 ± 0.7	94.0	4.7
	50	46.3 ± 5.0	92.6	10.8	50.8 ± 1.4	101.6	2.8
	250	234.9 ± 14.2	94.0	6.1	242.0 ± 5.3	96.8	2.2
	500	489.3 ± 32.0	97.9	6.5	452.3 ± 7.6	90.5	1.7

[a] Result at the detection limit of the method.

(2) Precision—as the relative standard deviation obtained from the analyses of seven replicate samples at each concentration.
(3) Limits of detection (LOD) and quantitation (LOQ). In addition, ELISA sensitivity was evaluated by means of I_{50} value (see below).

Table 2 summarises the repeatability and recovery values (the percentage ratio of ELISA or LC/MS/MS result to the spiked value) obtained. The term "recovery" discussed within this section should not be confused with the overall recovery of the extraction and clean-up method discussed in Section 3.1.

Irrespective of the spiked level, mean recovery values by immunoassay were 91.8, 97.6 and 89.2% for carbofuran, carbaryl and methiocarb, respectively. The RSDs for ELISA ranged from 3.6 to 27.6%. Nearly identical mean recoveries were obtained by LC/MS/MS (85.7, 96.0 and 96.2% for carbofuran, carbaryl and methiocarb, respectively) with lower RSDs ranging from 1.7 to 8.2%. The obtained recoveries of spiked samples indicate that no sample matrix problems or interferences occurred in the tested samples. For immunoassays, the 1 ppb concentration is within the linear working range of the carbofuran standard curve. The sample was therefore not diluted prior to ELISA, which resulted in a content of organic solvent higher than the optimal one. As a result, reduced carbofuran recovery value and higher RSD value (40%) was obtained. For LC/MS/MS, the lowest measured concentration corresponded to the LOD of the method (see below). Although relatively good results were obtained, in practice quantification should not be performed at levels lower than 4 ng ml^{-1}, which corresponds to the LOQ.

Fig. 1 shows the correlation analysis between the results obtained by ELISA and LC/MS/MS methods for carbofuran in spiked samples. For clarity reasons, the concentration range was divided into two charts for each pesticide. The values are presented with their corresponding confidence intervals. Similar results were found also for carbaryl and methiocarb. The linear regression equations for the whole concentration range were: $y = 0.85x + 1.83$, $y = 0.98x + 0.34$ and $y = 0.92x + 0.57$ for carbofuran, carbaryl and methiocarb, respectively. The excellent correlation

Table 3
Overview of the results obtained by analysis with ELISA of crude and purified extracts spiked with carbaryl, carbofuran and methiocarb at 10 and 250 ng ml^{-1} ($n = 7$ replicates)

Analyte	Spiked level (ng ml^{-1})	Crude extract			Purified extract		
		Mean ± S.D. (ng ml^{-1})	Recovery (%)	RSD (%)	Mean ± S.D.	Recovery (%)	RSD (%)
Carbofuran	10	8.5 ± 1.1	85.0	21.9	8.7 ± 0.8	87.0	14.6
	250	225.4 ± 35.6	90.1	14.4	199.1 ± 33.7	79.6	14.8
Carbaryl	10	9.5 ± 2.2	95.5	23.7	8.6 ± 1.4	86.4	16.6
	250	245.8 ± 20.7	98.3	8.8	216.6 ± 11.0	86.7	5.3
Methiocarb	10	9.6 ± 1.6	96.0	16.8	10.4 ± 1.3	104.4	12.7
	250	247.1 ± 24.6	98.8	10.3	253.1 ± 18.7	101.2	7.6

coefficients found (0.991, 0.999 and 0.998, respectively) indicate a high degree of correlation between both techniques.

The influence of sample pre-treatment on the analytical performance of immunoassay method was also assessed. For this experiment apple–strawberry baby food extracts spiked by *N*-methylcarbamates at two different concentrations (10 and 250 ng ml^{-1}, i.e. 10 and 250 μg kg^{-1}) were prepared in two duplicate sets—crude extracts and extracts purified by GPC. Table 3 summarises the obtained repeatability and recovery values. Good agreement between results obtained by analysis of crude extracts and purified extracts was found for carbofuran, carbaryl and methiocarb. The mean recoveries of carbaryl in crude and purified extracts were 96.9 and 86.6%, respectively, with RSDs ranging from 5.3 to 23.7%. The mean recoveries of methiocarb in crude extracts and purified extracts were 97.4 and 102.8%, respectively, with RSDs ranging from 7.6 to 16.8%. In the case of carbofuran the mean recoveries in crude and purified extracts were 87.0 and 84.0%, respectively, with RSDs ranging from 14.4 to 21.9%. As obvious from obtained results no significant influence of sample pre-treatment was observed for the mentioned carbamates.

The sensitivity of immunochemical methods is commonly expressed as a detection limit and by calculating the I_{50} value, i.e. the analyte concentration that decreases the assay signal to 50% of the maximum value. With respect to these criteria, the most sensitive of the three assays was ELISA for methiocarb, with an I_{50} value of 0.07 ng ml^{-1} (i.e. 0.07 μg kg^{-1}) and a detection limit of 0.02 ng ml^{-1} (i.e. 0.02 μg kg^{-1}). The carbaryl and carbofuran I_{50} values were 0.14 and 1.4 ng ml^{-1}, respectively. The detection limits for carbaryl and carbofuran were 0.04 and 0.3 ng ml^{-1}, respectively. For HPLC/MS/MS method the limits of detection for a calculated signal-to-noise ratio of 3 were 1 ng ml^{-1} (i.e. 1 μg kg^{-1}) for all three carbamates. In Table 4 linear ranges, LODs and I_{50} values of the compared methods are summarised. Fig. 2 shows the ELISA calibration curves for the three carbamates.

Table 5 gives an overview of time-demands for ELISA and LC/MS/MS methods. For LC/MS/MS ca. 40 min are required for the final analysis step. The ELISA technique usually includes overnight

Table 4
Comparison of linearity and limits of detection obtainable by ELISA and LC/MS/MS methods

	Linear range (ng ml^{-1})		Limit of detection (ng ml^{-1})		I_{50} (ng ml^{-1})
	ELISA	HPLC	ELISA	HPLC	ELISA
Carbofuran	10–0.4	500–4	0.3	1	1.4
Carbaryl	1.2–0.08	500–4	0.04	1	0.14
Methiocarb	0.4–0.02	500–4	0.02	1	0.07

Table 5
Comparison of time-demands for ELISA and LC/MS/MS methods

Analytical step	ELISA (min)	LC/MS/MS (min)
Extraction	15	15
Clean-up	35[a]	35[a]
Solvent exchange	10	10
Preparation of standards	10	20[b]
Dilution of samples	15	–
Quantitation step	240[c] (96 samples)	20 (1 sample)

[a] Can be omitted by ELISA.
[b] Matrix-matched standards are required for LC/MS/MS determination.
[c] Overnight incubation of the coating conjugate is not included.

incubation of coated microplate and thus requires a larger time of analysis. However, the problem of overnight incubation can be eliminated by storage of pre-coated plates in a refrigerator. In this way, the prepared plates are directly useable for analysis for 3 months. Using immunoassay, many samples can be analysed in each plate with minimum expense of reagents. Under the above described conditions, with a standard curve included in triplicate, 24 samples for one of mentioned pesticides can be determined in triplicate on each plate in less than 4 h. Considering the fact that four plates can be easily handled simultaneously, the number of samples handled per unit time is significant. Moreover, as discussed above, sample clean-up step can be omitted when using ELISA.

4. Conclusions

In the present work the potential of ELISA for the monitoring of selected pesticide residues in baby food samples was demonstrated. Immunoassay method provided excellent sensitivity and selectivity for the determination of carbaryl, carbofuran and methiocarb in baby food samples. Also a good correlation of results with the LC/MS/MS method has been proven in our experiments.

Whereas the initial price of an LC/MS/MS instrument is very high, ELISA technique can be performed at very low initial and operating costs. As regards time demands approximately 96 samples can be analysed in less than 4 h by ELISA, which considerably exceeds the time effectiveness of the LC/MS/MS method. In our study it has been demonstrated that practically no difference in the performance of ELISA exists between the analysis of purified and non-purified baby food samples. This is an additional advantage of the method, since the whole analytical procedure can be considerably simplified, which results in further savings in time and costs.

On the other hand, compared to LC/MS/MS, the number of compounds simultaneously analysed in one run is limited. However, the further development of multiresidue of immunoassay kits may overcome this particular disadvantage.

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Příloha 5:

Mičková B., Kovalczuk T., Rauch P., Moreno M. J., Abad A., Montoya A., Ferri E., Fini F., Girotti S.: Analytical performances of validated chemiluminescent enzyme immunoassays to detect *N*-methylcarbamate pesticides. *Anal. Chim. Acta* 528(2): 243-248, 2005.

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Analytical performances of validated chemiluminescent enzyme immunoassays to detect *N*-methylcarbamate pesticides

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Received 12 July 2004; received in revised form 24 September 2004; accepted 24 September 2004
Available online 8 December 2004

Abstract

In the present work, enzyme-linked immunosorbent assays (ELISAs) with chemiluminescent detection for the determination of carbofuran, carbaryl and methiocarb were developed and the analytical parameters of these assays were compared with those of ELISAs with colorimetric detection. Both were conjugate-coated formats based on identical monoclonal antibodies and homologous protein conjugates. In comparison with colorimetric ELISA, the ability of the chemiluminescent reagents to detect lower concentrations of horseradish peroxidase allowed to decrease the optimal antibody and conjugate concentrations and to reach better analytical parameters. The experimental comparison of the analytical performance of the ELISAs was carried out by analysing extracts of apple-strawberry baby food and simply diluted fruit juices, both spiked at different concentration levels with the above mentioned pesticides. Recovery values for both ELISAs were around 100% and no matrix effects were observed when fruit juices were diluted 1:20 or more. Results obtained by ELISAs correlated well, both in terms of accuracy and precision, with those obtained by a liquid chromatography–electrospray mass spectrometry (LC/ESI/MS/MS) analysis, used as reference method to validate the immunoassays results. The limits of detection reached by using the chemiluminescent assay were 0.03, 0.007 and 0.004 ng ml^{-1} for carbofuran, carbaryl and methiocarb, respectively.

Keywords: *N*-methylcarbamate pesticides; Chemiluminescent and colorimetric enzyme-linked immunosorbent assay; Liquid chromatography–electrospray mass spectrometry; Baby foods; Fruit juices

1. Introduction

N-methylcarbamates are an important class of pesticides now widely used in agriculture, instead of organochlorine pesticides, as insecticides, acaricides, nematocides and molluscicides for crop protection [1]. Carbaryl, carbofuran and methiocarb are the main compounds belonging to this class of pesticides that are acetylcholinesterase inhibitors; their residues may occur in fruits and vegetables and, therefore, pose a potential hazard for consumers [2]. As a result, international organisations relatively strict regulate maximum residue limits for pesticides in foods. Baby foods have a special status in legislative bodies to the issue of pesticides, since children are an extremely sensitive and vulnerable consumers group. In EU countries the total amount of pesticide residues in baby foods must be under the maximum residue limit of 10 μg kg^{-1} [3]. Because of the polarity and thermal instability of most of *N*-methylcarbamates, their determination by traditional gas chromatography (GC) techniques is difficult, then to detect the carbamates contamination the most widely used analytical technique is liquid chromatography (LC), using fluorescence, ultraviolet, diode array, and mass spectrometry detectors [4–7].

Liquid chromatography (LC) is a very sensitive and selective technique, but it involves some important drawbacks,

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doi:10.1016/j.aca.2004.09.066

such as the employment of complex and expensive instrumentation, highly qualified personnel, and time-consuming procedures for cleaning and concentrating the sample. All of these requirements often hinder the analysis of large number of specimens. The large scale monitoring activities imposed in several countries by environment concerned legislation requires the availability of rapid, sensitive and cost-effective analytical techniques [8]. Among others the immunoenzymatic assays aroused intense interest, complying with the characteristics mentioned above [9,10] and the enzyme-linked immunosorbent assay (ELISA) has been found to be a rapid, sensitive and cost-effective alternative to chromatographic methods [11]. Various polyclonal (PAb) or monoclonal antibody (MAb)-based assays have been developed to determine the carbamates presence in water and soil [12,13].

Colorimetric immunoassays for the determination of methiocarb, carbaryl and carbofuran in vegetables, contaminated water, fruits and fruit juices were previously developed. They demonstrated to be not only rapid and specific but also as suitable as the reference method for the quantitative determination of these compounds [14–17], which represents an analytical performance of remarkable importance.

The introduction of chemiluminescent (CL) reagents to reveal the formation of immunocomplexes in ELISAs already led to an improvement of the sensitivity, to a wider range of detected concentrations and to a lower consumption of immunoreagents when compared with the colorimetric endpoint detection [18,19].

The CL reagents, ready to use from commercial sources, are absolutely not toxic and the advantages related to the specific, sensitive and reliable immunoassays are further enhanced by the cheapness, simplicity, availability of portable and automatable of the instrumentation required for the luminescent detection.

Recently, we reported the application of colorimetric, MAb-based ELISAs for the determination of carbofuran, carbaryl and methiocarb in apple-strawberry baby foods [16]. In the present study, with the aim of demonstrating the possibility to perform very sensitive ELISAs of these *N*-methylcarbamates in non-fatty samples without any sample treatment, we introduced and optimised the chemiluminescent detection of our assays and we applied them to the determination of the above mentioned analytes both in extracts of non-fatty baby foods and in simply diluted, untreated fruit juices. The validation of the data obtained both by colorimetric and luminescent ELISAs on the extracts was done analysing them also by liquid chromatography–electrospray mass spectrometry (LC/ESI/MS/MS).

2. Experimental

2.1. Chemicals and immunoreagents

Certified standards of the three pesticides (purity 95–99%) were purchased from Dr. Ehrenstoffer (Germany). Individual stock standard solutions (concentration range 3.00–3.50 mg ml^{-1}) were prepared by dissolving neat pesticide standard in methanol. A stock standard solution of all analytes was made by mixing these individual pesticide solutions. Mixed working standard solution for ELISA and LC determinations was prepared fresh every day.

Gradient grade methanol was obtained from Merck, Germany. Deionised water for mixing of a mobile phase was produced in Milli-Q apparatus (Millipore, Germany). All other reagents were of analytical grade.

Bovine serum albumin (BSA), Tween 20, *o*-phenylenediamine (OPD), luminol and *p*-iodophenol (PIP) were obtained from Sigma Chemical Company (St. Louis, USA). Horseradish peroxidase (HRP)-labelled swine anti-mouse IgG (SwAM, HRP/SwIgG = 1.81, concentration, 8.9 mg ml^{-1}) was obtained from Seva Pharma, s.r.o. (Czech Republic).

MAbs specific for carbaryl, carbofuran and methiocarb, as well as the corresponding ovoalbumin (OVA)–hapten conjugates, were prepared in the Centro de Investigación e Innovación en Bioingenieria (Universidad Politécnica de Valencia, Spain). LIB-CNH45 MAb and OVA-2NAH conjugate were used for carbaryl analysis [20], LIB-BFNB67 MAb and OVA-BFNH conjugate were used for carbofuran analysis [21], and LIB-MXNB31 MAb and OVA-DPNH conjugate were used for methiocarb analysis [22].

2.2. Materials and instrumentation

Black, high binding, 96-well ELISA microplates with transparent bottom (Costar, cat. no. 3601) for chemiluminescent and colorimetric measurements were from Corning Incorporated (USA). ELISA plates were washed with a Labsystem Multiwash (USA) apparatus. Absorbances were read in a Labsystem Multiscan MCC/340 (Finland) and CL intensity was measured in a microplates reader "Victor" from Wallac (Turku, Finland). Data were processed using Microsoft Excel software (Microsoft Corporation, USA).

Liquid chromatography separation was carried out using a HP1100 liquid chromatograph (Hewlett Packard, USA) as previously described [16]. MS/MS analysis was performed as previously described [16] by LCQ Deca ion trap instrument and acquired data were processed by XCalibur software, both from Finnigan (USA).

2.3. Samples

Apple-strawberry baby foods and fruit juices were from a retail market.

Baby food extracts: The apple-strawberry baby foods, in which the absence of *N*-methylcarbamate residues had been verified by LC/MS analysis, were spiked with the mixed solution of the three carbamates at 1, 10 and 100 ng ml^{-1} and 12.5 g were mixed with 50 ml of acetonitrile and homogenised for 2 min in a Turrax tissumiser. The homogenate was evaporated and the residue was dis-

solved in methanol–water (1:4, (v/v)) and used for the analysis.

Fruit juices: *N*-methylcarbamates-free tomato and fruit juices, as verified by LC/MS analysis, were used without any sample treatment. For matrix effect and recovery studies both were spiked with the mixed standard solution of the three carbamates to obtain concentration levels of 0.5, 1 and 5 ng ml^{-1} for methiocarb, carbaryl and carbofuran, respectively. These concentrations were selected according to the respective LOD previously determined by ELISA on baby foods. For the analysis, the juices were simply diluted 1:5, 1:10, 1:20, 1:50 and 1:100 with phosphate-buffered saline solution (PBS: 0.14 M NaCl, 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4, 2.7 mM KCl, pH 7.4).

2.4. ELISA determinations

An indirect competitive ELISA format was chosen, that could work with colorimetric end-point detection (absorbance at 492 nm) [18] or with chemiluminescent detection (HRP catalysed luminol light emission). To compare the analytical performances of the colorimetric ELISA with those of the chemiluminescent one, both were used to analyse the crude extracts of baby foods and the fruit juices at all added concentrations. Samples were quantitatively analysed for a single pesticide in each plate, irrespective of the presence of the two other analytes. Working standard solutions for each immunoassay were prepared from the mixed standard stock solution by serial dilution in PBS. Each primary test sample was subjected to three independent dilutions to fit into the linear part of the calibration curve and subsequently analysed by ELISA. The dilution factors applied for the *N*-methylcarbamate analysis are shown in Table 1.

The immunoassays were performed by the following procedure: microplates were coated with the OVA–pesticide conjugate solution in coating buffer (0.05 M carbonate–bicarbonate buffer, pH 9.6; 100 μl/well) by overnight incubation at laboratory temperature, and then washed four times with washing solution (PBS containing 0.05% Tween 20). 50 μl/well of pesticide standards or sample extracts, diluted in PBS, and 50 μl/well of specific MAbs, diluted in PBS containing 0.1% BSA, were added to each well. After 2 h incubation at room temperature the microplates were washed as described above. The second antibody (SwAM) conjugated with peroxidase was added (100 μl/well) and incubated for 1 h at room temperature, then washed again. In the ELISA with photometric detection, 100 μl/well of a OPD solution in reaction buffer (4.6 mM OPD in 0.1 M phosphate–citrate buffer, pH 5.0, containing 0.1% (v/v) of 30% H_2O_2) was added. After 10 min incubation at room temperature, the reaction was stopped by adding 50 μl/well of 2.5 M sulfuric acid, and the absorbance at 492 nm measured. In the chemiluminescent ELISA, the peroxidase activity was revealed by adding 100 μl/well of the CL mixture (1 mM luminol, 0.5 mM *p*-iodophenol, 1 mM H_2O_2 in borate buffer, pH 8.5). Immediately after the addition, the chemiluminescent emission was measured for 1 s per well.

Table 1
Concentrations of immunoreagents and baby food sample dilutions applied in the ELISAs

Detection mode	Carbaryl	Carbofuran	Methiocarb
	Antibody concentration (μg ml^{-1})		
	LIB-CNH45	LIB-BFNB67	LIB-MXNB31
Colorimetric	0.060	0.030	0.060
Chemiluminescent	0.030	0.008	0.125
	Conjugate concentration (μg ml^{-1})		
	OVA-2NAH	OVA-BFNH	OVA-DPNH
Colorimetric	0.130	0.250	1.00
Chemiluminescent	0.130	0.030	0.250
	Pesticide added (ng ml^{-1})	Sample dilution	
Colorimetric			
	1	1/5	1/1	1/5
	10	1/25	1/5	1/50
	100	1/500	1/50	1/500
Chemiluminescent			
	1	1/15	1/5	1/20
	10	1/150	1/50	1/200
	100	1/1500	1/500	1/2000

Absorbance values or chemiluminescence intensity values from standards were mathematically fitted to a four-parameter logistic equation [19]. The analyte concentration in samples was determined by interpolation of the obtained values on the appropriate standard curve.

The limit of detection (LOD) for ELISAs was calculated as the analyte concentration that reduced signal to 90% of the maximum. The linear working ranges were determined as the concentrations producing 20–80% inhibition of the maximal assay signal. The I_{50} values were also calculated as the analyte concentrations that reduced the assay signal to 50% of the maximum one.

3. Results and discussion

Both colorimetric and chemiluminescent assays were performed by using black microplates with transparent bottom, i.e. those required for the chemiluminescent detection. Since the adsorption capacities of the various kinds of microplates could be different, the reagent concentrations were again selected for the colorimetric ELISA and optimised first time for the chemiluminescent one, to achieve the best immunoassay performances. The optimum concentrations of the specific monoclonal antibodies and of the corresponding OVA–hapten conjugates were found using a checkerboard titration. The optimum reagent concentrations, listed in Table 1, were defined as those giving the maximum inten-

Table 2
Comparison between chemiluminescent and colorimetric assays

	Linear working range (ng ml^{-1})	Limit of detection (ng ml^{-1}) ± S.D.	I_{50} (ng ml^{-1}) ± S.D.
Carbofuran			
Colorimetric	50 – 2	1.3 ± 0.4	6 ± 1
Chemiluminescent	0.96 – 0.04	0.03 ± 0.02	0.28 ± 0.09
Carbaryl			
Colorimetric	0.83 – 0.06	0.04 ± 0.01	0.15 ± 0.05
Chemiluminescent	0.52 – 0.02	0.007 ± 0.003	0.063 ± 0.004
Methiocarb			
Colorimetric	0.56 – 0.02	0.016 ± 0.004	0.08 ± 0.02
Chemiluminescent	0.18 – 0.01	0.004 ± 0.001	0.038 ± 0.005

sity of assay signal with minimum reagent expense. These concentrations were used to perform all the experiments. It is clear from Table 1 that: (i) the chemiluminescent assay, as it is able to detect lower amount of HRP, requires in quite all cases lower concentrations of both the immunoreagents; and (ii) CL-ELISA is able to measure samples diluted from 3 to 10 times more than those used in the colorimetric immunoassay.

In Table 2 the detection limits, the linear working ranges and the I_{50} values obtained by colorimetric and chemiluminescent ELISAs are compared: all analytical parameters were greatly improved by using the CL detection. In particular, the detection limits were significantly lower in comparison with those previously obtained by colorimetric ELISAs for fruit juices [23,24]. By using the chemiluminescent assay here described it is possible to detect the mentioned carbamates even at the low levels established by the European legislation as maximum residue limits (MRL) for drinking water, that in the case of carbofuran is 0.1 ng ml^{-1}.

The concentration ranges of the added pesticides were chosen taking into account the MRLs for pesticides in baby foods (10 μg kg^{-1}, corresponding to the 10 ng ml^{-1}) and the MRLs established for fruit and vegetables (about 3–0.3 mg kg^{-1} and 1–0.1 mg kg^{-1}, respectively). The influence of extracts purification on the analytical performance of ELISAs was assessed in our previous work on baby food samples [16] and negligible differences were observed in results obtained from purified and not-purified extracts. The latter were also suitable to be analysed by LC/MS/MS. For these reasons crude extracts were directly used in this work.

Table 3 summarises the repeatability and recovery values obtained for baby food extracts. Irrespective of the spiked level, mean recovery values obtained by both ELISAs were in the range 80–120% for all the analytes. The precision of the assay was better for the chemiluminescent ELISA, since coefficients of variation (CV) ranging from 1.2 to 19.2% were obtained. These values were lower than those of the colorimetric assay, and similar to those of LC/ESI/MS/MS determinations (range 1.4–18%).

In order to assess the possibility to analyse non-fatty samples without pre-treatment or extraction procedures, tomato and orange juices, as representative examples of possible contaminated products, were simply diluted with PBS. Table 4 shows the recovery values obtained for both fruit juices at the different dilutions applied: these values were very good also in this case. The samples were analysed only by chemiluminescent ELISA, since the better sensitivity of this detection method was already established during the assays of the baby food extracts. A slight matrix effect can be observed in tomato juice samples at the lower dilution factors (1:5 and 1:10). This effect led to false positive results with non-spiked samples and to low or too high recovery values in spiked juices when analysed at low dilutions. These effects could be ascribed to the turbidity of the tomato juice in comparison with the orange juice that, in fact, allows to obtain good results at any dilution assayed. Anyhow, this is not a problem, since at higher dilutions all matrix effects disappeared and an accurate determination of the pesticides content was still possible. Data showing the reproducibility and

Table 3
Overview of the results obtained by analysis with colorimetric and chemiluminescent ELISAs and LC/ESI/MS/MS of crude strawberry baby food extracts spiked with carbaryl, carbofuran and methiocarb at 1, 10 and 100 ng ml^{-1} (n = 3)

ng ml^{-1}	Colorimetric			Chemiluminescent			LC/ESI/MS/MS		
	Mean	Recovery (%)	CV (%)	Mean	Recovery (%)	CV (%)	Mean	Recovery (%)	CV (%)
Carbaryl									
1	0.8	80.0	24.2	1.0	97.5	10.8	1.2	121.0	5.2
10	9.3	92.5	14.8	10.0	100.0	10.0	8.9	89.3	7.8
100	91.4	91.4	9.4	110.3	110.3	8.7	100.1	100.1	2.5
Carbofuran									
1	1.2	119.6	26.4	1.0	100.0	14.1	1.1	112.4	8.7
10	9.5	95.0	22.3	9.0	90.0	19.2	8.8	88.0	18.0
100	93.0	93.0	3.0	114.0	114.0	1.2	99.7	99.7	1.4
Methiocarb									
1	0.9	85.5	23.2	1.0	96.0	10.9	1.1	105.4	14.2
10	9.2	92.0	23.2	11.0	110.0	18.1	9.3	93.2	8.3
100	100.3	100.3	4.9	100.3	100.3	6.1	98.6	98.6	3.1

Table 4
Influence of the matrix dilution on reliability of the determinations by CL-ELISA

Dilution factor	Tomato juice				Orange juice			
	0 ng ml^{-1}	5 ng ml^{-1}			0 ng ml^{-1}	5 ng ml^{-1}		
	Found[a]	Found[a]	Recovery (%)	CV (%)	Found[a]	Found[a]	Recovery (%)	CV (%)
Carbofuran								
5	3	2.6	51.0	13.9	n.d.	5.1	102.0	4.2
10	2	3.4	68.2	4.1	n.d.	4.7	94.7	3.9
20	n.d[b]	5.0	99.6	3.2	n.d.	5.4	108.2	3.1
50	n.d	5.1	102.2	5.1	n.d.	4.9	97.0	6.2
100	n.d	5.1	101.4	8.4	n.d.	4.9	98.9	9.7
		1 ng ml^{-1}				1 ng ml^{-1}		
		Found[a]	Recovery (%)	CV (%)		Found[a]	Recovery (%)	CV (%)
Carbaryl								
5	0.5	2.5	250	23.0	n.d	0.9	92.1	6.4
10	0.5	1.5	150	9.4	n.d.	1.1	105.6	9.2
20	n.d.	1.1	110.1	6.1	n.d.	1.0	100.9	4.5
50	n.d.	1.0	97.0	5.6	n.d.	0.9	94.3	4.1
100	n.d.	1.1	109.5	5.9	n.d.	1.0	98.1	6.0
		0.5 ng ml^{-1}				0.5 ng ml^{-1}		
		Found[a]	Recovery (%)	CV (%)		Found[a]	Recovery (%)	CV (%)
Methiocarb								
5	n.d.	0.44	87.5	8.3	n.d.	0.52	103.1	9.9
10	n.d.	0.51	101.5	5.4	n.d.	0.56	111.1	5.1
20	n.d.	0.48	95.0	6.3	n.d.	0.50	99.1	5.0
50	n.d.	0.55	109.3	7.4	n.d.	0.53	105.7	8.9
100	n.d.	0.50	99.5	14.1	n.d.	0.49	98.4	10.6

[a] (ng ml^{-1}); data are the average of 4 independent determinations.
[b] not detectable.

accuracy of the CL immunoassay on juices are reported in Table 5.

The correlation between the LC/ESI/MS/MS data, or the added amounts of carbamates, and ELISA results was very good: correlation coefficients and regression slopes were very close to 1. For methiocarb, the linear regression equations for the whole concentration range in crude extracts were: $y = 0.98x + 0.25$ for data obtained by colorimetric ELISA and $y = 0.99x + 0.69$ for the chemiluminescent one. For carbaryl the corresponding equations were $y = 1.10x + 0.46$ and $0.91x + 0.07$, respectively. For carbofuran the equations: $y = 1.08x + 0.80$ and $y = 1.01x + 0.56$ were obtained for the colorimetric and the chemiluminescent ELISA, respectively.

Table 5
Reproducibility and accuracy of the carbamate CL-ELISAs for spiked juice samples

	Mean (ng ml^{-1})	CV (%)	Recovery (%)
Carbofuran (5 ng ml^{-1})			
Tomato juice	5.1	4.9	101.1
Orange juice	5.0	5.2	99.9
Carbaryl (1 ng ml^{-1})			
Tomato juice	1.1	9.7	109.0
Orange juice	1.0	8.5	98.0
Methiocarb (0.5 ng ml^{-1})			
Tomato juice	0.49	8.1	98.8
Orange juice	0.52	5.3	104.0

Data are the average of 6 determinations. Spiked samples were measured at the 1:20 dilution ratio.

The reported results show how the chemiluminescent detection can improve the analytical performances of carbamates ELISAs. Moreover, the employment of assays with lower detection limits, such as the chemiluminescent ones, can allow to analyse various kinds of non-fatty samples simply by diluting them. This fact could avoid the time-consuming pre-treatment or extraction procedures, since the problems related with possible matrix effects can be easily overcome by using highly diluted samples. The time required to prepare the samples can be greatly reduced and their number increased significantly in each analytical session, as well as the reagents cost per assay is reduced.

Acknowledgements

This work was supported by the Grant No. 6 CH 1 of the Project of collaboration Italy-Czech Republic, by the MSM of Czech Republic No. 223300006 and by grants from the University of Bologna (Fundamental Oriented Research). Thanks to the Centro Interdipartimentale di Ricerche Biotecnologiche for the use of the "Victor" luminometer.

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Příloha 6:

Blažková, M., <u>Mičková-Holubová, B.</u>, Rauch, P., Fukal, L.: Immunochromatographic colloidal carbon-based assay for detection of methiocarb in surface water. *Biosens. Bioelectron.* 25(4): 753-758, 2009.

Biosensors and Bioelectronics 25 (2009) 753–758

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Immunochromatographic colloidal carbon-based assay for detection of methiocarb in surface water

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ARTICLE INFO

Article history:
Received 25 June 2009
Received in revised form 13 August 2009
Accepted 15 August 2009
Available online 22 August 2009

Keywords:
Immunochromatographic assay
Carbon particle
Methiocarb
Surface water
Environmental analysis

ABSTRACT

A simple and rapid immunochromatographic assay for a sensitive and inexpensive monitoring of methiocarb in surface water was developed using a binding inhibition format on a membrane strip. In the assay, detection reagent consisted of anti-methiocarb antibody and colloidal carbon-labelled secondary antibody. Methiocarb-ovalbumin conjugate was immobilized in a test line of the strip as a capture reagent. Colour intensity of the test line in methiocarb-positive assay was visually distinguishable from that of negative sample within 10 min. The optimized semi-quantitative method provided a visual detection limit of 0.5 ng mL^{-1}. Cross-reactions with other carbamate pesticides were not found (<1%). Only a negligible matrix effect of surface water was recognized. In parallel analyses of spiked water samples, the assay results were in a good agreement with those of ELISA. The stability test indicated the strips could be used at least 2 months without change in performance. All characteristics of the visually evaluated assay mentioned above were verified by instrumental quantification of colour intensity in test lines. The developed immunochromatographic assay offers potential as a useful on-site screening tool for environmental analysis.

1. Introduction

Although the use of pesticides has had a range of benefits, these compounds also can cause adverse environmental effects, including degradation of water quality. Monitoring the concentration of pesticides in surface water is important for maintaining aquatic health and eventually ensuring safe drinking water supplies.

Methiocarb [3,5-dimethyl-4-(methylthio) phenyl methylcarbamate] belongs to chemical class of N-methylcarbamate pesticides. As a broad-spectrum insecticide, molluscicide, acaricide, and bird repellent, it is commonly used in agriculture and household practice throughout the world. Therefore, contamination of water and agricultural products becomes imminent, and consequently, adverse health effects are possible in humans and animals. Due to its action as a potent acetylcholinesterase inhibitor and other toxic activity, methiocarb is considered to be highly hazardous according to WHO classification. Nevertheless, a more pressing concern is the toxicity to aquatic organisms. For some kinds of aquatic invertebrates, commonly used as bioindicators of water contamination in environment, values of LC_{50} or EC_{50} (acute) were found extremely low, in the range of 1.6–19 μg L^{-1} (Munn et al., 2006; Péry et al., 2004). U.S. Environmental Protection Agency declared that the methiocarb value of an acute aquatic life benchmark for invertebrates is 3.5 μg L^{-1} (Anonymous, 2007).

The use of methiocarb, as well as all pesticides, has been regulated for a long time in many countries. However, unwanted amounts of certain pesticides still are found in environmental media and residues exceeding regulatory limits still sometimes occur in agricultural produce. Until now, European Union legislation has established maximum residue levels for food (Commission Regulations (EC) No. 149/2008) and drinking water (Council Directive 98/83/EC) but not for surface water. However, the European Commission introduced the Water Framework Directive (2000/60/EC) as an instrument to sustain and improve quality of environmental waters. The directive aims to achieve good chemical and ecological status for all waters by 2015. The backbone of the directive implementation is monitoring of chemical substances and values of LC_{50} or the acute life benchmark for aquatic invertebrates are important reference concentrations for assessing good ecological status of surface water. Although the total volume of methiocarb used is low relative to some other pesticides, it could have major impacts in localized areas if there is concentrated outdoor use. Then, pesticide concentration in runoff may approach or exceed LC_{50} value for aquatic organisms (Primus et al., 2001; Schäfer et al., 2007; Vecchia et al., 2008; Wilson et al., 2005). Despite recommendations for pesticide application in good agriculture practices, contamination of fruit food (Blasco et al., 2005; Schulze et al., 2002) and surface water with methiocarb has been reported in the last years (Anonymous, 2005; Borkovcova et al., 2004; Fytianos et al.,

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doi:10.1016/j.bios.2009.08.023

2006; Garcia de Llasera and Bernal-Gonzales, 2001; Primus et al., 2001). Therefore, developments of analytical methods that are fast, inexpensive and may be used on-site are needed.

Current methods applied to analyze pesticides in water samples are mainly based on high-performance liquid chromatography or gas chromatography coupled to various selective detectors. Such procedures are also described for methiocarb (Huertas-Pérez and García-Campaña, 2008; Rodrigues et al., 2007; Saraji and Esteki, 2008). Although highly sensitive and reliable, they are time-consuming, involve multiple steps in sample preparation and analysis, and require expensive equipment and skilled analysts.

Nowadays, with increasing development of immunoassays, these methods have been shown to be useful alternative for analysis of pesticides in environmental samples (Anand et al., 2007; Jiang et al., 2008; Krämer et al., 2007; Mauriz et al., 2007). Currently, enzyme-linked immunosorbent assay (ELISA) carried out in a microtitre plate is the most common technique used for immunoassays. This technique has been successfully applied for the analysis of methiocarb (Abad et al., 1999; Mickova et al., 2003, 2005). However, for non-specialized laboratories and for field-use, it can be difficult to perform labour intensive operations including repeated incubation and washing, and enzyme reaction for final signal generation in ELISA. Immunosensors have also become increasingly practical tools in environmental monitoring (Marchesini et al., 2007). With the aim of real-time output, various kinds of immunosensors have been developed for pesticide analysis (Kim et al., 2007; Long et al., 2008; Zacco et al., 2007). In terms of field-use, these methods are often considered to be requiring expensive equipment and specialized personnel. With the demand for overall speed and simplicity, a lateral-flow immunochromatographic assay (ICA) could be a more suitable alternative (Posthuma-Trumpie et al., 2009). It combines several benefits including a user-friendly format, short assay time, and cost-effectiveness. These characteristics make it well suited for on-site screening. In the last years, several research groups have performed an ICA for some of pesticides (Gui et al., 2008; Kaur et al., 2007; Shi et al., 2008; Shim et al., 2006; Wang et al., 2005; Zhou et al., 2004; Zhu et al., 2008) and other environmental contaminants (Li et al., 2009; Zhou et al., 2009). In these works, the detection reagent was typically colloidal gold-labelled antibody. To our best knowledge, any use of ICA principles for methiocarb detection has not been published up to now.

In our previous studies, we described validation of the ELISA for control of methiocarb residues in some food samples (Mickova et al., 2003, 2005). Here we reported a development of ICA test for this pesticide using colloidal carbon nanoparticles as a label. The optimized test has been compared with ELISA results in analysis of spiked surface water samples.

2. Experimental

2.1. Reagents and materials

Standards of methiocarb (98.5%, HPLC/DAD), carbaryl (99%, HPLC/DAD), carbofuran (99.5%, HPLC/DAD), were from Dr. Ehrenstorfer GmbH, Augsburg, Germany. Aldicarb (99.9%, HPLC, Fluka), bendiocarb (99.5%, HPLC, Riedel-de Haën), ethiofencarb (99.0%, HPLC, Riedel-de Haën), fenoxycarb (99.6%, HPLC, Fluka), and methomyl (99.9%, HPLC, Fluka) were supplied by Sigma–Aldrich Inc. (St. Louis, USA). Individual stock standard solutions containing 10 mg mL^{-1} of each compound were prepared by dissolving accurately weighted amounts in ethanol and stored in darkness at 4 °C. Working standard solutions were freshly prepared by serial dilution in deionised water.

The mouse anti-methiocarb monoclonal antibody as well as the methiocarb hapten-ovalbumin conjugate (methiocarb-OVA) was purchased from Centro de Apoyo a la Innovación, la Investigación y la Transferencia de Tecnología (CTT), Universidad Politécnica de Valencia, Spain. Producer indicated these immunoreagents as LIB-MXNB31 and OVA-DPNH, respectively (Abad et al., 1998). Rabbit anti-swine antibody (RASw) as well as the swine anti-mouse antibody (SwAM) was obtained from Nordic Immunological Laboratories, Tilburg, The Netherlands (product codes RASw/IgG(H + L)/7S and SwAM/IgG(H + L)/7S). Both antibodies were supplied as purified IgG fraction of polyclonal antiserum.

Carbon nanoparticles (Spezial Schwartz 4, Degussa AG, Germany) were kindly provided from Agrotechnology & Food Sciences Group (Wageningen University and Research Centre, The Netherlands) as a dry powder (particles of amorphous shape with average size of 120 nm). Bovine serum albumin (BSA), Tween 20, polyethylene glycol (PEG, MW 3350) and *o*-phenylenediamine (OPD) were purchased from Sigma–Aldrich Inc. (St. Louis, USA). Horseradish peroxidase (HRP)-labelled swine anti-mouse IgG (SwAM, HRP/SwIgG = 1.81, concentration 8.9 mg mL^{-1}) was obtained from Seva Pharma, Czech Republic.

Other common chemicals were of the highest purity available and purchased from Sigma–Aldrich. Deionised water for standards and buffer solutions was prepared on apparatus RO-TFM-5SV (Fresh Water Systems, Inc., Greenville, USA).

Whatman GmbH (Dassel, Germany) supplied various types of nitrocellulose membranes (PRIMA 80, PRIMA 125, AE 98 FAST, AE 98, AE 99, AE 100, FT 020, FT 060, Protran BA 79, Protran BA 83, and Protran BA 85) tested in strip assay. Vinyl backing ARcare® 7823 was from Adhesives Research Inc. (USA), and the absorbent pad CFSP from Millipore Corp. (USA). Ninety-six-well ELISA polystyrene microtitre plates Costar (catalogue no. 9018) were obtained from Corning Inc. (USA).

2.2. Water samples

Samples of surface water were collected from streams in lowland agricultural area of central part of Czech Republic. Freshly collected samples were filtered through nylon filter to remove suspended particulate matter and than stored in darkness at 4 °C until analysis.

Water samples containing methiocarb (concentration range of 0.01–1000 ng mL^{-1}) were prepared as follows: methiocarb-free samples, as verified by LC/MS analysis, were spiked with a known amount of methiocarb derived from stock solution and used immediately for analysis.

2.3. Labelling of swine anti-mouse antibody with carbon nanoparticles

Carbon nanoparticles bind proteins non-covalently without changing their bioactivity (Van Amerongen et al., 1993). The swine anti-mouse antibody was labelled with colloidal carbon nanoparticles according to the O'Keeffe et al. (2003) with gentle modification. A colloidal carbon suspension (carbon nanoparticles 2 mg mL^{-1} of 5 mM borate buffer, pH 8.8) was sonicated for 10 min on ice using a Sonic 1 (Polsonic, Poland). Subsequently, with a simultaneous gentle stirring the SwAM was added to give a final protein concentration of 350 μg mL^{-1}. Then, this mixture was stirred gently at 4 °C overnight. In the end, the suspension was washed three times in a 5 mM borate buffer, pH 8.8 (containing 1% BSA and 0.02% NaN_3) using centrifugation (10 000 g, 15 min, 10 °C). Final sediment was resuspended to the initial volume. Prepared stock suspension of the SwAM-carbon conjugate was stored at 4 °C in the dark.

2.4. Preparation of immunochromatographic test strips

Each strip contained methiocarb-ovalbumin conjugate (methiocarb-OVA) and a rabbit anti-swine antibody immobi-

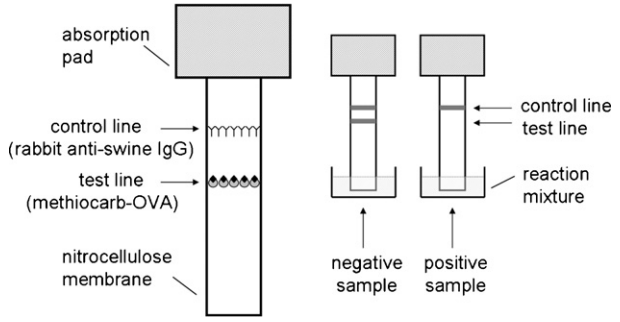

Fig. 1. Schematic diagram of the immunochromatographic strip test. Details are described in the text.

lized in a narrow lower zone and upper zone, respectively. A schematic representation is shown in Fig. 1. The strips were prepared as follows: firstly, nitrocellulose membrane sheet was cut to the desired size (20 cm × 2.5 cm) and pasted to a vinyl backing. Using the Linomat V (Camag AG, Switzerland) methiocarb-OVA and RASw solutions (5 mM borate buffer, pH 8.8) were separately dispensed (1 μL per 5 mm) as the test and control lines (20 cm long) on this plastic-backed membrane. Subsequently, the membrane sheet was dried at 37 °C for 2 h and cut into test strips (25 mm × 5 mm) using a programmable strip cutter (Economic Cutter ZQ2000, Shanghai Kinbio Tech Co., Ltd., Shanghai, China). An absorbent pad was cut in sections of 10 mm × 20 mm and pasted to the far end of the strip (Fig. 1). Strips prepared in such way were stored in sealed bags under dry conditions at laboratory temperature until used.

2.5. Procedure of immunochromatographic assay (ICA)

In a well of an ELISA microplate, 50 μL of methiocarb standard solution in deionised water (or water sample) was mixed with 30 μL of the running buffer, 10 μL of SwAM-carbon conjugate (stock suspension diluted with the running buffer), and 10 μL of anti-methiocarb antibody (diluted in the running buffer). Subsequently, the membrane strip was dipped into this reactant mixture vertically (Fig. 1). After 10 min, test results were observed.

2.6. Detection and quantification using ICA

The colour intensity of test line could be assessed visually by naked eyes. Samples producing the test line appearance of the same intensity as a negative control were considered to be negative (−). If the colour intensity of the test line was weaker than that of the negative control, the result was evaluated as weakly positive (±). When there was no band at the test line, the sample was positive (+). For quantification of the colour intensity, greyscale densitometry was used after the strip was fully air-dried. Greyscale digital scanned images of the strips were recorded using the Epson Perfection V700 Photo Scanner (Seiko Epson Corporation, Nagano, Japan), and the intensity of the test lines quantified using the TotalLab TL100 software (Nonlinear USA Inc., NC, USA). Average values were calculated from quadruplicate runs for each methiocarb concentration using three measurements for each strip. Standard curves were obtained by plotting the mean intensity value against the logarithm of methiocarb concentration. Experimental points were fitted to a four-parameter logistic equation using the Microsoft Excel software (Microsoft Corp., USA).

2.7. Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as we described previously (Mickova et al., 2003). Briefly, the methiocarb-OVA conjugate solution in 0.05 M carbonate-hydrogen carbonate buffer, pH 9.6 (0.5 μg mL^{-1}, 100 μL per well) was pipetted into wells of polystyrene microplate and left to incubate overnight at 4 °C. Then microplates were washed four times with 0.01 M PBS (phosphate-buffered saline), pH 7.4, and containing 0.05% Tween 20, by the use of a microplate washer (Columbus Pro, Tecan Group Ltd., Männedorf, Switzerland). The aliquots (50 μL per well) of methiocarb standard in deionised water or samples, and the anti-methiocarb antibody (0.06 μg mL^{-1}), diluted in 0.02 M PBS, pH 7.4, containing 0.1% BSA (50 μL per well) were pipetted to the microplate wells coated with the conjugate. Microplates were incubated for 2 h at laboratory temperature and then washed as described above. The second antibody conjugated with peroxidase (SwAM-HRP), diluted 1:2000 in 0.01 M PBS containing 0.05% Tween 20, was added, left to incubate for 1 h at laboratory temperature, and washed as described above. Then, the peroxidase substrate in reaction buffer (4.6 mM OPD in 0.1 M phosphate-citrate buffer, pH 5.0, containing 0.1% of 30% H_2O_2) was added to each well (100 μL per well). The enzyme reaction was stopped after 10 min by adding 2.5 M sulphuric acid (50 μL per well), and the absorbance was measured with a microplate reader (μQuant Microplate Spectrophotometer, BioTek Instruments, Inc., Winooski, USA). Data from ELISA were processed using the Microsoft Excel software (Microsoft Corp., USA). Absorbance values from standards were mathematically fitted to a four-parameter logistic equation. Parameters of standard curve were the same as we described previously (Mickova et al., 2005): limit of detection (LOD) was 0.016 ng mL^{-1}, the working range of immunoassay was from 0.02 to 0.56 ng mL^{-1}, and the IC_{50} value was 0.08 ng mL^{-1}.

3. Results and discussion

3.1. Development of ICA

A schematic description of the strip format is illustrated in Fig. 1. The assay was based on two coexistent phenomena: (1) capillary migration of reactants through a nitrocellulose strip followed by affinity capturing; (2) inhibition of immunochemical interaction between methiocarb immobilized in a test line and specific antibody, which was a part of a moving detection complex, by free methiocarb in a sample.

In designing the assay strip, three types of antibody (Ab) which differed in their specificity were used: primary Ab, the mouse anti-methiocarb antibody; secondary Ab, the swine anti-mouse antibody, which was labelled with colloidal carbon nanoparticles (SwAM-carbon conjugate); and tertiary Ab, the rabbit anti-swine Ab, which was immobilized in the control line of the strip. In arrangement of ICA, an unusual procedure was applied, which consisted in the indirect labelling of primary Ab with the aid of the labelled secondary Ab, instead of the use of directly labelled primary Ab. It made it possible to optimize a concentration of primary Ab more conveniently and minimize a consumption of primary Ab in the assay.

When mixed in a reaction mixture, the carbon-labelled secondary Ab bound the primary Ab forming the detection complex. In the assay, the reaction mixture migrated from the well through the strip into the absorbent pad. In the absence of methiocarb in the sample, the solution of the detection complex and the excess of labelled Ab moved upward across the strip. In the test line, detection complex was captured by immobilized methiocarb-OVA conjugate to form a visible black-colour line. However, in the presence of methiocarb in the sample, free methiocarb molecules bound to primary Ab, thereby inhibiting the interaction of the detection complex with the immobilized methiocarb. Thus, the colour intensity of the test line inversely correlated with methiocarb concentration in the sample.

The control line acted as a positive control to assure that the labelled secondary Ab migrated throughout the system. In the control zone, it was captured by immobilized tertiary Ab to form the visible control line. If intensive colour was not present in the line, the assay was considered as invalid.

3.2. Preliminary optimization of immunoreagent concentrations

Most of components necessary for the immunostrip construction as well as the experimental conditions have a key influence on both the performance and final sensitivity.

Firstly, in this experimental study the concentrations of reagents were preliminary optimized to satisfy the following assay criteria: good sensitivity, minimum immunoreagent consumption, and appearance of the test line with good colour intensity and sharpness (for negative sample).

Therefore, checkerboard titration experiments were carried out. Several amounts of methiocarb-OVA conjugate immobilized on the membrane (10–500 ng per strip) against different amounts of primary Ab (1–100 ng per strip) were investigated in assays of standard solutions containing methiocarb at concentrations of 0, 1.0, 10, and 100 ng mL^{-1}. The same experiments were performed for different concentrations of carbon-labelled secondary Ab.

Preliminary optimized concentrations of reagents were finally specified after the following optimization of other assay conditions.

3.3. Optimization of assay conditions

Subsequently, some other factors affecting strip test performance were also evaluated: the type of membrane, the type of running buffer, and the incubation time of reactant mixture before the strip is dipped inside.

Firstly, we tested eleven various types of nitrocellulose membrane. The type of membrane influenced flow time and sharpness of detection lines. Faster-flowing membranes reached endpoint more quickly but required higher reagent concentrations and provided lower sensitivity. Secondly, the composition of the running buffer markedly influenced flow rate, line intensity, and test sensitivity. Phosphate buffer and borate buffer of various pH values and ion concentrations were tested. Moreover, additives such as BSA (0.1–5%), gelatine (0.1–1%), polyethylene glycol (0.01–2%), sucrose (0.5–5%), and their combinations, with and without surfactant Tween 20 (0.01–0.5%) were tested to further improve the test performance. These factors affected mainly the character of reactant flow, level of background colour, sharpness, and intensity of the test line. Thirdly, various intervals of incubation time (0, 5, and 30 min) of methiocarb analyte with primary Ab and labelled secondary Ab were tested. Using methiocarb concentrations in standard samples of 0, 2, and 10 ng mL^{-1}, the test line intensities were not significantly influenced by incubation time.

Optimized conditions for ICA test were chosen by visual evaluation of strips as a compromise among a good colour perception of lines, good test sensitivity, minimum immunoreagent consumption and rapid test performance. They are collected in Table 1.

Table 1
Optimized parameters of immunochromatographic assay.

Parameter	Chosen as optimal
Type of nitrocellulose membrane	AE 98 (Whatman)[a]
Amount of methiocarb-OVA conjugate per strip	100 ng
Amount of primary antibody in assay	6.25 ng
Dilution of stock suspension of SwAM-carbon conjugate prior addition into assay	10-times
Composition of running buffer	0.2 M borate buffer, pH 8.8 (containing 2% BSA, 0.1% Tween 20, 1% polyethylene glycol, and 0.02% NaN_3)
Incubation time of reaction mixture	0 min

[a] Pore-size 5 μm, capillary rise in water 160–210 s per 4 cm.

3.4. Sensitivity of ICA

The sensitivity of the ICA strip was determined by testing the methiocarb standard samples in the concentration range from zero to 1000 ng mL^{-1} (Fig. 2). The visual limit of detection of the assay was defined here as the minimum methiocarb concentration producing the colour density of the test line significantly weaker than that at zero concentration (it means absolute agreement between result assessments of six observers for five repetitions of the same test). As shown in Fig. 2, the methiocarb concentration of 0.5 ng mL^{-1} caused a slight but visually distinguishable difference in the test line intensity compared to the negative control. Thus, 0.5 ng mL^{-1} of methiocarb was considered to be the visual detection limit for the ICA test. At concentrations of methiocarb >5 ng mL^{-1}, the test line was invisible.

The ability of the developed ICA strip to detect lower methiocarb concentrations than the value of an acute aquatic life benchmark for invertebrates illustrated the potential of this technology for environmental analyses. The value of visual LOD observed in our work is comparable with several published ICA tests for other low-molecular-weight contaminants, which used a colloidal gold label (Kaur et al., 2007; Shi et al., 2008; Shim et al., 2006). Furthermore, the developed ICA test is 5–100 times more sensitive than those described for pesticide detection by many other authors (Gui et al., 2008; Wang et al., 2005; Weetall and Rogers, 2002; Zhou et al., 2004; Zhu et al., 2008). However, Li et al. (2009) presented the LOD value of ICA with gold label for indomethacin detection in water as low as 0.1 ng mL^{-1}.

Finally, to obtain qualitative results and verify the visual LOD, colour intensities of the test lines were evaluated using scanning densitometry. The mean signal values were fitted to a sigmoid equation and displayed as the standard curve (Fig. 3). To express the assay sensitivity, the LOD of 0.14 ± 0.03 ng mL^{-1} was estimated as the analyte concentration providing a 10% decrease of the blank signal. Subsequently, the linear working range of 0.31–3.38 ng mL^{-1} was determined as the concentrations causing 20–80% inhibition of the maximal assay signal, and the 50% inhibition (IC_{50}) value of 1.01 ± 0.13 ng mL^{-1} related to the midpoint of the curve was calculated.

3.5. Specificity of ICA

To determine the selectivity of the developed ICA test for methiocarb, a study of cross-reactivity was carried out under the

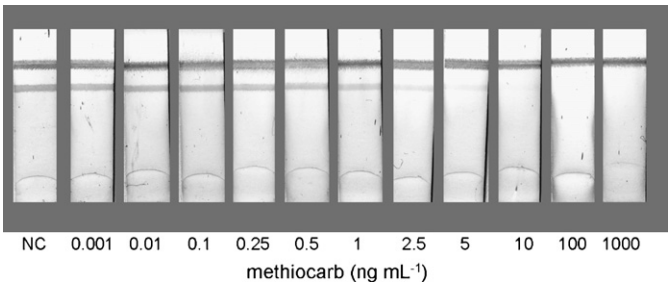

Fig. 2. Concentration range of methiocarb standard assayed by the immunochromatographic strip tests. NC, negative control. Compared to the negative control the concentration of 0.5 ng mL^{-1} caused a considerable weak test line still visually distinguishable.

Table 2
Characteristics of methiocarb standard curves for immunochromatographic assay in different water matrixes[a].

Water matrix	Linear working range[b] (ng mL^{-1})	Limit of detection ± SD[c] (ng mL^{-1})	IC_{50} ± SD[c] (ng mL^{-1})
Deionised water	0.31–3.38	0.14 ± 0.03	1.01 ± 0.13
Surface water	0.28–4.35	0.14 ± 0.04	1.15 ± 0.11

[a] Values were extracted from the four-parameter equations used to fit the standard curves.
[b] Expressed as the concentration range causing 20–80% inhibition of the maximal assay signal.
[c] Standard deviation.

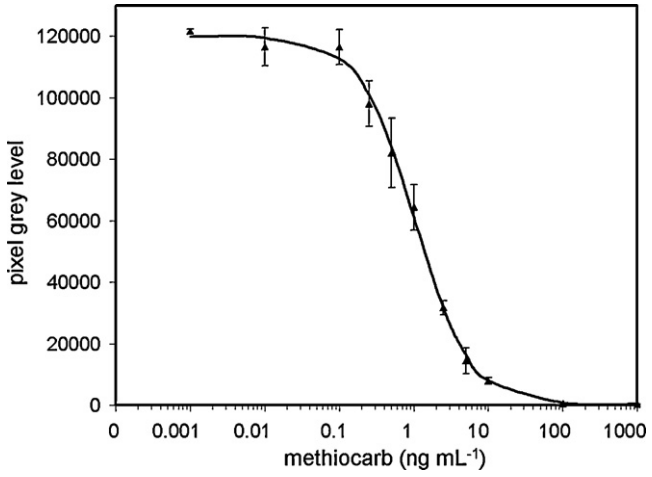

Fig. 3. Standard curve of methiocarb in optimized immunochromatographic assay coupled with densitometry. (−) Four-parameter logistic fit. The error bars correspond to the standard deviations of the data points ($n = 4$).

optimized conditions. Cross-reactants tested were the following carbamate pesticides: carbaryl, carbofuran, aldicarb, bendiocarb, ethiofencarb, fenoxycarb, and methomyl. Standard solutions of each compound in concentrations of 10 and 100 ng mL^{-1} were applied into reactant mixture of ICA test instead of methiocarb. No significant inhibition of the test line intensity was found. These results indicated that the cross-reactions of mentioned pesticides were <1%. Similarly, Abad et al. (1998) and Mickova et al. (2005) described only negligible cross-reactions of some structurally related compounds in ELISA for methiocarb using the same monoclonal anti-methiocarb antibody and methiocarb-OVA conjugate as used in this work.

3.6. Matrix effects

Water samples can usually be used directly for immunochemical analysis without prior cleanup. However, immunochemical interaction is not completely free from interferences caused by unidentified compounds of the sample matrix. Therefore, it is advisable to determine the importance of these matrix effects before the application of immunoassay to real samples. To investigate the potential effect of sample matrix on strip test, we compared standard curves performed with methiocarb in deionised water and surface water, respectively. The obtained curves were nearly identical as can be seen from their analytical features shown in Table 2. Accordingly, the developed ICA test seemed to be directly applicable to the surface water screening without any sample treatment, as matrix effects were minimal.

3.7. Comparison of ICA with ELISA in analysis of water samples

Methiocarb-free samples of surface water were used for spiking study. Samples were spiked at methiocarb levels of 0.5, 2.5 and 10 ng mL^{-1} and assayed by ICA and validated ELISA (Table 3).

In the visually assessed ICA, negative results (−) were obtained for non-spiked samples. Weakly positive results (±) were obtained for samples spiked at concentrations of 0.5 and 2.5 ng mL^{-1}, while samples spiked at level of 10 ng mL^{-1} were assessed as positive (+). All ELISA results were very close to the spiked levels. These experiments demonstrated the high correlation between visually assessed ICA and ELISA.

Using the quantitative ICA, the accuracy and repeatability of the assay were evaluated through recovery study. In the first quantitative analysis, samples with a high spiked concentration were recognized as requiring dilution with deionised water for repeated analysis (they fell out of the working range of the standard curves in ICA or ELISA). In ICA, the recovery of the spiked concentrations was satisfactory with all water samples, as all data fell into the range of 90–106%. In ELISA, recoveries ranged from 91 to 117%. Moreover, method reproducibility was also very similar for both analytical systems, with relative standard deviation (RSD) ranging from 8.2 to 16.5% for the ICA and from 5.3 to 16.9% for the ELISA. The results showed the high correlation of the quantitatively evaluated ICA with ELISA.

Table 3
Comparison of ICA and ELISA in analysis of water samples spiked by methiocarb ($n = 5$).

Type of water	Spiked concentration (ng mL^{-1})	ICA results				ELISA results		
		Visual detection[a]	Mean[b] (ng mL^{-1})	Recovery[b] (%)	RSD[b,c] (%)	Mean (ng mL^{-1})	Recovery (%)	RSD[c] (%)
Surface water I	0	−−−−−	<LOD[d]	–	–	<LOD[d]	–	–
	0.5	±±±±±	0.51	102.0	11.8	0.54	108.0	12.9
	2.5	±±±±±	2.58	103.2	12.0	2.62	104.8	8.4
	10.0	+++++	10.30	106.0	15.1	11.70	117.0	16.9
Surface water II	0	−−−−−	<LOD[d]	–	–	<LOD[d]	–	–
	0.5	±±±±±	0.49	98.0	8.2	0.55	110.0	14.6
	2.5	±±±±±	2.25	90.0	10.2	2.28	91.2	5.3
	10.0	+++++	10.28	102.8	16.5	11.24	112.4	8.5

[a] Visual assessment of the test line; (−) negative result (concentration <0.5 ng mL^{-1}); (+) positive result (concentration >5 ng mL^{-1}); (±) weakly positive result (concentration in the range of 0.5–5.0 ng mL^{-1}.
[b] Quantitative assessment of ICA using densitometry.
[c] RSD, relative standard deviation.
[d] LOD, limit of detection.

3.8. Stability of ICA test

The stability of ICA tests was studied using methiocarb standard solution of 2.0 ng mL^{-1} and blank solution. Strips were stored at laboratory temperature under desiccated conditions. The running buffer, diluted stock suspension of SwAM-carbon conjugate, and anti-methiocarb antibody solution were stored at 4 °C. Three assays were performed once a week during the period of 2 months. Based on the visual assessment of ICA strips, no significant differences in the test line intensity were recognized. Quantitative values of the assay stability were expressed as percentage of the test line intensity at the day zero. All these values were in the range of 88–122% without any notable trend in time. It could be assumed that ICA tests were stable for at least 2 months.

4. Conclusions

We have successfully demonstrated an approach to carbon particle-based ICA for rapid and sensitive monitoring of methiocarb in surface water. The methiocarb immunostrip test was developed, which used colloidal carbon nanoparticles as a tracer to provide visual evidence of the presence of methiocarb at environmentally relevant levels within 10 min. This semi-quantitative assay is easy to perform, allowing for non-specialized personnel on-site application without requirement for intensive labour and any device for sample analysis. This characteristic makes it useful for incorporation into environmental monitoring programmes. Moreover, by the use of a scanner for quantitative strip evaluation, the ICA allowed the analysis of surface water with acceptable accuracy. It showed a potential to be a rapid and inexpensive quantitative method for methiocarb assay in laboratory. Research is currently focused on the on-site application of the ICA test and on the use of ICA for detection of methiocarb in fruit juices and baby-food.

Acknowledgements

This work was supported by the Czech Grant Agency (project No. 525/07/0618), and by Ministry of Education of Czech Republic (project No. MSMT 6046137305).

Authors are thankful to Dr. Aart Van Amerongen from Wageningen University and Research Centre for providing carbon particles.

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Příloha 7:

<u>Holubová-Mičková, B.</u>, Blažková, M., Fukal, L., Rauch, P.: Development of colloidal carbon-based immunochromatographic strip for rapid detection of carbaryl in fruit juices. *Eur. Food Res. Technol.* 231(3): 467-473, 2010.

Eur Food Res Technol (2010) 231:467–473
DOI 10.1007/s00217-010-1301-z

ORIGINAL PAPER

Development of colloidal carbon-based immunochromatographic strip for rapid detection of carbaryl in fruit juices

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Received: 1 April 2010 / Revised: 19 May 2010 / Accepted: 20 May 2010 / Published online: 6 June 2010

Abstract A simple immunochromatographic assay for sensitive detection of insecticide carbaryl in fruit juices was developed. The test is based on inhibition format on a nitrocellulose membrane strip. The strip was separately coated with rabbit anti-swine antibody (control line) and carbaryl hapten-OVA conjugate (test line). Colour intensity on the test line was possible to recognize visually from that of negative sample within 10 min, with the detection limit of 5 ng mL^{-1}. All characteristics of the visually evaluated strips were also measured quantitatively, and then the detection limit was 1.5 ng mL^{-1}. Cross-reactions with other carbamate pesticides were not found (<1%). No matrix effects were observed when fruit juices (orange, apple, pear, banana) were diluted tenfold times before analyses. The results from immunochromatographical assay were in a good agreement with those obtained by enzyme-linked immunosorbent assay as reference method. All these facts indicate the potential of the immunochromatographic strip for the quality control of fruit juices.

Keywords Carbaryl · Immunochromatographic assay · Colloidal carbon-based immunoassay · Fruit juice

Introduction

The extensive use of pesticides formulations in agriculture, together with the increasingly rigorous regulations of the presence of contaminants in the environment, crop protection, and food products, has brought about the need to develop sensitive and accurate methods for the detection and determination of pesticide residues [1].

Carbaryl (1-naphthyl *N*-methylcarbamate) is one of the most frequently detected pesticide residues in food analyses worldwide. The presence of traces of carbaryl in fruits and vegetables poses a potential hazard for consumers [2]. European Union legislation has established maximum residue level for food (Commission Regulations (EC) No. 149/2008) [3]. In the case of carbaryl, the maximum residue limit (MRL) is 0.05 mg kg^{-1}.

Numerous analytical procedures have been developed for the determination of carbaryl and its metabolites in various matrices, including water, soil, fruits, vegetables, and other crops. The preferred analytical technique for this pesticide is high-performance liquid chromatography, using ultraviolet [4], diode array [5], fluorescence [6], chemiluminescence [7], or mass spectrometric detection [8]. These chromatographic techniques are highly sensitive and reliable. However, they involve multiple steps in the sample preparation and analysis, they require specialized instrumentation, and they are expensive and time-consuming.

With an increasing importance of the immunochemical methods for pesticide screening and quantification, several formats of enzyme-linked immunosorbent assays (ELISA) were developed for monitoring carbaryl in water [9], fruits, or vegetables [10, 11].

Nowadays, the instrumental techniques combined with an immunochemical approach for the carbaryl detection were described: disc-based immunoassay microarrays [12], a piezoelectric immunosensor [13], plasmon resonance flow-through immunosensor [14], portable optical immunosensor [15], and capillary electrophoresis-base immunoassay with laser-induced fluorescence [16]. The last technique has extremely low detection limit for carbaryl (0.05 ng mL^{-1}).

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The described methods for carbaryl detection also require skilled personnel, and their application is usually restricted to the well-equipped laboratories. Recently, various immunochromatographic techniques have given rise to some very popular commercially available tools mainly in the diagnostic [17–20] and have proved to be cheap, robust and user-friendly and thus could be incorporated into the food inspection, surveillance, and monitoring programmes of regulatory agencies [21, 22]. Such immunochromatographic techniques were developed also for the carbaryl detection in agricultural products and biological samples [23–25]. In these techniques, the authors based their experiments on the polyclonal antibodies and as a marker they used colloidal gold nanoparticles.

Our previous studies described validation of ELISA for control of carbaryl residues in some food samples [26, 27]. Here, we reported a development of the sensitive immunochromatographic strip test for the carbaryl detection using monoclonal antibodies and colloidal carbon nanoparticles as a label. The optimized test has been compared with ELISA results in the analysis of spiked fruit juice samples.

Materials and methods

Reagents and materials

Standards of carbaryl (99%, HPLC/DAD), carbofuran (99.5%, HPLC/DAD), methiocarb (98.5%, HPLC/DAD) were from Dr. Ehrenstorfer GmbH, Augsburg, Germany. Aldicarb (99.9%, HPLC, Fluka), bendiocarb (99.5%, HPLC, Riedel-de Haën), ethiofencarb (99.0%, HPLC, Riedel-de Haën), fenoxycarb (99.6%, HPLC, Fluka), and methomyl (99.9%, HPLC, Fluka) were supplied by Sigma–Aldrich Inc. (St. Louis, USA). Individual stock standard solutions containing 1 mg mL^{-1} of each compound were prepared by dissolving accurately weighted amounts in ethanol and stored in darkness at 4 °C. Working standard solutions were freshly prepared by serial dilution in the running buffer (0.2 M borate buffer, pH 8.8, containing 2% BSA, 0.2% Triton).

The mouse anti-carbaryl monoclonal antibody as well as the carbaryl hapten-ovalbumin conjugate (carbaryl-OVA) was purchased from Centro de Apoyo a la Innovación, la Investigación y la Transferencia de Tecnología (CTT), Universidad Politécnica de Valencia, Spain. Producer indicated these immunoreagents as MAb/LIB-CNH45 and OVA-2NAH, respectively [27]. Rabbit anti-swine antibody (RASw) as well as the swine anti-mouse antibody (SwAM) was obtained from Nordic Immunological Laboratories, Tilburg, the Netherlands (product codes RASw/IgG(H+L)/7S and SwAM/IgG(H+L)/7S). Both antibodies were supplied as purified IgG fraction of polyclonal antiserum.

Carbon nanoparticles (Spezial Schwartz 4, Degussa AG, Germany) were kindly provided from Agrotechnology & Food Sciences Group (Wageningen University and Research Centre, the Netherlands) as a dry powder (particles of amorphous shape with average size of 120 nm).

Bovine serum albumin (BSA), Tween 20, polyethylene glycol (PEG, MW 3350), and *o*-phenylenediamine (OPD) were purchased from Sigma–Aldrich Inc. (St. Louis, USA). Horseradish peroxidase (HRP)-labelled swine anti-mouse IgG (SwAM, HRP/SwIgG = 1.81, concentration 8.9 mg mL^{-1}) was obtained from Seva Pharma, Czech Republic.

Other common chemicals were of the highest purity available and purchased from Sigma–Aldrich.

Deionized water for standards and buffer solutions was prepared on apparatus RO-TFM-5SV (Fresh Water Systems, Inc., Greenville, USA).

Various types of nitrocellulose membranes were supplied by Whatman GmbH, Dassel, Germany (PRIMA 80, PRIMA 125, AE 98 FAST, AE 98, Protran BA 79, Protran BA 83, Protran BA 85), by Millipore Ltd., Praha, Czech Republic (Immunopore FP, Immunopore RP, HiFlow Plus HF 135, HiFlow Plus HFB180, HiFlow Plus 090), by Sartorius Stedim Biotech S.A., Aubagne Cedex, France (Unisart 140), and tested in strip assay. Vinyl backing ARcare® 7823 was from Adhesives Research Inc. (USA), and the absorbent pad CFSP was from Millipore Corp. (USA). Ninety-six well ELISA polystyrene microtitre plates Costar (catalogue no. 9018) were obtained from Corning Inc. (USA).

Fruit juices samples

Samples of fruit juices were from a retail market. Carbaryl-free fruit juices, as verified by ELISA and by LC/MS analysis [26], were used without any sample treatment.

Spiked juice samples containing carbaryl were prepared as follows: carbaryl-free samples were spiked with a known amount of carbaryl derived from stock solution (1 mg mL^{-1} in ethanol) and used immediately for the matrix effect experiments and recovery studies. The concentration of ethanol in the analysed sample was very low and had no negative effect on nitrocellulose membrane.

Labelling of swine anti-mouse antibody with carbon nanoparticles

Carbon nanoparticles bind proteins non-covalently without changing their bioactivity [28]. The swine anti-mouse antibody (SwAM) was labelled with colloidal carbon nanoparticles, as described elsewhere [29]. The procedure was slightly modified as follows: a colloidal carbon suspension (carbon nanoparticles 2 mg mL^{-1} of 5 mM borate buffer,

pH 8.8) was sonicated for 10 min on ice using a Sonic 1 (Polsonic, Poland). Subsequently, with a simultaneous gentle stirring, the SwAM was added to give a final protein concentration of 350 μg mL^{-1}. Then, this mixture was stirred gently at 4 °C overnight. In the end, the suspension was washed three times in a 5 mM borate buffer, pH 8.8 (containing 1% BSA and 0.02% NaN_3) using centrifugation (10,000*g*, 15 min, 10 °C). Final sediment was resuspended to the initial volume. Prepared stock suspension of the SwAM-carbon conjugate was stored at 4 °C in the dark until used.

Preparation of immunochromatographic test strips

The carbaryl-ovalbumin conjugate (carbaryl-OVA) and rabbit anti-swine antibody (RASw) were used as immobilized capture reagents. The strips were constructed as follows: First, nitrocellulose membrane sheet was cut to the desired size (20 cm × 2.5 cm) and pasted to a vinyl backing. Then, using the Linomat V (Camag AG, Switzerland), carbaryl-OVA and RASw solutions (5 mM borate buffer, pH 8.8) were separately dispensed (1 μL per 5 mm) as the test and control lines (20 cm long) on this plastic-backed membrane. Subsequently, the membrane sheet was dried at 37 °C for 2 h and cut into test strips (25 × 5 mm) using a programmable strip cutter (Economic Cutter ZQ2000, Shanghai Kinbio Tech Co., Ltd., Shanghai, China). An absorbent pad was cut in sections of 10 × 20 mm and pasted to the far end of the strip (Fig. 1). Strips prepared in such way were stored in sealed bags under dry conditions at laboratory temperature until used.

Procedure of immunochromatographic strip

In a well of an ELISA microplate, 30 μL of the running buffer (0.2 M borate buffer, pH 8.8 (containing 2% BSA, 0.2% Triton) was mixed with 10 μL of SwAM-carbon conjugate (stock suspension diluted with the running buffer), 10 μL of anti-carbaryl antibody (diluted in the running buffer), and 50 μL of carbaryl standard solutions or fruit juice samples (tenfold diluted in running buffer). Subsequently, the membrane strip was dipped into this reactant mixture vertically (Fig. 1). After 10 min, test result was observed.

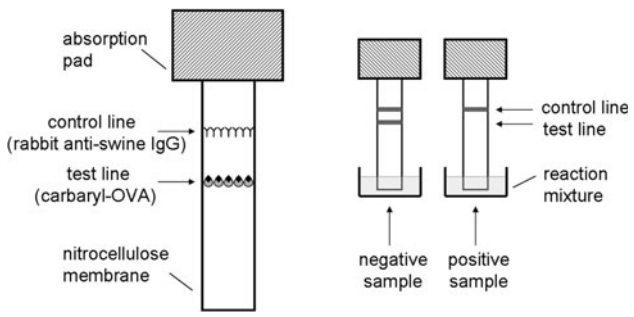

Fig. 1 Schematic diagram of the immunochromatographic strip test. Details are described in the text

Detection and quantification using immunochromatographic strip

The colour intensity of test line could be assessed visually by naked eyes. For quantification of the colour intensity, greyscale densitometry was used after the strip was fully air-dried. Greyscale digital scanned images of the strips were recorded using the Epson Perfection V700 Photo Scanner (Seiko Epson Corporation, Nagano, Japan), and the intensity of the test lines quantified using the TotalLab TL100 software (Nonlinear USA Inc., NC, USA). Average values were calculated from five parallel runs for each carbaryl concentration using three measurements for each strip. Standard curves were obtained by plotting the mean intensity value against the logarithm of carbaryl concentration. Experimental points were fitted to a four-parameter logistic equation using the Microsoft Excel software (Microsoft Corp., USA).

Enzyme-linked immunosorbent assay (ELISA)

ELISA, validated by LCMS analysis, was performed as we described previously [27].

Results and discussion

Description of the immunochromatographic strip

The scheme of the immunochromatographic strip is shown in Fig. 1. The rabbit anti-swine antibody is immobilized onto control line and conjugate carbaryl-ovalbumin on the test line. The indirect format was used in the detection procedure. Each analysed sample contains, besides carbaryl standard, or fruit juice diluted by buffer, the primary mouse anti-carbaryl antibody and the secondary swine anti-mouse antibody labelled by carbon nanoparticles. Both antibodies form detection complex. In the assay, the detection complex in the reaction mixture migrated from the well through the strip into the absorbent pad. In the absence of carbaryl in the sample, the solution of the detection complex and the excess of labelled Ab moved upward across the strip. In the test line, detection complex was captured by immobilized carbaryl-OVA conjugate to form a visible black colour line. However, in the presence of carbaryl in the sample, free carbaryl molecules bound to primary Ab, thereby inhibiting the interaction of the detection complex with the immobilized carbaryl hapten. Thus, the colour intensity of the test

line inversely correlated with carbaryl concentration in the sample.

The control line acted as a control to assure that the labelled secondary Ab migrated throughout the system. Here, it was captured by immobilized rabbit anti-swine antibody to form a visible line. If intensive colour was not present in the line, the assay was considered as invalid.

The stability of immunochromatographic strip tests was studied using carbaryl standard solution of 5 ng mL^{-1} and blank solution. Strips were stored at laboratory temperature under desiccated conditions. The running buffer, diluted stock suspension of SwAM-carbon conjugate, and anti-carbaryl antibody solution were stored at 4 °C. Three assays were performed once a week during the period of 2 months. Stability values were expressed as percentage of the test line intensity at the day zero. All these values were in the range of 82–121% without any notable trend in time. It could be assumed that immunochromatographic strip test were stable for at least 2 months.

Selection of immunoreagent concentrations

Most of components necessary for the immunochromatographic strip construction as well as the experimental conditions have a key influence on both the performance and final sensitivity.

First, in this experimental study, the concentrations of reagents were preliminary optimized to satisfy the following assay criteria: good sensitivity, minimum immunoreagent consumption, and appearance of the test line with good colour intensity and sharpness (for negative sample). Therefore, checkerboard titration experiments were carried out. Several amounts of carbaryl-OVA conjugate immobilized on the membrane (10–500 ng per strip) against different amounts of primary Ab (1–100 ng per strip) were investigated in assays of standard solutions containing carbaryl at concentrations of 0, 5, 10, and 50 ng mL^{-1}, respectively. The same experiments were performed for different concentrations of carbon-labelled secondary Ab.

Subsequently, some other factors affecting strip test performance were also evaluated as follows: the type of membrane, the type of running buffer, and the incubation time of reactant mixture before the strip is dipped inside. Thirteen various types of nitrocellulose membrane were tested. The type of membrane influenced flow time and sharpness of detection lines. Faster-flowing membranes reached endpoint more quickly but required higher reagent concentrations and provided lower sensitivity. Also, the composition of the running buffer markedly influenced flow rate, line intensity, and test sensitivity. Phosphate buffer and borate buffer of various pH values and ion concentrations were tested. Moreover, additives such as BSA (0.1–5%), gelatine (0.1–1%), polyethylene glycol (0.01–2%), sucrose (0.5–5%), and their combinations, with and without surfactants Tween 20 (0.01–0.5%) and Triton (0.01–0.5%), were tested to further improve the test performance. These factors affected mainly the character of reactant flow, level of background colour, sharpness, and intensity of the test line.

Table 1 Selected parameters of carbaryl immunochromatographic strip test

Parameter	Chosen as optimal
Type of nitrocellulose membrane	AE 98, Whatman[a]
Amount of carbaryl-OVA conjugate per strip	100 ng
Amount of primary antibody in assay	12.5 ng
Dilution of stock suspension of SwAM-carbon conjugate prior addition into assay	10-times
Composition of running buffer	0.2 M borate buffer, pH 8.8 (containing 2% BSA, 0.2% Triton)
Incubation time of reaction mixture	0 min

[a] Pore size 5 μm, capillary rise in water 160–210 s per 4 cm

Optimized conditions for immunochromatographic strip test were chosen by visual evaluation of strips as a compromise among a good colour perception of lines, good test sensitivity, minimum immunoreagent consumption, and rapid test performance. They are collected in Table 1.

Sensitivity of immunochromatographic strip test

The sensitivity of the immunochromatographic strip test was determined by checking both the carbaryl standard solutions and spiked fruit juice samples in the concentration range from zero to 100 ng mL^{-1} (Fig. 2). The visual limit of detection of the assay was defined here as the minimum carbaryl concentration producing the colour density of the test line significantly weaker than that at zero concentration (it means absolute agreement between result assessments of four observers for five repetitions of the same test). As shown in Fig. 2, the carbaryl concentration of 5 ng mL^{-1} caused a slight, but visually distinguishable, difference in the test line intensity compared to the negative control. Thus, 5 ng mL^{-1} of carbaryl was considered to be the visual detection limit for the immunochromatographic strip test. The test line was invisible at concentrations of carbaryl >10 ng mL^{-1}.

Finally, to obtain semiquantitative results and verify the visual limit of detection, greyscale densities of the test lines were recorded using the Epson Perfection V700 Photo Scanner (Seiko Epson Corporation, Nagano, Japan). The mean signal values were fitted to a sigmoid equation and displayed as the standard curve (Fig. 3). To express the assay sensitivity (Table 2), the limit of detection for

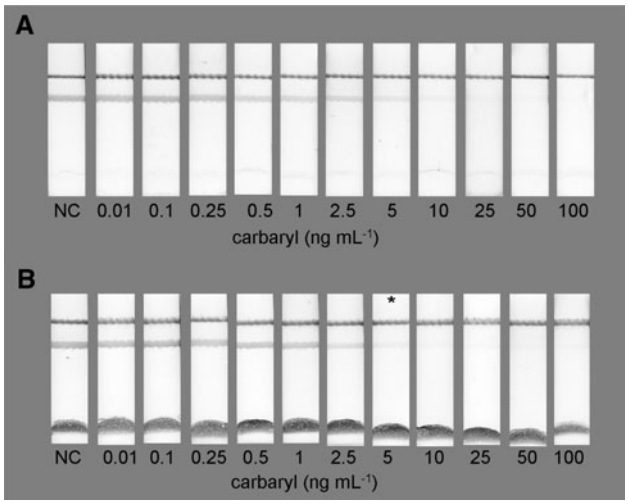

Fig. 2 The concentration range of carbaryl standard (**a**) and carbaryl in 10% orange juice (**b**) assayed by immunochromatographic strip test. *NC*, negative control. The concentration 5 ng mL^{-1} caused considerably weak test line still visually distinguishable from the negative control

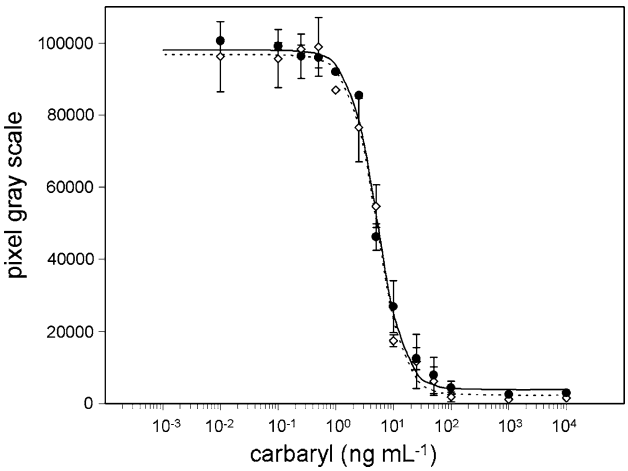

Fig. 3 Standard curves of carbaryl in buffer (*open diamond*) and in 10% orange juice (*closed circle*). The optimized immunochromatographic strip tests were measured by the Epson Perfection V700 Photo Scanner and evaluated by four-parameter logistic fit. The *error bars* correspond to the standard deviations of the data points ($n = 5$)

carbaryl in buffer (1.58 ± 0.22 ng mL^{-1}) was estimated as the analyte concentration providing a 10% decrease in the blank signal. Subsequently, the linear working range of 2.5–11.6 ng mL^{-1} was determined as the concentrations causing 20–80% inhibition of the maximum assay signal, and the 50% inhibition (IC_{50}) value of 5.12 ± 0.40 ng mL^{-1} related to the inflection point of the curve was calculated.

The ability of the developed immunochromatographic strip to detect lower carbaryl concentrations than the levels established by the European legislation as maximum residue limit (MRL) 0.05 mg kg^{-1} [3] illustrated the potential of this technology for the analysis of fruit juices. The value of visual detection limit observed in our work is comparable or even lower than those described for carbaryl immunochromatographic detection by the other authors who used polyclonal antibodies and colloidal gold as a label [23–25]. Our results are also comparable with the sophisticated instrument immunoassays [13–15].

Table 2 The characteristics of carbaryl standard curves for immunochromatographic strip in buffer and orange juice obtained using the Epson Perfection V700 Photo Scanner

Sample	Linear working range[a] (ng mL^{-1})	Limit of detection ± SD[b] (ng mL^{-1})	IC_{50} ± SD[b] (ng mL^{-1})
Buffer	2.5–11.6	1.58 ± 0.22	5.12 ± 0.40
10% Orange juice	2.5–12.2	1.63 ± 0.37	5.16 ± 0.96

Values were extracted from the four-parameter equations used to fit the standard curves

[a] Expressed as the concentration range causing 20–80% inhibition of the maximal assay signal

[b] Standard deviation

Specificity of immunochromatographic strip test

To determine the selectivity of the developed immunochromatographic strip test for carbaryl, a study of cross-reactivity was carried out under the optimized conditions. Figure 4 shows that even two-order higher concentration of cross-reacting pesticide (e.g. carbofuran) has no influence on the result of the assay. Standard solution of carbofuran in concentration 10 µg mL^{-1} was applied into reactant mixture of immunochromatographic strip test together with carbaryl (strip 2) and instead of carbaryl (strip 3). The similar results (data not shown) were obtained with the following pesticides: methiocarb, aldicarb, bendiocarb, ethiofencarb, fenoxycarb, methomyl. No inhibition of the test line intensity

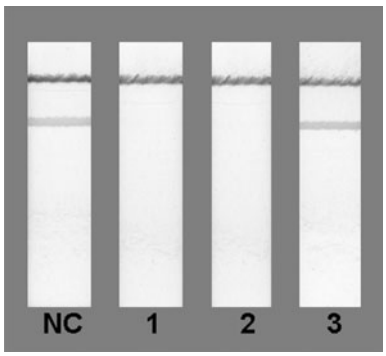

Fig. 4 Cross-reactivity of carbofuran in the carbaryl assay. *NC* negative control, *1* carbaryl 0.1 µg mL^{-1}, *2* carbaryl 0.1 µg mL^{-1} and carbofuran 10 µg mL^{-1}, *3* carbofuran 10 µg mL^{-1}

was found even at instrumental assessment of strips. These results indicated that the cross-reactions of mentioned pesticides were <1%. Similarly, other authors [9] described only negligible cross-reactions of some structurally related compounds in ELISA for carbaryl, using the same monoclonal anti-carbaryl antibody and carbaryl-OVA conjugate as used in this work.

Matrix effects

Immunochemical methods for the detection of pesticides have many advantages. On the other hand, these methods are susceptible to the matrix interference of food samples [24]. Therefore, it is advisable to determine the importance of possible matrix effects before the application of immunochromatographic strip test to real samples. The matrix effect, in case of fruit juices, can be overcome by their simple dilution with buffer [27]. To investigate the potential effect of sample matrix on strip test, we compared standard curves performed with carbaryl in buffer and juices, respectively. No matrix effect of orange juice was observed at tenfold dilution in borate buffer, pH 8.8. This result is demonstrated on Fig. 3. The obtained curves were nearly the same as can be seen from their analytical values shown in Table 2. Accordingly, the developed immunochromatographic strip test seemed to be directly applicable to the fruit juices screening after tenfold dilution by buffer as matrix effects were negligible. The same results were obtained with the other tested fruit juices: pear, apple, and banana (data not shown).

Comparison of immunochromatographic strip test with ELISA in recovery study

The accuracy and repeatability of the optimized immunochromatographic strip test were evaluated through recovery studies with samples of fruit juices spiked at carbaryl levels from 50 to 500 ng mL^{-1}. The spiked samples were tenfold diluted before analysis. The immunochromatographic strip test results (Table 3) found by naked eye and using quantification by photo scanner were compared with those found by ELISA validated by LCMS analysis [26]. By visual detection, all carbaryl-spiked samples were assessed as positive.

Using the quantitative immunochromatographic strip test, the recovery of the spiked concentrations in the fruit juices was satisfactory, as all data fell into the range of

Table 3 Overview of the results obtained by immunochromatographic strips (ICS) and enzyme immunoassay (ELISA) of juice samples spiked by different carbaryl concentration ($n = 5$)

Type of sample	Carbaryl concentration[a] (ng mL^{-1})	ICS results				ELISA results		
		Visual detection[b]	Found[c] (ng mL^{-1})	Recovery (%)	RSD (%)	Found[c] (ng mL^{-1})	Recovery (%)	RSD (%)
Banana juice	0	− − − − −	<LOD	–	–	<LOD	–	–
	5	± ± ± ± ±	4.2	82.0	18.2	4.1	82.1	15.9
	10	± ± ± ± ±	8.1	81.0	16.7	8.8	88.0	18.4
	50	+ + + + +	39.0	78.0	12.0	41.3	82.6	12.9
Pear juice	0	− − − − −	<LOD	–	–	<LOD	–	–
	5	± ± ± ± ±	4.2	84.0	16.9	4.3	86.0	14.8
	10	± ± ± ± ±	9.2	92.0	15.6	9.5	95.1	16.4
	50	+ + + + +	40.5	81.0	12.1	42.3	84.6	11.9
Apple juice	0	− − − − −	<LOD	–	–	<LOD	–	–
	5	± ± ± ± ±	4.4.	88.0	16.6	4.5	90.0	14.9
	10	± ± ± ± ±	8.9	89.0	12.1	9.0	90.0	13.7
	50	+ + + + +	46.1	92.2	10.9	44.3	88.6	10.2
Orange juice	0	− − − − −	<LOD	–	–	<LOD	–	–
	5	± ± ± ± ±	4.1	82.0	15.2	4.5	90.0	14.3
	10	± ± ± ± ±	8.6	86.0	12.0	9.1	92.1	13.9
	50	+ + + + +	42.5	90.0	9.6	45.0	90.0	9.6

[a] To avoid matrix effect, all analysed samples were tenfold diluted before analysis

[b] Visual assessment of the test line; (−) negative result (concentration <2.5 ng mL^{-1}); (+) positive result (concentration >10 ng mL^{-1}); (±) weakly positive result (concentration in the working range 2.5–12.2 ng mL^{-1})

[c] Quantitative assessment of ICS using densitometry

RSD Relative standard deviation

LOD Lmit of detection

78.1–92.2%. In ELISA, the recoveries ranged from 82.1 to 95.1%. Moreover, the method reproducibility was also very similar for both analytical systems, with relative standard deviation (RSD) ranging from 9.6 to 18.2% for the immunochromatographic strip test and from 9.6 to 18.4% for the ELISA.

Conclusions

We have successfully demonstrated an approach to carbon particle-based immunochromatographic strip test for the rapid and sensitive monitoring of carbaryl in fruit juices. The carbaryl test, which used colloidal carbon nanoparticles as a tracer to provide visual evidence of the presence of this insecticide in fruit juices within 10 min, was developed. This assay is simple, easy to perform, allowing for non-specialized personnel on-site application without requirement for intensive labour and any device for sample analysis. These characteristics make the immunochromatographic strip test useful for incorporation into food analysis. Moreover, by the use of a scanner for quantitative strip evaluation, the immunochromatographic strip test allowed the analysis of fruit juices with acceptable accuracy. It showed a potential to be a rapid and inexpensive method for carbaryl assay in laboratory.

Acknowledgments The authors are grateful to financial support from the Grant Agency of the Czech Republic (project No. 525/07/P273) and to Ministry of Education of the Czech Republic (project No. MSMT 6046137305).

Conflict of interest statement The authors declare that they have no conflict of interest.

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Příloha 8:

Fojtíková L., Göselová S., <u>Holubová B</u>.: Anabolické androgenní steroidy – nebezpečí v doplňcích stravy. *Chem. Listy* 109(12): 913-917, 2015.

ANABOLICKÉ ANDROGENNÍ STEROIDY – NEBEZPEČÍ V DOPLŇCÍCH STRAVY

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Došlo 28.4.15, přijato 23.6.15.

Klíčová slova: anabolické androgenní steroidy, testosteron, doplňky stravy, doping

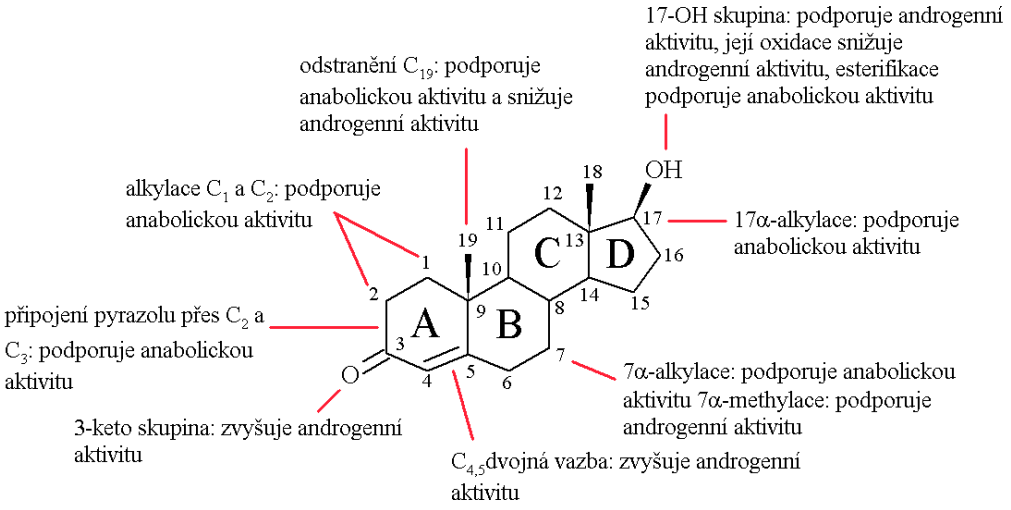
Obsah

1. Úvod

Anabolické androgenní steroidy (AAS) jsou v dnešní době využívány nejen v lékařství na podporu léčebných postupů, ale i ve sportu jako nepovolený doping na podporu rychlého nárůstu svalové hmoty a celkového zesílení organismu. Volný prodej těchto látek je zakázaný, přesto se mohou nelegálně dostat do prodeje například ve formě doplňků stravy, aniž je to uvedeno na etiketě obalu. Toto jednání ohrožuje především zákazníky, kteří kupují produkt bez deklarovaného obsahu anabolik, a tudíž tyto látky užívají nevědomky. Užívání AAS s sebou nese závažné vedlejší účinky jak po stránce fyziologické, tak psychologické. Kromě toho se spotřebitelé vystavují riziku pozitivního dopingového testu. Z těchto důvodů je vhodné, aby existoval dohled nad zdravotní nezávadností doplňků stravy a byly k dispozici metody, jež by umožnily AAS v doplňcích stravy spolehlivě odhalit.

2. Anabolické androgenní steroidy

Anabolické androgenní steroidy (AAS) jsou syntetické deriváty testosteronu. Ovlivňují vývoj a funkci mužských pohlavních orgánů, spermatogenezi v Sertoliho buňkách a vývoj sekundárních pohlavních znaků, což je souhrnně označováno jako „androgenní“ působení. Vyznačují se však i tzv. „anabolickým“ efektem, kdy působí periferně a stimulují retenci dusíku v těle a syntézu proteinů především ve svalech[1].



Obr. 1. **Vliv změn struktury anabolických androgenních steroidů na biologickou funkci** (převzato z cit.[2])

Strukturní modifikací testosteronu je možné ovlivnit poměr anabolických a androgenních vlastností (zvýšit účinnost a biologickou aktivitu) a způsob, jakým bude daný steroid aplikován do organismu (obr. 1)[2]. V současné době však není dostupný žádný steroid, který by měl pouze anabolické nebo pouze androgenní vlastnosti.

Obecně lze říci, že rozeznáváme tři typy analogů testosteronu. První typ je charakterizován esterifikací 17β-hydroxylové skupiny karboxylovou kyselinou, která má za následek zvýšenou odolnost vůči biodegradaci a delší biologickou aktivitu steroidů. Esterifikované steroidy jsou lépe rozpustné v nepolárním prostředí, čehož je využito např. při intramuskulárním podání. Délka působení jednotlivých preparátů závisí na druhu kyseliny použité k esterifikaci. Může se pak jednat o acetát, propionát, fenylpropionát, cyklopentylpropionát, dekanoát aj. příslušného steroidu. Nejznámějším zástupcem této skupiny je testosteron propionát. Druhý typ látek se vyznačuje alkylací atomu uhlíku v pozici 17α, která umožňuje příjem steroidů orální cestou. Bez této modifikace by byly orálně podávané steroidy prakticky neúčinné, neboť by část podané dávky byla ještě před vstupem do systémové cirkulace metabolizována v játrech a došlo by ke snížení účinku steroidu[3]. Pomalejší odbourávání je ovšem spjato s jejich zvýšenou jaterní toxicitou. Jako příklad lze uvést methyltestosteron, stanozol, či oxandrolon. Poslední typ vzniká modifikací A, B, nebo C kruhu. Příkladem může být boldenon, který obsahuje dvojnou vazbu v poloze C_1. Druhý a třetí typ látek je polárního charakteru, což má za následek renální exkreci.

2.1. Lékařské využití AAS

AAS jsou součástí tradiční medicíny již řadu let a hrají důležitou roli v léčbě mnoha chorob. Od 40. let 20. století jsou předepisovány především při stavech celkového oslabení organismu v důsledku lidské imunitní nedostatečnosti (HIV), při rozsáhlých popáleninách, po operacích a radiační léčbě, při anemiích, jaterních a ledvinových selháních, plicních obtížích i při onemocnění rakovinou[4,5]. Asi nejčastěji jsou aplikovány u pacientů dlouhodobě trpících svalovou dystrofií, kdy je anabolická funkce steroidů nesporným kladem ve srovnání s jejich vedlejšími účinky. Jsou známy i případy podávání anabolických steroidů pro zlepšení psychického stavu pacientů trpících depresemi[4].

Vzhledem k tomu, že s užíváním AAS se pojí široké spektrum negativních vedlejších účinků (jak bude popsáno v kapitole 2.3.), je při terapii AAS nutné dobře zvážit, zdali příznivý účinek na zdravotní stav pacienta dostatečně převyšuje negativní dopady a velmi důkladně hlídat dávkování a stav pacienta během léčby[4].

2.2. AAS a sport

Původním účelem syntézy anabolických steroidů bylo jejich využití v medicíně při léčbě závažných onemocnění. Pro své již zmiňované účinky však našly uplatnění i v řadě sportovních odvětví. Očekávání sportovců od užívání AAS se odlišuje podle sportu, který provozují. Vzpěrači chtějí zvýšit svou fyzickou sílu, kulturisté zvýšit nárůst objemu svalové hmoty a snížit obsah tuku v těle, rychlostní sportovci, jako plavci a běžci, hledají možnost zvýšit intenzitu tréninku na maximum bez fyzického kolapsu[6]. Otázka anabolik se ovšem netýká pouze vrcholových sportovců, čím dál více po nich sahají zejména mladší vyznavači bodybuildingu, jejichž cílem je snadné dosažení „vysněných tvarů“ svalových partií těla.

Užívání AAS

Nejběžněji se AAS užívají orálně a intramuskulárně. V dnešní době bývají dostupné také ve formě krémů či gelů. Použití těchto neobvyklých aplikačních forem souvisí se snahou přelstít antidopingové testy. Uživatelé často aplikují několik různých typů AAS najednou, čímž se snaží předejít vypěstování si tolerance vůči jednomu z nich. Ve snaze získat co největší objem svalové hmoty dochází velmi často k „předávkování“. Jsou podávány dávky 10× až 100× větší než pro léčebné účely[7].

Legislativa AAS ve sportovní oblasti

Za zachování ducha fair play ve sportu začal mezi prvními bojovat Mezinárodní olympijský výbor a to již v roce 1967, kdy vydal první listinu zakázaných látek a metod dopingu ve sportu. Dnes na dodržování sportovních ustanovení daných dokumenty, jako jsou „Světový antidopingový kodex“ či „Směrnice pro kontrolu a postih dopingu ve sportu“, dohlíží na mezinárodním poli Světová antidopingová agentura (WADA, z angl. World Anti-Doping Agency), na poli tuzemském Antidopingový výbor České republiky (ADV ČR). Ty také provádí samotné antidopingové testy u sportovců. Pro ně je každoročně vydáván aktualizovaný „Seznam zakázaných látek a metod dopingu“. Anabolické steroidy na tomto seznamu figurují od roku 1975.

Plošné omezení anabolik pouze pro terapeutické užití bylo před sedmi lety začleněno i do českého trestního zákoníku. Přesto i dnes velké množství webových stránek obhajuje „bezpečné“ užívání steroidů, obsahuje mylné informace o AAS a bagatelizuje rizika jejich užívání. Mnohé z nich také nabízí internetový prodej AAS bez lékařského předpisu.

2.3. Vedlejší účinky při užívání AAS

Nebezpečí AAS spočívá v řadě vedlejších účinků a rizik z nich plynoucích. Rizika užívání jsou závislá na věku, pohlaví, fyzických dispozicích, individuální toleranci, typu AAS, celkové dávce expozice a reverzibilitě či ireverzibilitě změn.

AAS jsou podle mezinárodní agentury pro výzkum rakoviny IARC (z angl. International Agency for Research on Cancer) zařazeny do třídy 2A (cit.[8]). Do této kategorie patří látky pravděpodobně karcinogenní pro člověka, tzn., že epidemiologické důkazy nejsou jednoznačně průkazné, avšak karcinogenita těchto látek byla dostatečně prokázána na experimentálních zvířatech.

K nejzávažnějším vedlejším účinkům patří poruchy plodnosti, hypertenze[9], poruchy koagulace[10], poškození jater[11], poruchy lipidového metabolismu[12], ruptury svalů a šlach[13].

Nadměrné dávky AAS mohou u mužů vlivem přeměny na estrogeny podnítit růst prsní tkáně a vznik gynekomastie[14]. U žen přílišné dávky steroidů snižují endogenní tvorbu progesteronu a estrogenů, s čímž souvisí poruchy menstruačního cyklu, které mohou vyústit v neplodnost[15].

Byly prováděny také studie zabývající se vlivem AAS na lidskou psychiku, přičemž byla zaznamenána souvislost mezi agresivitou, úzkostí a dlouhodobým užíváním AAS. Osoby na počátku užívání popisovaly stavy euforie a mánie, dlouhodobí uživatelé pociťovali úzkost, podrážděnost, pocity nepřátelství a tendence negativně hodnotit sami sebe i své okolí[16,17].

3. Doplňky stravy

Vzhledem k současnému trendu v oblasti životního stylu a masivní reklamě zaměřené na užívání široké škály preparátů „zajišťujících“ formování postavy nebo hubnutí dochází v posledních letech k značnému rozvoji obchodu s těmito produkty.

3.1. Legislativa doplňků stravy

Podle zákona o potravinách č. 456/2004 Sb. (úplné znění zákona č. 110/1997 Sb.) jsou doplňky stravy definovány jako potraviny určené k přímé spotřebě, které se odlišují od potravin pro běžnou spotřebu vysokým obsahem vitaminů, minerálních látek nebo jiných látek s nutričním nebo fyziologickým účinkem a které byly vyrobeny za účelem doplnění běžné stravy spotřebitele na úroveň příznivě ovlivňující jeho zdravotní stav[18].

Požadavky na doplňky stravy stanovuje Vyhláška č. 352/2009 Sb.[19]. V doplňcích stravy nesmí být přítomny anabolické látky.

Od začátku roku 2010 platí nový trestní zákoník, podle kterého spadá mezi trestné činy přechovávání, výroba, dovoz, nabízení a prodej látek s anabolickými nebo jinými hormonálními účinky za jiným než léčebným účinkem. V minulosti byly nálezy příměsi anabolických steroidů v doplňcích stravy řešeny jako správní delikt distribuce nebezpečné potraviny, avšak podle současné legislativy hrozí tomu, kdo neoprávněně distribuuje ve větším množství anabolické steroidy, odnětí svobody až na jeden rok. V České republice podléhají doplňky stravy, stejně jako potraviny, dozoru Státní zemědělské a potravinářské inspekce (SZPI). Při řešení případů nálezů AAS v doplňcích stravy spolupracuje SZPI s Národní protidrogovou centrálou.

Tabulka I
Přehled zakázaných potravinových doplňků v ČR, v kterých byla Státní zemědělskou a potravinářskou inspekcí potvrzena přítomnost steroidních látek (sledované období 2004–2014)[26]

Rok	Název výrobku	Zakázaná látka	Typ	Množství
2006	MULTIPOWER MUSCLE PROFESSIONAL	nandrolon, testosteron, DHEA[a]	AAS[b]	neuvedeno
	Creatine Pyruvate	19-norandrostendion a androstandion	AAS	neuvedeno
2007	Young Star	dehydroepiandrosteron	AAS	13000 mg/kg
2008	San ATTITUDE	androstendion a DHEA	AAS	5460 mg/kg a 630 mg/kg
2009	Animal cuts	progesteron	SH[c]	neuvedeno
2014	Tribulus Terrestris 90% saponins	1-dehydroandrostenedion	AAS	0,103 mg/kg
	Amix Per4max booster	progesteron	SH	13,8 mg/kg
	WEIDER TRIBULUS TERRESTRIS	1-dehydroandrostenedion	AAS	1,34 mg/kg
	TRIBULUS FORTE	1-dehydroandrostenedion	AAS	0,256 mg/kg
	VEMOHERB BULGARIAN TRIBULUS	1-dehydroandrostenedion	AAS	0,103 mg/kg
	COMPRESS FEVER	progesteron	SH	1,76 mg/kg
	AMIX Shake 4 Fit & Slim	progesteron	SH	4,70 mg/kg
	AMIX Anabolic Masster	progesteron	SH	3,16 mg/kg
	TRIBU	1-dehydroandrostenedion	AAS	neuvedeno
	SURVIVAL Tribulus Terrestris 90%	1-dehydroandrostenedion	AAS	0,476 mg/kg

[a] Dehydroepiandrosteron, [b] anabolický androgenní steroid, [c] steroidní hormon

3.2. Otázka kontaminovaných doplňků stravy ve světě a u nás

Stále častěji je v literatuře diskutována otázka potravinových doplňků, zejména doplňků stravy, u kterých byl nalezen obsah nepovolených anabolických steroidů[20–23]. V rámci první velké studie probíhající v letech 2001 a 2002 bylo testováno 634 doplňků stravy, které byly volně dostupné ke koupi ve 13 různých zemích. Třináct procent z těchto nehormonálních přípravků bylo kontaminováno anabolickými androgenními steroidy. Od roku 2002 byla celá řada dalších přípravků označena za nebezpečné, protože u nich byla prokázána kontaminace vysokými koncentracemi AAS např. methandienonem, stanazolem, boldenonem, testosteronem a oxandrolonem[24]. Tyto látky samozřejmě nebyly deklarovány na etiketě výrobku a mohly způsobit nejen pozitivní dopingový test, ale především velké množství zdravotních problémů[25].

V ČR byl opakovaně zaznamenán výskyt doplňků stravy určených pro sportovce, které obsahovaly anabolické steroidy. SZPI se začala doplňky stravy zabývat jako samostatnou skupinou od roku 2004. Od té doby zakázala během svého šetření prodej hned několika doplňků stravy[26]. V tab. I je přehledně zpracován seznam zakázaných výrobků doplňků stravy, ve kterých byla od roku 2004 potvrzena SZPI přítomnost AAS.

Výše uvedené výsledky zmíněných studií poukazují na nutnou obezřetnost při koupi potravinových doplňků, zejména doplňků stravy pro sportovce. Je třeba mít na paměti bezpečnost, resp. možnou nebezpečnost související s jejich užíváním.

4. Metody stanovení

Na analýzu anabolických androgenních steroidů lze obecně pohlížet dvěma způsoby. Buď z pohledu epidemiologických studií, které jsou spíše zaměřeny na analýzu konkrétní látky, nebo alespoň konkrétní skupiny látek, anebo z pohledu „screeningu“ (antidopingové kontroly, kontrola potravin), kdy je cílem identifikovat co možná nejvíce zakázaných látek ze vzorku.

Kontrola výskytu steroidů v různých biologických matricích se běžně provádí pomocí plynové chromatografie (GC) nebo kapalinové chromatografie (LC) v kombinaci s vhodným detektorem. Běžně používané UV detektory nejsou schopny splnit požadavky kladené na detekční limity metod, a proto jsou při analýzách nejčastěji používány detektory hmotnostní spektrometrie (MS), nebo tandemové hmotnostní spektrometrie (MS/MS).

V posledních letech získala metoda LC-MS/MS oproti GC-MS či GC-MS/MS na popularitě. Důvodem jejího zvýšeného používání je poměrně snadná automatizace, která činí tuto metodu ideální pro rutinní diagnostiku. Další výhodou je možnost detegovat při analýze vzorků krve a moči konjugáty steroidů i nekonjugované steroidy bez předchozí derivatizace, s čímž souvisí nižší časová náročnost metody. Oproti tomu při plynové chromatografii je derivatizace nutná[27]. Běžně je možno dosáhnout limitu kvantifikace v řádech desítek pg ml^{-1} (cit.[28]).

V České republice je při kontrole potravin na průkaz anabolických steroidů využívaná laboratoří SZPI metoda plynové chromatografie, konkrétně dvojrozměrné plynové chromatografie[29].

K detekci nízkomolekulárních látek (mezi které patří AAS) lze rovněž aplikovat imunochemické metody, jako je enzymová imunoanalýza (ELISA) nebo imunochromatografie v laterálním toku na membráně (LFIA)[30,31]. Metody pro stanovení AAS jsou založeny na specifické interakci steroid-protilátka. K jejich výhodám patří citlivost (mez detekce pro stanovení steroidů se pohybuje v rozmezí 10 až 500 pg ml^{-1}), jednoduchost, rychlost a možnost použití přímo v terénu. Tyto výhody z nich činí vhodnou screeningovou metodu. Publikována byla například imunoanalýza pro detekci anabolického steroidu nortestosteronu v doplňcích stravy[32]. Metoda ELISA byla využita také ve studiích pro stanovení anabolického steroidu boldenonu[33,34].

5. Závěr

V současné době je povoleno, aby anabolické androgenní steroidy mohly být pouze součástí léčiv, jejichž užívání je možné výhradně na lékařský předpis. Přesto jsou v ČR i v zahraničí opakovaně zaznamenávány nezákonné výskyty těchto látek v doplňcích stravy určených nejen pro sportovce. Jak bylo zmíněno v tomto přehledu, AAS jsou pro lidský organismus velmi škodlivé, a proto je potřeba tyto nebezpečné kontaminované výrobky rychle odhalit a zamezit jejich prodeji. Instituce dohlížející na zdravotní nezávadnost doplňků stravy mají k dispozici chromatografické metody, jež tyto látky spolehlivě prokáží. Požadavky na rychlou a spolehlivou detekci splňují i nově vyvíjené imunochemické metody. Tyto metody jsou mnohem méně náročné na přístrojové vybavení a kvalifikaci personálu než chromatografické. V budoucnu by je v podobě uživatelsky přívětivých testů mohly využít nejen zákonodárné instituce, ale i laická veřejnost.

Autoři děkují Grantové agentuře MV ČR za finanční podporu projektu MV0 VG20112015045.

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L. Fojtíková, **S. Göselová, and B. Holubová** *(Department of Biochemistry and Microbiology, University of Chemistry and Technology, Prague):* **Anabolic Androgenic Steroids – Hazard in Food Supplements**

In the last decade, several immunoassays have been published as the alternative/complementary rapid methods for steroid analysis in food supplements. The present review shows a significant amount of food supplements containing banned anabolic androgenic steroids that are not declared as ingredients thus presenting risk for consumers and may lead to positive results in anti-doping controls. Traditional methods for analysis of steroids such as LC/MS and GC/MS were used for monitoring suspect food supplements.

Příloha 9:

Fojtíková L., Fukal L., Blažková M., Sýkorová S., Kuchař M., Mikšátková P., Lapčík O., Holubová B.: Development of enzyme-linked immunosorbent assay for determination of boldenone in dietary supplements. *Food Anal. Methods* 9(11): 3179-3186, 2016.

Food Anal. Methods (2016) 9:3179–3186
DOI 10.1007/s12161-016-0511-9

Development of Enzyme-Linked Immunosorbent Assay for Determination of Boldenone in Dietary Supplements

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Received: 26 January 2016 / Accepted: 11 April 2016 / Published online: 15 April 2016

Abstract There is an increasing interest in the investigation and implementation of methods for the analysis of anabolic steroids in dietary supplements. In this study, a competitive indirect enzyme-linked immunosorbent assay (ELISA) has been developed for the detection of boldenone. For this purpose, an antiserum against boldenone was raised in a rabbit using boldenone-17-hemisuccinate-bovine serum albumin as an immunization conjugate. Based on the ELISA standard curve, the detection limit was as low as 0.014 ± 0.007 ng mL^{-1} with the IC_{50} value of 0.293 ± 0.084 ng mL^{-1} and linear working range of 0.065–1.529 ng mL^{-1}. The intra- and inter-assay variations were found to be satisfactory. Relative standard deviations were calculated in the range of 3.8–10.5 and 7.3–12.9 %. The developed ELISA was applied in the analysis of extracts obtained from spiked samples of dietary supplements. Ethanol extracts were applied into the immunoassay without a cleanup procedure (only diluted) to minimize the effect of the matrix. Recoveries from spiked samples were from 86.0 to 115.7 %. An excellent correlation between ELISA and UHPLC-MS/MS was obtained with the linear equation of $y = 1.0256\,x - 0.7772$ ($R^2 = 0.9999$). This ELISA as proposed here could be successfully applied for the simple monitoring of boldenone in dietary supplements.

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Keywords ELISA · Boldenone · Anabolic steroid · Dietary supplement

Introduction

Dietary supplements with strength and muscle bulk-enhancing effects usually contain amino acids, proteins, prohormones, and creatine as their active components. A lot of these dietary supplements are freely available on numerous internet websites, which leads to huge expansion in their applications. In recent years, there have been growing concerns about the illicit presence of anabolic steroids in dietary supplements (Abbate et al. 2015; Baume et al. 2006; Geyer et al. 2004; Maughan 2005; Odoardi et al. 2015; Petroczi et al. 2011). Anabolic steroids are added into dietary supplements due to their effect enhancing the physical condition, body mass, and muscle strength, although the application of these steroids is banned in EU countries, except for medical treatment (Schanzer and Donike 1992).

Boldenone, with the correct systematic name androsta-1,4-diene-17-ol-3-one (Fig. 1), ranks second among the most frequently appearing anabolic steroids in cases when these compounds were identified in dietary supplements (Van Poucke et al. 2007). Together with other anabolic steroids, the International Agency for Research on Cancer (IARC) classifies boldenone as class 2A (growth promoters—steroids), i.e., as a possible human carcinogen (e.g., prostate and liver tumors) (Oda and El-Ashmawy 2012). Adverse effects of boldenone applications were already assessed in several studies (de Souza and Hallak 2011; Garcia et al. 1987; Groot and Biolatti 2004; Tousson et al. 2012).

The most frequent method applied in boldenone determination is liquid chromatography (Buiarelli et al. 2005; Destrez et al. 2009; Draisci et al. 2003; Van Poucke et al. 2007) or gas chromatography coupled with mass spectrometry (Gaillard et al. 1999;

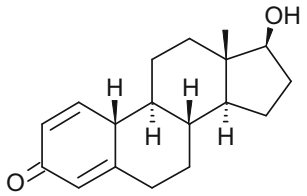


Fig. 1 Structure of boldenone

Gambelunghe et al. 2007; Stepan et al. 2008). Chromatographic analyses provide sensitive and specific techniques, but they also require highly skilled operators and expensive equipment. Moreover, laborious sample pretreatment procedures involve numerous extraction (cleanup) steps that are quite time-consuming and not suitable for the routine analysis of large amounts of samples.

In the light of recent reports on the contamination of dietary supplements with anabolic steroids and due to serious health risks associated with their use, it is desirable to have a simple, rapid, cost-effective and sensitive method to monitor the compliance of dietary supplements with the ban imposed on any addition of anabolic compounds (to check for any forbidden addition of boldenone). Immunochemical methods just possess such desired properties. The possibility of analyzing liquid samples or solid sample extracts without any purification is one of the most outstanding advantages the immunoassays have over the instrumental methods commonly applied. However, immunochemical interactions are not completely free from interferences caused by unidentified compounds of matrices. Although enzyme-linked immunosorbent assay (ELISA) for boldenone detection in urine and blood has already been described (Hagedorn et al. 1994; Lu et al. 2006) and commercial kits are available, no assay has been designed especially for the boldenone detection with respect to dietary supplements. As is well known, body fluid samples (urine, blood) have lower matrix effects in simple immunoassays that do not use the cleaning of samples in comparison with the analysis of solid sample extracts (dietary supplements). On the other hand, commercial kits are designed for animal tissue analyses and their procedures involve complex sample pretreatment with extraction, solvent evaporation and residual reconstitution. Therefore, the aim of this study was to develop a sensitive immunochemical method that would be as simple as possible to be applied by the state authorities for the monitoring of boldenone in dietary supplements.

Materials and Methods

Chemicals and Materials

The boldenone standard and all the other steroid standards used in cross-reactivity studies were obtained from Steraloids, Newport, Rhode Island, USA. Individual stock standard solutions containing 1 mg mL^{-1} of each compound were prepared by dissolving precisely weighed amounts in 96 % ethanol and stored at −28 °C. Working standard solutions were freshly prepared by gradual dilution in an assay buffer. Bovine serum albumin (BSA), ovalbumin (OVA), dimethyl sulfoxide (DMSO), 3,3′,5,5′-tetramethylbenzidine (TMB), and H_2O_2 (30 % *v/v*) were purchased from Sigma-Aldrich, St. Louis, USA. Peroxidase-labelled goat anti-rabbit IgG (GAR-Po) antibody was supplied by Nordic-MUbio, Netherlands, and 96-well polystyrene microtiter plates Costar 9018 were purchased from Corning Inc., USA. Applied microplate washer Columbus Pro was from Tecan Group, Austria, and microplate reader uQuant BIO-TEK was provided by Inc. Winooski, USA. The following buffers were used for the immunoassay: coating buffer (0.01 M carbonate/bicarbonate buffer pH 9.6); assay buffer (0.01 M phosphate-buffered saline (PBS), pH 7.4); wash buffer (0.01 M PBS pH 7.4 containing Tween 20 (0.05 % *v/v*)); substrate solution for enzyme (9 mL 0.05 M citrate/phosphate buffer pH 5.0, 1 mg of TMB, 1 mL of DMSO, and 2 μl of 30 % H_2O_2 (*v/v*))); and stopping solution (2 M sulfuric acid in distilled water). The following samples of dietary supplements were purchased from local shops: 100 % WHEY PROTEIN (apple-cinnamon) (Scitec Nutrition, Orlando, USA), Egg amino 6000 (tablets) (Amix™, Manchester, UK), and GS ExtraStrong Multivitamin (Green-Swan Pharmaceuticals CR, a.s., Prague, CZ).

Synthesis of Hapten and General Procedure of Conjugate Preparation

Reagents and solvents were acquired from commercial sources and used after distillation. Reactions were monitored with the aid of thin-layer chromatography (TLC silica gel 60F254), and visualization was carried out with UV light. Here, chromatography refers to the flash column chromatography and it was performed with the indicated solvents on a silica gel (particle size 0.040–0.060 mm) or in a reverse phase column (Redisep RF Gold C18). NMR spectra were recorded in CDCl3 or CD3OD at room temperature by Varian Gemini 300 MHz (1H NMR 300 MHz, 13C NMR 75 MHz) and Agilent 400 MR DDR2 (1H NMR 400 MHz, 13C NMR 100 MHz). The spectra were referenced to residual solvent protons in the 1H NMR spectra (7.26 ppm for CDCl3 and 4.84 and 3.31 ppm for CD3OD) and to solvent carbons in the 13C NMR spectra (77.0 ppm for CDCl3 and 49.05 ppm for CD3OD). The complete assignment of 1H and 13C chemical shifts of all compounds was realized on the basis of the combination of correlation spectroscopy and heteronuclear single quantum coherence experiments. The peak intensity is given as strong (s), medium (m), or weak (w). MS and high-

resolution MS were carried out either with ESI ionization (EI, 70 eV) or in a fast atom bombardment mode.

For the derivatization of boldenone in position C_{17}, boldenone was treated with succinic anhydride in the standard procedure (Chen and Zhang 2008). Succinic anhydride (630 mg, 6.28 mmol) was added to the solution of boldenone (180 mg; 0.63 mmol) in dry pyridine (6 mL). After 6 h of stirring at 90 °C, the reaction mixture was allowed to cool down to the ambient temperature and subsequently treated with saturated aqueous sodium bicarbonate. The final solution was rinsed with ethyl acetate (3 × 5 mL), and the water layer was acidified (2 M HCl) until the solid product precipitated. The water layer with the solid phase was extracted with ethyl acetate (3 × 10 mL). Combined organic layers were dried over anhydrous $MgSO_4$ and evaporated to dryness. Finally, boldenone 17-hemisuccinate was obtained as white crystalline powder (196 mg, 80 % yield).

Conjugates were prepared with an activated ester method. A conjugation step was carried out in a reversed micellar solution. Boldenone-17-hemisuccinate was dissolved in *N*, *N*-dimethylformamide (DMF). *N*,*N*′-dicyclohexyl carbodiimide (DCC) and *N*-hydroxysuccinimide (NHS) were added, and the mixture was allowed to react at room temperature overnight. The formed precipitate of *N*,*N*′-dicyclohexylurea was separated, and the supernatant containing the activated ester of boldenone was used in the subsequent reaction with OVA for the preparation of a coating conjugate (BOL-17-OVA) or with BSA for the preparation of an immunization conjugate (BOL-17-BSA).

A solution of BSA or OVA in the bicarbonate buffer (pH 8.5) was gradually added to the solution of dioctyl sulfosuccinate in octane to form a reversed micellar solution. A boldenone activated ester was added into this solution while stirring. The reaction mixture was stirred overnight at room temperature. The modified BSA or OVA was precipitated after an addition of cooled acetone, then collected by centrifugation and air dried. The resulting conjugates were dissolved in water and subsequently frozen and lyophilized. The stock solution of the conjugate with OVA was prepared by dissolving 1 mg of lyophilisate in 1 mL of the coating buffer and stored at −28 °C. Before the application in ELISA, this solution was appropriately diluted in the coating buffer.

Preparation of Antiserum

An antiserum against boldenone (Anti-BOL) was raised in a rabbit using BOL-17-BSA as an immunization conjugate. The rabbit was immunized and the antiserum was then collected with the standard procedure. The immunogen was dissolved in a sterile isotonic saline and emulsified with the identical volume of complete Freud's adjuvants. Subsequently, it was applied subcutaneously into three or four spots on the rabbit's back and legs. Immunization was repeated four times in 4-week periods; one dose represented 0.3 mg of the immunogen in 0.3 mL of the emulsion. The final serum harvesting was realized 10 days after the last immunization by the cardiac puncture under complete anesthesia. The antiserum collected was lyophilized and stored at −28 °C. A stock solution of the antiserum was prepared by dissolving 1 mg of lyophilisate in 1 mL of an assay buffer and stored at −28 °C. Before being used in ELISA, these solutions were appropriately diluted in the assay buffer.

ELISA General Protocol

Concentrations of immunoreagents and composition of the buffers used are listed in Table 1. Microtiter plates were coated with BOL-17-OVA (100 μL/well) at a suitable concentration in a coating buffer and incubated at 4 °C overnight. Afterwards, the unbound conjugate was removed with the wash buffer (four times, 350 μL/well). The standards of boldenone (the concentration range of 0–500 ng/mL diluted from the stock solution (concentration 1 mg mL^{-1} in 96 % ethanol)) or sample extracts (extraction in 96 % ethanol) were diluted in the assay buffer and added to microtiter plates (50 μL/well) followed by the relevant solution of the antiserum in the assay buffer (50 μL/well) and incubated at 37 °C for 1 h. Microtiter plates were washed again (four times, 350 μL/well), and an enzyme-labelled antibody GAR-Po (100 μL/well) was added and incubated at 37 °C for 1 h. After the washing step, a substrate solution for the enzyme was added (100 μL/well) and incubated at room temperature for 10 min. The enzyme reaction was stopped by adding the stopping solution (50 μL/well), and the absorbance was measured at 450 nm.

Calibration Curves and Interpretation of Results

Sigmoid calibration standard curves were obtained by plotting the mean values of absorbance against the logarithm of

Table 1 Optimized conditions for boldenone immunoassay

	Selected conditions
Antiserum	Anti-BOL
Dilution of antiserum	1:32,000
Coating conjugate	BOL-17-OVA
Concentration of conjugate (μg mL^{-1})	0.008
Secondary antibody	GAR-Po
Dilution of secondary antibody	1:10,000
Buffer of 1st incubation	PBS
Buffer of 2nd incubation	PBS-Tw-0.1 % milk

BOL-17-OVA conjugate of boldenone with ovalbumin at C_{17} position, *GAR-Po* goat anti-rabbit antibody conjugated to horseradish peroxidase, *PBS* 0.01 M phosphate-buffered saline, pH 7.4, *PBS-Tw-0.1 % milk* 0.01 M PBS, pH 7.4 containing Tween 20 (0.05 % *v/v*) and milk (0.1 % *w/v*)

boldenone concentrations through a four-parameter logistic equation:

$$y = \left\{ (A-D) \Big/ \left[1 + \left(x \Big/ C \right)^{B} \right] \right\} + D$$

where parameter A is the y-value of the upper asymptote, B is the curve slope at the inflection point, C is the concentration of the analyte giving 50 % inhibition of the asymptotic maximum (IC_{50}), and D is the y-value of the lower asymptote.

The limit of detection (LOD) was defined as the concentration of an analyte corresponding to the maximum assay signal minus 3× standard deviation (SD) in accordance with the calibration curve (the blank was calculated from six parallel determinations with the absence of an analyte).

The linear working range corresponded to the analyte concentration causing the 20–80 % inhibition of the maximal assay signal.

Specificity

The specificity of ELISA was defined as the ability of an antiserum to interact with various steroid substances. Each steroid standard (stock solution 1 mg mL^{-1} in 96 % ethanol) was diluted in the assay buffer, and calibration curves were constructed (the concentration range of 0–500 ng mL^{-1}). The cross-reactivity (CR %) was calculated as (IC_{50} of boldenone) / (IC_{50} of tested steroid) × 100.

Sample Preparation—Artificially Contaminated Samples

All the dietary supplements used in this study were freely available commercial samples obtained from local supermarkets. Matrices were in the form of powder or tablets. The absence of anabolic steroids in tested matrices was verified with gas chromatography (Stepan et al. 2008).

Spiking of Dietary Supplements

Dietary supplements containing different concentrations of boldenone were prepared as follows: 1 g of each boldenone-free matrix (powder or ground tablets) was spiked with boldenone from the stock solution (concentration 1 mg mL^{-1} in 96 % ethanol) in order to obtain following concentrations: 50, 5, 0.5, and 0.05 μg g^{-1}. Spiked samples were left to dry at room temperature for 0.5 h.

Preparation of Extracts for ELISA

After drying, spiked samples were mixed and extracted in 10 mL of 96 % ethanol (*v/v*) at room temperature for 15 min. The samples were allowed to stand for at least 15 min, and supernatants were collected and stored at −20 °C. Before assaying, supernatants were at least 10× diluted with the assay buffer.

Accuracy

Artificially contaminated samples of dietary supplements were used to determine the accuracy of the developed methods. The recovered amount of boldenone was subsequently calculated as (concentration in extracts measured by ELISA) / (spiked concentration) × 100.

Correlation with UHPLC-MS/MS

To evaluate the correlation of the developed ELISA with UHPLC-MS/MS, four solutions with different concentrations of boldenone in ethanol (concentration range of 0.25–250 μg mL^{-1}) were assayed. Before the ELISA analysis, the solutions were diluted with the assay buffer.

The following set of devices was used for the UHPLC-MS/MS analysis: Agilent 1290 Infinity UHPLC system coupled with Agilent 6460 Triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was performed in Agilent Zorbax Eclipse Plus C18 column (2.1 × 50 mm; 1.8 μm). Ammonium fluoride in water (1 mM) (A) and methanol (B) acted as mobile phases for gradient elution. The flow rate was 0.25 mL min^{-1} with the column temperature of 35 °C. The gradient elution was carried out as follows: 0 min, 80:20 (A:B); 4 min, 0:100; 5.5 min, 0:100; 5.8 min, 80:20; and 7 min, 80:20.

The MS/MS apparatus was operated in a positive mode. The applied conditions of the electrospray ion source were as follows: drying gas temperature 340 °C; drying gas flow 4 L min^{-1}; sheath gas temperature 380 °C; sheath gas flow 12 L min^{-1}; nebulizer pressure 45 psi; nozzle voltage 900 V; and capillary voltage set to 3500 V. Multiple reaction monitoring mode was used for the detection. Three transitions of m/z were used: 287.2 → 121.1, 135.1, and 77.1.

Standard stock solutions were prepared in ethanol at the concentration of 1 mg mL^{-1} and subsequently diluted in 20 % methanol at seven concentration levels (ranging from 0.1 to 500 ng mL^{-1}). The peak areas of external standards (at each concentration) were plotted against the corresponding standard concentrations using linear regression to generate the standard curve. Stock solutions of the samples in ethanol were 1000× diluted by 20 % methanol. Agilent MassHunter (Agilent Technologies, Inc.) was used for the data acquisition and quantification of the samples.

Results and Discussion

Conjugates

Boldenone was treated with succinic anhydride under the standard procedure to obtain boldenone-17-hemisuccinate with 80 % yield. Boldenone-17-hemisuccinate was then used as an initial molecule for the preparation of conjugates with proteins (BSA and OVA). The preparation of a coating conjugate is shown in Scheme 1. Conjugates were confirmed by UV–VIS spectroscopy. These spectra showed qualitative differences between the conjugate and the corresponding carrier protein and revealed that the hapten and carrier protein had been coupled successfully. For the molar ratios of hapten, carrier protein was estimated as 7:1 and 9:1 for the immunization conjugate (BOL-17-BSA) and coating conjugate (BOL-17-OVA), respectively.

Development and Optimization of Assay Protocol

The assay was developed as an indirect competitive ELISA using a polyclonal antiserum targeted at boldenone. Checkerboard titrations were performed and optimal immunoreagent concentrations were determined when the maximum absorbance ranged from 1.2 to 1.9 and the calibration curve reached the lowest IC_{50} values. The assay was optimized for time intervals and temperatures of incubations and for the composition of dilution buffers. The optimized conditions for the boldenone immunoassay are summarized in Table 1.

Standard Curve

To assess significant analytical parameters of the assembled format, a boldenone calibration curve (concentration range of 0–500 ng mL^{-1}) was constructed (Fig. 2). The assay was carried out under the optimized conditions. Based on the standard curve, LOD was 0.014 ± 0.007 ng mL^{-1} with the IC_{50} value of 0.293 ± 0.084 ng mL^{-1} and the linear working range being 0.065–1.529 ng mL^{-1}. The results show that the assay developed is very sensitive. Hagedorn et al. 1994 reported that a method of ELISA detected anabolic steroid boldenone in equine blood and urine. Another ELISA method was used to screen for boldenone and methyl boldenone in bovine urine (Lu et al. 2006). Compared with the methods published previously, our assay newly developed has almost twice as low IC_{50} value (15 pg/well) as the IC_{50} value in Hagedorn's study (Hagedorn's $IC_{50} = 26$ pg/well) and almost 50 times lower LOD than the ELISA published by Lu et al. 2006 (Lu's LOD $= 0.681 \pm 0.016$ ng mL^{-1}). The higher sensitivity of the method is important for its use in the rapid analysis of dietary supplement samples. The samples are diluted multiple times before the ELISA analysis (Section 3.6) in order to eliminate the influence of the matrix effect.

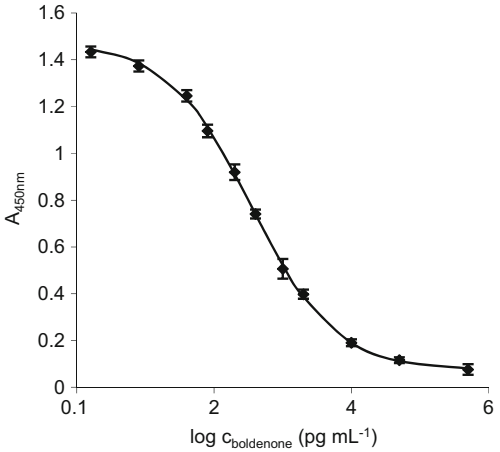
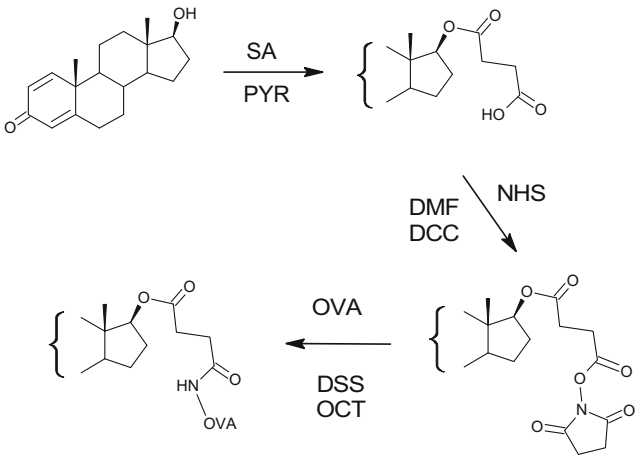


Fig. 2 Typical ELISA standard curve using optimized assay protocol (mean value ± SD, $n = 5$), LOD $= 0.014 \pm 0.007$ ng mL^{-1}, $IC_{50} = 0.293 \pm 0.084$ ng mL^{-1}, and linear working range = 0.065–1.529 ng mL^{-1}



Scheme 1 Synthesis of coating conjugate. *SA* succinic anhydride, *PYR* pyridine, *NHS* *N*-hydroxysuccinimide, *DMF* *N*,*N*-dimethylformamide, *DCC* *N*,*N*′-dicyclohexyl carbodiimide, *DSS* dioctyl sulfosuccinate sodium salt, *OCT* octane

Specificity and Precision

The reactivity with structurally and functionally similar substances was tested to verify the specificity of the format assembled. The study of cross-reactivity was carried out under the optimized conditions. The results for cross-reactant substances are reported in Table 2. The antiserum obtained does not react with boldenone only but also with its esters. It can be seen that the reactivity to boldenone esters decreases with the extension of their acyl chain (e.g., boldenone acetate CR = 26.2 %, boldenone propionate CR = 17 %, boldenone sulfate CR = 3.1 %, boldenone undecylenate CR = 2.1 %, and boldenone undecanoate CR = 0.5 %).

To evaluate the precision of the developed ELISA, three standard samples were assayed with boldenone concentrations in the range of 0.1–0.5 ng mL^{-1}. For the intra-day precision

Table 2 Cross-reactivity data for antiserum against boldenone used in ELISA

Steroids standards	CR (%) Anti-BOL
Boldenone	100
Boldenone acetate	26.2
Boldenone benzoate	1.7
Boldenone propionate	17.0
Boldenone sulfate	3.1
Boldenone undecylenate	2.1
Boldenone undecanoate	0.5
Dihydrotestosterone	10.9
Ethisterone	0.9
Cortisone	0.3
Cortisol	2.2
Methandienone	9.1
Oxandrolone	0.2
Oxymetholone	0.9
Progesterone	0.4
Testosterone	2.1

Other tested steroids (11-deoxycorticosterone, 21-deoxycortisol, deoxycholic acid, dehydroepiandrosterone, dehydromethyltestosterone, epitestosterone, estriol, ethinylestradiol, fluoxymesterone, hydroxypregnenolone, methyltestosterone, methyldihydrotestosterone, phenylpropionate)—all with cross-reactivity 0.001 % in developed ELISA

study, one set of analyses ($n = 6$) was performed for every sample on the same day. Similarly, one set of analyses was carried out with each sample daily in five consecutive days for the inter-day precision study. The intra- and inter-day relative standard deviations (RSD) were calculated in the range of 3.8–10.5 and 7.3–12.9 %, respectively, indicating the acceptable precision.

Solvent Tolerance

The preparation of real samples for analyses often requires extraction with organic solvents. In case of immunoassays, solvents may negatively affect sensitivity. Basically, they can change the native structure of antibodies and/or influence conditions of their non-covalent interaction with the hapten molecule. In this work, the effect of three water soluble solvents—methanol, ethanol, and isopropyl alcohol on the ELISA performance was evaluated by preparing standard curves in the assay buffer and in the assay buffer containing various amounts (1, 5, 10, and 20 % (*v/v*)) of an organic solvent. The solvent tolerance of the method was evaluated on the basis of IC_{50} value. A similar trend in the effect of three solvents was observed. The decrease in the maximum signal of absorbance and deterioration of sensitivity were acquired with the increase of the solvent concentration (results not presented here). Ethanol was selected for the extraction step with regard to its safer use when compared with methanol and isopropyl alcohol. It was found that ethanol not exceeding 10 % (*v/v*) does not significantly affect any parameters of the method.

Evaluation of Matrix Effect and Sample Preparation

A possibility to analyze solid sample extracts without complicated purification is one of the most valued advantages of immunoassays over the instrumental methods commonly used today. In this study, the influence which the dietary supplements selected had on the assay was tested by comparing standard curves created by the assay buffer and in the presence of various amounts of individual dietary supplements. All matrices were diluted 100× (10× in extraction step with 96 % ethanol and 10× with assay buffer to reduce ethanol effect). The analytical data obtained are nearly identical as shown in Table 3. Hence, this sample preparation method is suitable for the ELISA analysis.

Analysis of Spiked Samples

Artificially contaminated samples were used to determine the accuracy of the ELISA developed. The preparation of the samples is specified in Section 2.6. The recovered amount of boldenone (Table 4) was calculated by subtracting the spiked dose from the value obtained for each spiked sample. Extracts were appropriately diluted with the assay buffer to fall into the linear working range on the standard curve. The recovery of the spiked samples was outstanding for all the dietary supplements tested. All calculated data were in the range of 86.0 to 115.7 %. Method reproducibility, expressed as relative

Table 3 Matrix effect

Type of matrix	$IC_{50} \pm SD$ (ng mL^{-1})	Linear working range[a] (ng mL^{-1})	$LOD \pm SD$ (ng mL^{-1})
Assay buffer[b]	0.27 ± 0.02	0.05–1.56	0.014 ± 0.007
I	0.29 ± 0.03	0.04–1.72	0.019 ± 0.006
II	0.30 ± 0.02	0.05–1.64	0.021 ± 0.009
III	0.32 ± 0.02	0.06–1.63	0.023 ± 0.011
IV	0.36 ± 0.02	0.07–1.69	0.018 ± 0.012

Parameters of boldenone standard curves were created in different matrices ($n = 3$). Before analysis, all matrices were diluted 100 times (10× in extraction step with 96 % ethanol and 10× with assay buffer to reduce ethanol effect)

IC_{50} the 50 % intercept value of sigmoid curve, *SD* standard deviation, *LOD* limit of detection, *I* GS Extra Strong Multivitamin, *II* 100 % Whey Protein, apple-cinnamon, *III* 100 % Whey Protein, vanilla, *IV* Amix™ Egg Amino 6000

[a] Expressed as concentration range causing 20–80 % inhibition of asymptotic maximum

[b] 0.01 M PBS, pH 7.4

Table 4 Accuracy data obtained with ELISA

Dietary supplements	Spiked concentration (ng g^{-1})	Mean value (ng g^{-1})	Recovery (%)	RSD (%)
I	0	LOD	–	–
	50	44	88.0	8.2
	500	462	92.4	7.8
	5000	4540	90.8	11.3
	50,000	49,300	98.6	10.9
II	0	LOD	–	–
	50	45	90.0	15.8
	500	478	95.6	7.2
	5000	5130	102.6	11.4
	50,000	46,250	92.5	8.2
III	0	LOD	–	–
	50	48	96.0	14.7
	500	576	115.2	15.6
	5000	5430	108.6	7.8
	50,000	57,850	115.7	9.4
IV	0	LOD	–	–
	50	43	86.0	9.5
	500	561	112.2	16.2
	5000	4710	94.2	5.8
	50,000	43,600	87.2	6.9

Recovery of boldenone standard was added to different dietary supplements ($n = 3$). Before analysis, all spiked samples were extracted in 10 mL of 96 % ethanol and subsequently diluted in the appropriate volume of assay buffer

RSD relative standard deviation, *LOD* limit of detection, *I* GS Extra Strong Multivitamin, *II* 100 % Whey Protein, apple-cinnamon, *III* 100 % Whey Protein, vanilla, *IV* Amix ^{Tm} EGG Amino 600

standard deviation (RSD) ranging from 5.8 to 16.2 %, was also satisfactory. The results demonstrate a good potential of the developed ELISA to be applied in routine analyses.

Correlation with UHPLC-MS/MS

UHPLC-MS/MS was selected for the realization of a comparison study. Four solutions of boldenone in ethanol (concentration range of 0.25–250 μg mL^{-1}) were assayed three times by UHPLC-MS/MS and the newly developed ELISA. The results of these two methods are shown in Table 5. A linear relationship was observed. The linear equation is $y = 1.0256\,x - 0.7772$ ($R^2 = 0.9999$, $n = 3$).

Conclusion

A highly sensitive ELISA method was devised and optimized for the determination of boldenone in dietary supplements.

Table 5 Correlation data obtained with developed ELISA and UHPLC-MS/MS ($n = 3$)

	ELISA results		UHPLC results	
Spiked concentration (μg mL^{-1})	Mean (μg mL^{-1})	RSD (%)	Mean (μg mL^{-1})	RSD (%)
0	LOD	–	LOD	–
0.25	0.24	1.6	0.26	4.3
2.5	2.53	6.6	2.56	4.9
25	24.2	0.8	25.9	5.1
250	255.9	5.0	250.1	0.6

RSD relative standard deviation, *LOD* limit of detection

The achieved sensitivity of the method allows omitting the cleanup procedure of extracts before assaying. Matrix effects were avoided by the extract dilution before analysis. Correlation between ELISA and UHPLC-MS/MS was found to be excellent. The ELISA developed in this research is primarily suitable for simple monitoring of boldenone in dietary supplements. If identified as positive during such extensive monitoring, the samples could subsequently be analyzed with more sophisticated instrumental methods such as chromatography.

Acknowledgments This work was supported by the Grant of the Ministry of the Interior of the Czech Republic (VG20112015045).

Compliance with Ethical Standards

Funding This study was funded by Ministry of the Interior of the Czech Republic (grant number VG20112015045).

Conflict of Interest Lucie Fojtíková has no conflict of interest. Ladislav Fukal has no conflict of interest. Martina Blažková has no conflict of interest. Sandra Sýkorová has no conflict of interest. Martin Kuchař has no conflict of interest. Petra Mikšátková has no conflict of interest. Oldřich Lapčík has no conflict of interest. Barbora Holubová has no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of laboratory animals were followed.

Informed Consent Informed consent was obtained from all the individual participants involved in the study.

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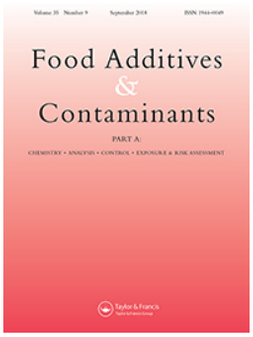
Příloha 10:

Sýkorová, S., Fojtíková, L., Kuchař, M., Mikšátková, P., Karamonová, L., Fukal, L., Lapčík, O., Holubová, B.: Sensitive Enzyme Immunoassay for Screening Methandienone in Dietary Supplements. *Food Addit Contam A* 35(9): 1653 – 1661, 2018.



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Food Additives & Contaminants: Part A

ISSN: 1944-0049 (Print) 1944-0057 (Online) Journal homepage: http://www.tandfonline.com/loi/tfac20

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To cite this article: Sandra Sýkorová, Lucie Fojtíková, Martin Kuchař, Petra Mikšátková, Ludmila Karamonová, Ladislav Fukal, Oldřich Lapčík & Barbora Holubová (2018) Sensitive enzyme immunoassay for screening methandienone in dietary supplements, Food Additives & Contaminants: Part A, 35:9, 1653-1661, DOI: 10.1080/19440049.2018.1459876

To link to this article: https://doi.org/10.1080/19440049.2018.1459876

FOOD ADDITIVES & CONTAMINANTS: PART A
2018, VOL. 35, NO. 9, 1653–1661
https://doi.org/10.1080/19440049.2018.1459876

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Sensitive enzyme immunoassay for screening methandienone in dietary supplements

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ABSTRACT

Methandienone is a synthetic exogenous steroid which, like other anabolic steroids, is strictly regulated in many countries. In recent years, increasing numbers have been detected of illegal additions into dietary supplements of methandienone and other anabolic androgenic steroids (AAS). In this work, a competitive indirect enzyme-linked immunosorbent assay (ELISA) has been constructed for the detection of methandienone using an antiserum against methandienone. Under optimal experimental conditions, the ELISA achieved a limit of detection of 0.04 ± 0.01 μg. g^{-1}. The obtained intra- and inter-day coefficients of variation were less than 8%. The developed ELISA was applied in the analysis of real dietary supplement samples. To minimise the effect of the sample matrix, the sample extracts were simply diluted before addition into the immunoassay. The achieved recovery values were around 100%. Results obtained from the ELISA correlated well, both in terms of accuracy and precision, with those obtained by UHPLC-MS/MS (reference method). The presented ELISA could be successfully applied for the simple screening of dietary supplements.

ARTICLE HISTORY
Received 6 October 2017
Accepted 11 March 2018

KEYWORDS
Methandienone; anabolic steroid; enzyme-linked immunosorbent assay (ELISA); screening method; dietary supplements

Introduction

An exogenous anabolic androgenic steroid methandienone (17β-hydroxy-17α-methylandrosta-1,4-dien-3-one; also known as dianabol or methandrostenolone) was first synthesised in 1955 by Vischer et al. Methandienone is a derivate of the male sex hormone testosterone associated with both skeletal muscle-building (anabolic) and masculinising (androgenic) effects (Prendergast et al. 2003; Orlando et al. 2007; Barceloux and Palmer 2013). This oral steroid was designed for the treatment of a variety of medical conditions but because of deleterious side effects it is strictly regulated in many countries (Stang-Voss and Appell 1981; Evans 2004; Bond et al. 2016). Currently, methandienone is legally available on prescription only.

Since 1999 several studies have shown evidence of some dietary supplements containing anabolic androgenic steroids (AASs), which were not declared on the label (Geyer et al. 2004; Maughan 2005; Baume et al. 2006; Petroczi et al. 2011; Abbate et al. 2015; Odoardi et al. 2015). Methandienone was found in significant amounts of analysed dietary supplements, although the law prohibits the inclusion of AASs (and their precursors) in these products. The highest concentrations were in the tens of mg/g. For comparison, the effective daily dose of methandienone for therapeutic purposes is about 30 mg/day (Geyer et al. 2003; Greyer et al. 2008).

For effective control of AAS content in a large collection of dietary supplements, screening methods are important thanks to their ability to select suspect samples at considerably reduced cost and time, so that well-timed decisions can be taken. Fast and cheap screening methods are especially demanded for official methandienone/AAS control in dietary supplements. However, the current screening strategies are based on chromatographic methods (Hagedorn et al. 1992; Hooijerink et al. 1998; He et al. 2008; Wang et al. 2011; Gómez et al. 2013; Odoardi et al. 2015; Polet et al. 2016). Chromatographic methods are very suitable for confirmation but, due to the expensive, time-consuming and laborious analyses, not for screening large

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numbers of test samples (Galarini et al. 2014). In contrast, immunoassays may be portable and cost-effective, with appropriate sensitivity, high selectivity, and minimal sample extraction process. Therefore, immunotechniques have become favoured and more considered as methods for the rapid steroid monitoring in the area of dietary supplements (Popii and Baumann 2004).

During recent years, some authors have described the production of antibody (polyclonal or monoclonal) against methandienone for the preparation of immunoaffinity columns and their potential application to the selective extraction of methandienone residues from animal tissue and feed samples (Wang et al. 2011, 2013, 2014). To the best of our knowledge, to date no immunoassay has been published for the detection of methandienone in dietary supplements or routinely applied for the screening of official controls. Our aim was to develop a sensitive competitive immunoassay that would be as simple as possible to be applied by the state authorities for the screening of methandienone in dietary supplements.

Materials and methods

Materials, reagents and apparatus

Standards of anabolic steroids were obtained from Sigma-Aldrich Inc. (St. Louis, USA) or STERALOIDS, Inc. (Rhode Island, USA). Stock solutions were prepared in 96% ethanol and stored at −28°C. Working standard solutions were freshly prepared by serial dilution in an assay buffer. Tween 20, sulphuric acid, 3,3′,5,5′-tetramethylbenzidine (TMB), hydrogen peroxide (H_2O_2; 30%), dimethylsulphoxide (DMSO), *N*-hydroxysuccinimide (NHS), ovalbumin (OVA), bovine serum albumin (BSA), gelatine and dried milk were obtained from Sigma-Aldrich Inc. (St. Louis, USA). *N,N*'-dicyclohexylcarbodiimide (DCC) was obtained from Fluka, *N,N*-dimethylformamide (DMF) extra dry was purchased from Acros. All the other solvents were obtained from Penta. Peroxidase labelled goat anti-rabbit IgG antibody (GAR-Px) was purchased from Norvic Immunology (Tilburg, the Netherlands). 96-well polystyrene microtiter plates Costar 9018 were supplied from Corning Inc., USA.

Enzyme-linked immunosorbent assay (ELISA) plates were washed with a Columbus Pro instrument from Tecan Group (Austria) and the absorbance was read in Labsystem Multiscan MCC/340 (Helsinki, Finland). The data were processed using Microsoft Excel software (Microsoft Corporation, Tulsa, USA).

Preparation of immunoreagents

Synthesis of immunogen and coating conjugates

First, methandienone-17-hemisucuccinate (MET-17-HS) was synthesised by a modified procedure published by Evans et al. (1963). Commercial reagents and solvents were used after distillation. Reactions were monitored with the aid of thin-layer chromatography (TLC Silica gel 60 F254) and visualisation was carried out with UV light (Fojtíková et al. 2016). Methandienone was dissolved in a dried flask under an inert atmosphere (180 mg, 0.6 mmol) in dry diethyl ether (20 mL). A 3 M solution of ethyl magnesium bromide in diethyl ether (220 µL, 0.66 mmol) was added with stirring and followed by slow dropwise addition of 3-(carbomethoxy)propionyl chloride (147 µL, 1.2 mmol). After 5 h reflux, a saturated solution of $NaHCO_3$ (20 mL) was carefully added to the reaction mixture and the organic layer was separated. After the extraction of organic layer with diethyl ether (2 x 20 mL), the combined organic extracts were washed with saturated aqueous sodium chloride (2 x 10 mL) and dried over anhydrous $MgSO_4$. The desiccant was filtered out and the solvent evaporated at the rotary evaporator (RVO). The product as methyl ester was dissolved in methanol (6 mL) and a solution of K_2CO_3 (90 mg/1.5 mL of water, 0.65 mmol) was added. The reaction mixture was stirred at ambient temperature overnight. Methanol was evaporated; water (15 mL) and diethyl ether (10 mL) were added and carefully acidified with 2 M hydrochloric acid. The organic layer was retained and the aqueous layer was extracted by diethyl ether (2 x 10 mL). The combined organic extracts were washed with brine (2 x 10 mL) and dried over anhydrous $MgSO_4$. After filtering out the drying agent and evaporation of solvent, the desired methandienone-17-hemisuccinate (115 mg, 48%, white crystalline solid) was afforded.

Sequentially, the methandienone-protein conjugates MET-17-HS-BSA and MET-17-HS-OVA were synthesised in a reversed micellar system for steroid immunogen synthesis by Yatsimirskaya et al. (1993) with minor modifications. Ten percent solutions (w/w) of *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC) and MET-17-HS in anhydrous dimethylformamide (DMF) were prepared. In brief, MET-17-HS was left to react with DCC and NHS (molar ratio 3:4:5) in anhydrous mixture dimethylformamide/dimethylsulphoxide (1:1). After 4 h, the reaction mixture was centrifuged to sediment the crystals of dicyclohexylurea and the supernatant used for conjugation with BSA or OVA in a reversed micellar system. The conjugates dissolved in water were frozen and then lyophilised. The stock solutions of the conjugates were prepared by dissolving 1 mg of lyophilisate in 1 mL of the coating buffer and stored at −28°C. MET-17-HS-BSA was used as immunogen for immunisation and MET-17-HS-OVA was used as coating conjugate in ELISA procedure.

Production of polyclonal antiserum

An antiserum specific for methandienone (Anti-MET) was prepared in Biotest a.s., (Konárovice, Czech Republic). Antiserum was raised in a rabbit using MET-17-HS-BSA. The rabbit was immunised and the antiserum was then collected with the standard procedure. The immunisation protocol was described previously in Fojtíková et al. (2016). The antiserum collected was lyophilised and stored at −28°C. A stock solution of the antiserum was prepared by dissolving 1 mg of lyophilisate in 1 mL of an assay buffer and stored at −28°C.

ELISA

Buffers

The buffers and solutions used in this study: coating buffer (0.01 M carbonate/bicarbonate buffer, pH 9.6); assay buffer (0.01 M phosphate-buffered saline (PBS), pH 7.4); wash buffer (PBS containing Tween 20 (0.05% *v/v*)); substrate solution for enzyme (1 mg of TMB, 1 mL of DMSO, 2 µl of 30% H_2O_2 (*v/v*) and 9 mL 0.05 M citrate/phosphate buffer pH 5.0); and stop solution (2 M sulphuric acid).

ELISA protocol

The indirect competitive ELISA was performed as follows: 96-well ELISA plates were coated with the MET-17-HS-OVA conjugate solution in the coating buffer (100 µL/well), left to incubate at 4°C overnight, then washed with the wash buffer (four times, 350 µL/well). Aliquots (50 µL/well) of methandienone (in the concentration range of 0–5,000 ng/mL diluted from the stock solution) or sample extracts, diluted in the assay buffer, and aliquots (50 µL/well) of the Anti-MET, diluted in the assay buffer containing BSA (1% *w/v*), were pipetted into the wells. Microtiter plates were left to incubate at 37°C for 1.5 h and washed as described above. Subsequently, GAR-Px was added (100 µL/well) and left to interact at 37°C for 1.5 h. After the final washing step, the substrate solution for enzyme was added (100 µL/well) and incubated at room temperature for 10 min. Finally, the enzyme reaction was stopped by adding the stop solution (50 µL/well), and the absorbance was measured at 450 nm. To construct standard curves, absorbance values were fitted to a four-parameter logistic equation (Fojtikova et al. 2016). The IC_{50} values represented the methandienone concentration that produced 50% inhibition of antibody binding to the hapten conjugate. The limit of detection (LOD) was defined as the concentration of an analyte corresponding to the maximum assay signal minus 3x standard deviation (SD) in accordance with the calibration curve (the blank was calculated from six parallel determinations with the absence of an analyte). The linear working range corresponded to the analyte concentration causing the 20–80% inhibition of the maximal assay signal (A_{max}).

Cross-reactivity study

Calibration curves with various steroid substances were constructed and their IC_{50} values were compared to those from a standard curve for methandienone run in the same plate. The value of cross-reactivity (CR) was calculated as follows: (IC_{50} of methandienone/IC_{50} of tested steroid) × 100 (%).

Recovery study

A recovery study was realised using three kinds of widely available commercial dietary supplements. Samples of dietary supplements – '100%

WHEY PROTEIN (apple-cinnamon)' (SCITEC NUTRITION, Orlando, USA); 'EGG AMINO 6000' (AmixTM, Manchester, UK) and 'GS ExtraStrong Multivitamin' (Green-Swan Pharmaceuticals CR, a.s., Prague, CZ) – were purchased from a local store. The absence of anabolic steroids in tested matrices was verified with UHPLC-MS/MS (described in chapter UHPLC-MS/MS).

Sample preparation

Artificially contaminated samples of dietary supplements were prepared as follows: 1 g of each sample (milled into powder) was spiked with methandienone from the stock solution (concentration 1 mg.mL^{-1} in 96% ethanol) to obtain the following concentrations: 0.05, 1 and 100 μg.g^{-1}. After drying (room temperature, 0.5 h), spiked samples were homogenised and extracted in 10 mL of 96% ethanol (*v/v*) (room temperature, 15 min). Supernatants were collected and stored at 4°C. Before assaying, supernatants were diluted with the assay buffer. The recovered amount of methandienone was calculated as: (concentration in extracts measured by ELISA)/(spiked concentration) × 100 (%).

Analysis of counterfeit dietary supplement samples

Three counterfeit dietary supplements containing methandienone were supplied by the police of the Czech Republic (details are published in Jurášek et al. 2015). Real samples were supplied in the form of an ampoule (labelled sample no. 1) or tablets (sample no. 2 and 3).

Sample preparation

Sample no. 1: 0.25 mL of the liquid sample in the ampoule was homogenised and extracted in 2.25 mL of 96% ethanol (*v/v*). Sample no. 2: 0.1 g of tablet powder was mixed and extracted in 10 mL of 96% ethanol (*v/v*) (room temperature, 15 min). Sample no. 3: 0.13 g of tablet powder was mixed and extracted in 10 mL of 96% ethanol (*v/v*) (room temperature, 15 min). Subsequently, supernatants were collected and stored at 4°C. Before assay, the samples were appropriately diluted with the assay buffer.

UHPLC-MS/MS

To evaluate the accuracy of obtained ELISA results from prepared extracts of real samples, UHPLC-MS/MS were used. Samples were prepared as described previously (2.6.1) and analysed using an Agilent 1290 Infinity UHPLC system coupled with Agilent 6460 Triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separations were performed on an Agilent Zorbax Eclipse Plus C18 column (2.1 x 50 mm; 1.8 μm). The mobile phases for gradient elution were ammonium fluoride in water (1 mM) (A) and methanol (B). The chromatography conditions were described previously in Fojtikova et al. (2016).

The mass spectrometer was operated in positive electrospray mode. The drying gas temperature and the flow rate were 340°C and 4L/min, respectively, and the sheath gas temperature and flow rate were 380°C and 12L/min, respectively. The nebuliser pressure was 45 psi, the nozzle voltage 900V and the capillary voltage was 3500V. Multiple reaction monitoring (MRM) mode was used for the detection. Two transitions of m/z were used: 301.2 → 149.1 and 121.1. Agilent Mass Hunter (Agilent Technologies, Inc.) was used for data acquisition and quantification of samples.

A standard stock solution was prepared in ethanol at 1 mg.mL^{-1} and subsequently diluted in 20% methanol to obtain seven concentration levels ranging from 0.1 to 500 ng.mL^{-1}.The extract solution of the real samples in ethanol were 100,000 × diluted by serial dilution in 20% methanol and analysed in triplicate to minimise the errors. LOD for methandienone was determined 0.03 ng.mL^{-1} and LOQ was 0.1 ng.mL^{-1}.

Results and discussion

Methandienone-protein conjugates

The methandienone-protein MET-17-HS-BSA and MET-17-HS-OVA were synthesised in a reversed micellar system for steroid immunogen synthesis. The prepared conjugates were confirmed by UV-VIS spectroscopy in the range of 230–350 nm (not shown). The obtained spectra demonstrated successful coupling between the hapten and carrier protein. The molar ratios of

hapten:carrier protein were estimated as 8:1 and 12:1 for the immunisation conjugate (MET-17-HS-BSA) and coating conjugate (MET-17-HS-OVA), respectively.

Development of competitive ELISA

A competitive indirect ELISA was assembled for methandienone determination. The optimum concentrations of the Anti-MET and of the corresponding MET-17-HS-OVA conjugate were found using a checker-board titration. The assay was optimised for time intervals and temperatures of incubations and for the composition of dilution buffers. The effect of these parameters were determined by measuring the maximum absorbance (A_{max}, the absorbance value at zero analyte concentration) and the concentration of analyte at 50% specific binding (IC_{50}). It was necessary to find ELISA conditions that produced the lowest IC_{50} value and the optimum A_{max} values in the range from 1.5 to 2.0. The optimised conditions for the methandienone immunoassay are summarised in Table 1.

The standard curve of ELISA for methandienone determination (concentration range of 0–5000 ng.mL^{-1}) is illustrated in Figure 1. Based on this curve, the limit of detection (LOD) was 0.04 ± 0.01 ng.mL^{-1} (0.04 ± 0.01 µg.g^{-1}) with the IC_{50} value of 1.54 ± 0.03 ng.mL^{-1} and the linear working range was 0.2 – 12 ng.mL^{-1}. The high sensitivity of the method is important for its use in the rapid analysis of dietary supplement samples. As will be discussed in the next section, the sample extracts are diluted multiple times before the analysis in order to eliminate the influence of any matrix effect.

Intra- and inter-day precision studies were performed with three standard samples of methandienone (0.5; 1 and 100 ng.mL^{-1}). The intra- and inter-day relative standard derivations (RSD) were calculated in the range of 0.8–2.4% and 6.4–8%, respectively, confirming that the method is sufficiently accurate.

Cross-reactivity study

The specificity of the Anti-MET was evaluated using assembled ELISA under the optimised conditions. The reactivity with 59 structurally related compounds was tested and the obtained IC_{50} values were used to calculate the CRs. Table 2 reports the CR for tested compounds. In this work, group specificity of the Anti-MET was expected as the immunisation conjugate was prepared by binding BSA through C17 of methandienone.

Cross-reactivity was found with steroids which have the same structure as methandienone in A, B and C rings of the steroid skeleton. The main cross-reactant steroids for Anti-MET were boldenone and its esters (CR in range of 23–3%). The cross-reactivity of esters of boldenone decreases with increase in size of the side chain in position C17. In Figure 2, structure differences between the methandienone, boldenone and boldenone esters are demonstrated. As shown in Table 2, the group specificity was

Table 1. Optimised condition and parameters for methandienone ELISA.

	Selected condition
Coating conjugate	MET-17-HS-OVA
Concentration of MET-17-HS-OVA (µg.mL^{-1})	0.02
Antiserum	Anti-MET
Concentration of Anti-MET (µg.mL^{-1})	2.5
Buffer for Anti-MET dilution	PBS-1.0% BSA
Secondary antibody	GAR-Px
Dilution of secondary antibody	1:10.000
Buffer for secondary antibody dilution	PBS-0.01% gelatin
	Selected parameters
IC_{50} (ng.mL^{-1})	1.54 ± 0.03
Linear working range (ng.mL^{-1})	0.2 – 12
Detection limit (ng.mL^{-1})	0.04 ± 0.01

MET-17-HS-OVA conjugate of methandienone with ovalbumin at C17 position; *GAR-Px* goat anti–rabbit antibody conjugated to horseradish peroxidase; *PBS* 0.01 M phosphate-buffered saline, pH 7,4; *PBS-1.0% BSA* PBS buffer containing bovine serum albumin (1.0% *w/v*); *PBS-0.01% gelatin* PBS buffer containing gelatin (0.01% *w/v*)

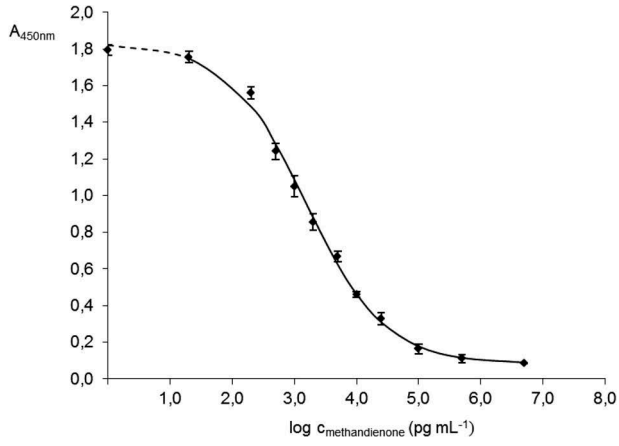

Figure 1. Representative methandienone ELISA standard curve using optimised assay protocol (mean value ± SD, *n* = 6), LOD = 0.04 ± 0.01 ng.mL^{-1} and IC_{50} = 1.54 ± 0.03 ng.mL^{-1}.

Table 2. Cross-reactivity data calculated for methandienone polyclonal antibody.

Steroid	CR (%)	Steroid	CR (%)
Methandienone	100.0	21-deoxycortisol	0.7
Boldenone	22.9	Dihydrotestosterone	0.7
Boldenone acetate	19.3	Progesterone	0.7
Boldenone propionate	8.9	17-Hydroxyprogesterone	0.6
Boldenone sulphate	4.9	Cortisol	0.5
17α-Methyltestosterone	3.2	4-Androsten-11β-ol-3,17-dione	0.3
Boldenone benzoate	2.4	Boldenone undecanoate	0.3
9- dehydromethyltestosterone	2.3	19-Nortestosterone	0.3
Testosterone	1.8	Nortestosterone acetate	0.2
4-Androstene-19-ol-3,17-dione	1.7	Testosterone propionate	0.2
Cortisone	1.5	Methyldihydrotestosterone	0.1
4-Androsten-3,17-dione	1.3	Nandrolone propionate	0.1
11-deoxycorticosterone	1.2	Oxymetholone	0.1
Corticosterone	0.8	Testosterone benzoate	0.1
5β-Androstan-3,17-dione	0.7	Testosterone decanoate	0.1

CR cross-reactivity

confirmed. The fact that the assembled ELISA is not specific only for methandienone, but to some extent also for anabolic steroid boldenone and its esters does not preclude its application for screening of methandienone in dietary supplements. If identified as positive during ELISA screening, the content of samples could subsequently be analysed with more sophisticated instrumental methods such as chromatography.

For other tested compounds with the steroid skeleton, CRs were less than 0.1% (not shown in Table 2; aldosterone, boldenone undecylenate, cholesterol, deoxycholic acid, dehydroepiandrosterone, dihydrotestosterone acetate, dihydrotestosterone benzoate, dihydrotestosterone enanthate, drostanolone, epitestosterone, estriol, ethinylestradiol, ethisterone, fluoxymesterone, hydroxypregnenolone, mesterolone, nandrolone benzoate, nandrolone phenylpropionate, nortestosterone cypionate, nortestosterone decanoate, oxandrolone, stanozolol, testosterone acetate, testosterone cypionate, testosterone undecanoate, trenbolone, trenbolone acetate, trenbolone enanthate, trenbolone hexahydrobenzyl carbonate).

Solvent tolerance

In immunoassay, the preparation of real food samples for analyses often requires an extraction using organic solvents. The presence of organic solvents can negatively affect assay sensitivity by modifying the presentation of the soluble analyte to the antibody or by changing the interaction of the antibody with the conjugated hapten used in the assay (Abad et al. 1999). The effects of several concentrations of methanol, ethanol and isopropyl alcohol on the performance of the methandienone ELISA were studied. Standard curves were prepared in the assay buffer and in the assay buffer containing various amounts of an organic solvent (1, 5, 10 and 20% (*v*/*v*)). A_{max} and IC_{50} changes were investigated as described above (results not presented here). The decrease in the A_{max} and the increase in the IC_{50} values were observed in connection with the increase of the solvent concentration. A marked difference was not found between solvents. Ethanol was used for the preparation of samples with regard to the safety of method users. It was found that ethanol was very well tolerated by the assay format up to a 10% concentration.

Recovery study

In this study, three artificially contaminated solid samples of dietary supplements were used for the recovery study. The preparation of sample extracts is specified in section Materials and methods. To minimise matrix effects, dilution as the most efficient method was applied. All matrices were diluted 100x (10x in extraction step with 96% ethanol and 10x with assay buffer to reduce ethanol effect). Parameters of the calibration curve were not influenced by 1% content of any tested matrix (results not shown). The recovered amount of methandienone was calculated by subtracting the spiked dose from the value obtained for each spiked sample. The

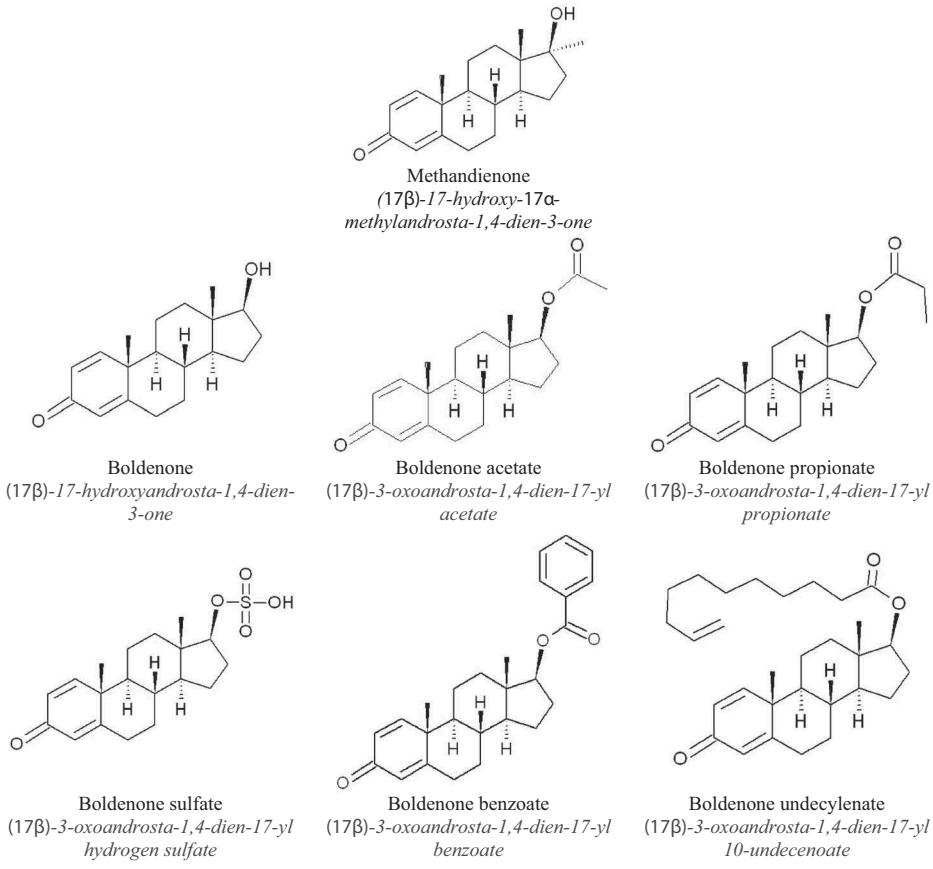

Figure 2. Structures of anabolic steroids with value of cross reactivity: methandienone (100.0%); boldenone (22.9%); boldenone acetate (19.3%); boldenone propionate (8.9%); boldenone sulphate (4.9%); boldenone benzoate (2.4%); boldenone undecylenate (less than 0.1 %).

recovery study was performed in three replicates and the results were satisfactory as seen in Table 3 (no false-negative results were obtained). All calculated data were in the range of 88 to 118%.

Table 3. Recovery of methandienone from spiked samples (as established by ELISA).

Dietary supplement	Spiked concentration (μg.g^{-1})	Mean value (μg.g^{-1})	Recovery (%)	RSD (%)
A	0	ND	-	-
	0.05	0.053	106	23
	1	0.99	99	10
	100	109.8	110	9
B	0	ND	-	-
	0.05	0.044	88	25
	1	0.97	97	12
	100	104	104	5
C	0	ND	-	-
	0.05	0.051	102	20
	1	0.98	98	15
	100	118.1	118	7

Set of analysis *n = 3; A* 100% Whey Protein – apple-cinnamon; *B* Amix Tm EGG Amino 6000; *C* GS Extra Strong Multivitamin; *RSD* relative standard deviation; *ND* not determined.

Analysis of counterfeit dietary supplement samples

As a part of this study, a comparison study was done using the developed ELISA and UHPLC-MS/MS (as a conventional method for food control quality). Three counterfeit dietary supplements containing methandienone were supplied by the police of the Czech Republic. The preparation of sample extracts is specified in section Materials and methods. The results of these two methods are shown in Table 4. A linear relationship was observed. The correlation between the UHLC-/MS/MS data and ELISA results was very good: correlation coefficient and regression slope were very close to 1 (linear equation: y = 1.1631 x; R^2 = 0.996, set of analysis: n = 3). Results demonstrate that the ELISA could be an important tool in food control as a reliable screening method for methandienone in dietary supplement samples.

Table 4. Comparison of methandienone determination by ELISA and UHPLC-MS/MS in real samples.

	Real dietary supplement sample		
	1	2	3
UHPLC-MS/MS results	8.58 ± 0.03 $mg.g^{-1}$	63.8 ± 0.1 $mg.g^{-1}$	19.4 ± 0.3 $mg.g^{-1}$
ELISA results	7.6.± 1.2 $mg.g^{-1}$	75.0 ± 3.0 $mg.g^{-1}$	21.0 ± 3.0 $mg.g^{-1}$

Three counterfeit dietary supplements were supplied by the Police of Czech Republic; liquid sample 1 was supplied in the ampoule form; samples 2 and 3 were supplied in the tablet form.

Conclusions

In this work, MET-17-HS-BSA was synthesised to generate an antiserum against methandienone (Anti-MET). An indirect competitive ELISA was established and optimised using Anti-MET and an excellent limit of detection was obtained. The method was able to detect methandienone in dietary supplements without a complicated clean-up step. To reduce or eliminate unwanted co-extracted matrix components, the sample extracts were diluted in buffer. A good correlation of results with UHPLC-MS/MS was demonstrated. The presented ELISA has a potential use for a screening of methandienone and other abused anabolic steroids in dietary supplements. The results suggest possible commercial test applications in food control.

Disclosure statement

No potential conflict of interest was reported by the authors.

Ethical Approval

All applicable international, national and/or institutional guidelines for the care and use of laboratory animals were followed.

Informed Consent

Informed consent was obtained from all individual participants included in the study.

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Příloha 11:

Holubová, B., Göselová, S., Ševčíková, L., Vlach, M., Blažková, M., Lapčík, O., Fukal, L.: Rapid Immunoassays for Detection of Anabolic Nortestosterone in Dietary Supplements. *Czech J. Food Sci.* 31(5): 514–519, 2013.

Rapid Immunoassays for Detection of Anabolic Nortestosterone in Dietary Supplements

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Abstract

Holubová B., Göselová S., Ševčíková L., Vlach M., Blažková M., Lapčík O., Fukal L. (2013): **Rapid immunoassays for detection of anabolic nortestosterone in dietary supplements**. Czech J. Food Sci., **31**: 514–519.

An enzyme immunoassay (ELISA) and an immunochromatographic strip were designed for a rapid detection of nortestosterone in dietary supplements. Two polyclonal antibodies and two types of nortestosterone-protein coating conjugates were tested to develop the most appropriate method. Under optimal experimental conditions, the most sensitive ELISA achieved the IC_{50} and the limit of detection values of 6.41 and 0.09 ng/ml, respectively. The assay specificity was tested measuring cross-reactivity of several steroids. The interference with the assay was negligible (< 0.1%), except for cross-reactivity with another frequently abused steroid testosterone (23%). The optimised gold particle-based immunochromatographic strip provided in semi-quantitative test a visual detection limit of 1 ng/ml. None of these methods showed the interference using a filtrate of the suspension of non-contaminated sample. After the validation for particular matrices, the ELISA and the strip test could be useful tools for a rapid analysis of nortestosterone in crude extracts of dietary supplements.

Keywords: 19-nortestosterone; ELISA; colloidal gold immunoassay; strip test

Dietary supplements with strength and muscle bulk enhancing effects contain usually amino acids, proteins, prohormones and creatine as active components. A lot of these dietary supplements are freely available through numerous internet sites which causes an expansion in the use of these supplements (Václavíková & Kvasnička 2013). Some studies have shown that many supplements contain banned substances that are not declared as ingredients and present risks for the consumer (Mikulcikova *et al*. 2008). The most common are anabolic steroids such as testosterone precursors, 19-nortestosterone, methandienone, or boldenone (Petroczi *et al.* 2011). These undeclared substances can cause health risks to consumers and may lead to positive results in sports doping control, especially with the nandrolone metabolite norandrosterone. 19-Nortestosterone (NTS) (17β-hydroxy-19-norandrost-4-en-3-one), also named nandrolone, is an anabolic steroid which occurs naturally in the human body, but only in tiny quantities. It is very similar in structure to the male hormone testosterone, and has many of the same effects in terms of increasing muscle mass. Ergogenic use for this steroid in sports, racing, and bodybuilding is controversial because of its adverse effects and the potential to gain an advantage. Its use is referred to as doping and is banned by all major sporting organisations.

Supported by the Ministry of the Interior of Czech Republic, Project No. MV0 VG20112015045.

The analysis of nutritional supplements for anabolic steroids has proven to be rather difficult due to the different matrices in various products. Traditional methods for the analysis of NTS such as LC/MS and GC/MS (Martello *et al.* 2007; Stepan *et al.* 2008) are highly sensitive and reliable. However, they involve multiple steps in the sample preparation and analysis, require expensive equipment and skilled analysts, and are therefore unsuitable for routine analysis of a large number of samples or on-site determinations. In contrast, immunoassays could be portable and cost-effective, with adequate sensitivity, high selectivity, and a simple sample extraction process. Therefore, immunotechniques have become popular and are increasingly considered as alternative/complementary methods for the residue analysis (Popii & Baumann 2004).

During the last decade, several immunoassays based on the enzyme-linked immunosorbent immunoassay (ELISA) for the detection of NTS in veterinary areas of animal health control have been described (Lu *et al.* 2006; Xu *et al.* 2006; Jiang *et al.* 2011b, 2012). Moreover, the strip immunoassays for the visual detection of NTS residues in unit of ng/ml in buffer or animal urine have been published by Liu *et al.* (2007), Tian *et al.* (2009), and Jiang *et al.* (2011a). To our best knowledge, no use of immunoassay for the detection of NTS in dietary supplements has been reported up to now. That is why we present here experiments leading to the design of ELISA and immunochromatographic test for these purposes.

MATERIAL AND METHODS

Chemicals and immunoreagents. The rabbit polyclonal antibodies (RAbs) specific for NTS were prepared in the Biotest s.r.o. (Konarovice, Czech Republic). Bovine serum albumin (BSA), Tween 20, polyethylene glycol (PEG, MW 3350), and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich Inc. (St. Louis, USA). The NTS-3-ovalbumin (NTS-3-OVA) and NTS-3-BSA conjugates were synthesised using 19-NTS-3-karboxymethyloxime in a reversed micellar system (Lapčík *et al.* 2004). Donkey anti-goat antibody (DAG), goat anti-rabbit antibody (GAR) as well as the goat anti-rabbit antibody with horseradish peroxidase (GAR/Px) were obtained from Nordic Immunological Laboratories (Tilburg, the Netherlands). All antibodies were supplied as purified IgG fractions of polyclonal antiserum. Gold colloid nanoparticles (an average diameter of 40 nm) were from BB International (Cardiff, UK). GAR labelled with gold nanoparticles was prepared according to the procedure described for carbon nanoparticles (Holubová-Mičková *et al.* 2010) with a slight modification. Steroids standards were from Steraloids Inc. (Newport, USA). Individual stock standard solutions containing 1 mg/ml of each compound were prepared by dissolving accurately weighed amounts in ethanol which was stored in darkness at 4°C. Working standard solutions were freshly prepared by serial dilutions in buffers. Other common chemicals were of the highest purity available and purchased from Sigma-Aldrich. Various types of nitrocellulose membranes were from Whatman GmbH, Dassel, Germany (PRIMA 80, PRIMA 125, AE 98 FAST, AE 98, Protran BA 79, Protran BA 83, Protran BA 85), Millipore Ltd., Praha, Czech Republic (Immunopore FP, Immunopore RP, HiFlow Plus HF 135, HiFlow Plus HFB180, HiFlow Plus 090), and Sartorius Stedim Biotech S.A., Aubagne Cedex, France (Unisart 140). Vinyl backing ARcare® 7823 and the absorbent pad CFSP both was from Millipore Corp. (Glen Rock, USA). ELISA polystyrene microtitre-plates Costar (catalogue No. 9018) were obtained from Corning Inc. (Corning, USA).

Instrumentation. ELISA plates were washed with Labsystem Multiwash (New York, USA) and the absorbance was read in Labsystem Multiscan MCC/340 (Helsinki, Finland). The data were processed using Microsoft Excel software (Microsoft Corporation, Tulsa, USA). For preparation of an immunochromatographic test the Linomat V (Camag AG, Muttenz, Switzerland) and a programmable strip cutter (Economic Cutter ZQ2000; Shanghai Kinbio Tech Co., Ltd., Shanghai, China) were used. Epson Perfection V700 Photo Scanner (Seiko Epson Corporation, Nagano, Japan) and the TotalLab TL100 software (Nonlinear USA Inc., Durham, USA) were used for the quantification of the colour intensity of lines on strips.

ELISA. The ELISA of NTS was carried out in an indirect competitive format as follows: the microplates were coated with the NTS-3-OVA conjugate solution in 0.05M carbonate-bicarbonate buffer, pH 9.6 (100 µl/well), left to incubate for 2 h at 37°C, and then washed four times with PBS containing Tween 20 (0.05%, v/v). The aliquots (50 µl/well) of NTS or others steroid standards, diluted in PBS, and the aliquots (50 µl/well) of RAbs were pipetted into the wells. Incubation for 2 h at 37°C and wash-

ing as described above. Subsequently, GAR/Px was added (100 µl/well), left to interact for 1.5 h at 37°C and washed. Peroxidase substrate in the reaction buffer was added to each well (100 µl/well). After 10 min incubation at 24°C, the enzyme reaction was terminated by adding 50 µl/well of 2.5M sulphuric acid, and the absorbance at 450 nm was measured.

To construct the standard curve, the absorbance values were fitted to a four-parameter logistic equation. The IC_{50} values represented the concentration of NTS that produced 50% inhibition of antibody binding to the hapten conjugate. The detection limit was defined as the lowest concentration of NTS that exhibits a signal of 10% inhibition. The linear working range was calculated as the concentrations of NTS providing a 20–80% inhibition rate of the maximum signal. The specificity of antibodies was expressed as cross-reactivity (CR) value with structurally related steroids. The CR was calculated as: (IC_{50} of NTS)/(IC_{50} of competitors) × 100.

Design of immunochromatographic strip. The strips were prepared similarly as in the procedure described previously (Holubová-Mičková *et al.* 2010), using nitrocellulose membrane sheet, vinyl backing, NTS-3-OVA (or NTS-3-BSA) and DAG solutions (5mM borate buffer, pH 8.8), Linomat V and cutter (Figure 1). Then, the strips were stored in sealed bags under dry conditions at laboratory temperature until used.

Procedure of immunochromatographic assay. In a microplate well, 50 µl of NTS standard solution in buffer (or filtrate of sample suspension) was mixed with 50 µl of the running buffer (0.2M borate buffer, pH 8.8), 20 µl of GAR-gold conjugate, and 1 µl of an anti-NTS antibody (1 µg/ml). Subsequently, the membrane strip was dipped into this reactant mixture vertically. The solution migrated toward the absorbent pad and after 10 min, the test result was observed. The colour intensity of test line could be assessed visually with naked eyes. For the quantification of the colour intensity, grayscale densitometry was used after the strip was fully air-dried and the intensity of the test lines was quantified using the TotalLab TL100 software.

Table 1. Significant parameters of optimised ELISA

Parameter	Chosen as optimal for antibody	
	No. 102	No. 120
Concentration of nortestosterone-3-OVA (µg/ml)	0.125	0.25
Concentration of antibody (µg/ml)	0.02	0.025
Additives in reaction buffer	0.1% BSA	0.1% gelatin
IC_{50} (ng/ml)	5.17 ± 0.2	6.41 ± 0.4
Linear working range (ng/ml)	1–32	1–67
Detection limit (ng/ml)	0.12 ± 0.03	0.09 ± 0.02

RESULTS AND DISCUSSION

Six rabbits were immunised with NTS-3-BSA and the antisera obtained were tested for reactivity with NTS. Checkerboard titrations were performed. Only two antibodies marked No. 102 and No. 120 were selected and with the NTS-3-OVA used for the development of ELISA.

The optimal reagent concentrations of the immunoreagents used were determined when the maximum absorbance ranged from 1.0 to 1.5, and the dose-response curve pursued the lowest IC_{50} values. Because inert proteins (such as BSA, gelatin, milk powder) and surfactants (such as Tween-20) are commonly used in ELISA to reduce nonspecific interactions, their influence on the assay performance was examined. It was observed that the addition of 0.1% BSA and 0.1% gelatin in PBS for the dilution of antibodies No. 102 and No. 120 had a positive effect on the curve parameters,

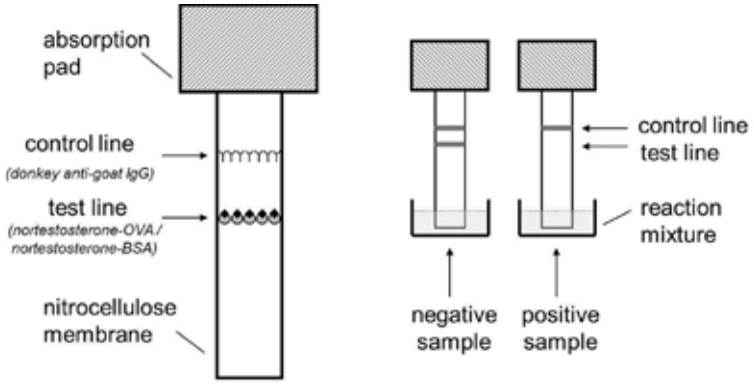

Figure 1. Schematic illustration of the strip test design

Table 2. Cross-reactivities (CR) of related structural or functional analogues in the nortestosterone ELISA

Analogue	CR (%) for antibody	
	No. 102	No. 120
Nortestosterone	100	100
Testosterone	9	23
DHEA	1	5
5-β-Androstane-3,17-dione	< 0.01	< 0.01
Epitestosterone	< 0.01	< 0.01
Progesterone	< 0.01	0.3
Cortisone	< 0.01	< 0.01
4-Androstene-11-β-ol-3,17-dione	< 0.01	< 0.01
17-OH progesterone	< 0.01	0.08
Cortikosteron	< 0.01	< 0.01
Aldosterone	< 0.01	< 0.01
17-β-Estradiol	0.06	0.02
Estriol	< 0.01	< 0.01
4-Androstene-3,17-dione	< 0.01	< 0.01
Cortisol	< 0.01	< 0.01
11-Deoxycortikosteron	< 0.01	< 0.01
21-Deoxycortisol	0.02	0.1

DHEA – dehydroepiandrosterone

respectively. Optimal combinations of concentrations are summarised in Table 1.

Figure 2 shows the representative calibration curves obtained using the optimised ELISA. The central section was accepted as the assay working range. In Table 1, the analytical parameters of the assay are shown. The limit of detection (LOD) was about 0.10 ng/ml. It is comparable with that obtained by other authors (Jiang *et al.* 2011b). From the metabolic fate of NTS in humans is it clear that the method used to analyse dietary supplements should have a detection limit of about 20 μg/g (De Cock *et al.* 2001). We can deduce that converted to solution (filtrate of suspended sample), the LOD value achieved with ELISA is sufficient in abundance.

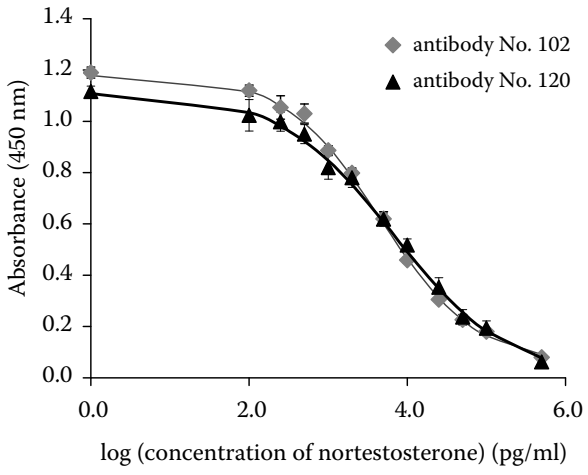

Figure 2. Standard curves of ELISA

Subsequently, the specificity of the described ELISA was evaluated by the determination of cross-reactivity (CR) based on the IC_{50} values of the individual chemicals. This was undertaken by adding various structurally or functionally related analogues instead of using NTS. When using antibody No. 120 the method exhibited slight cross-reactivity with structurally similar testosterone (23%) and DHEA (5%). Antibody No. 102 also interacted weakly (9%) with testosterone. The interferences by other structurally related analogs were negligible (≤ 0.1%), thus proving that the developed assay is highly specific for NTS (Table 2).

In the immunochromatographic method, the colloidal particles, which are indirectly conjugated to the analyte-specific antibody, serve as the label for the immunoassay. The principle of the test is as follows: The target analyte in the reactant mixture is bound by colloid-antibody (RAb-GAR-gold) and migrates due to capillary effects along the membrane (Figure 1). The test line, impregnated with NTS-protein conjugate, captures any free colloid-antibody as a narrow pink coloured band which is visible by the eye. Therefore, the samples free of NTS will result in just free colloid antibodies in the reaction mixture, which will give a pink coloured test line of maximum intensity. Alternatively, analyte-positive samples, resulting in binding to the colloid-antibody, are identified by a decrease or the absence of colour intensity in the test line. The control line acts as a positive control to assure that the labelled secondary Ab migrated through the system.

Most of the components necessary for the immunochromatographic strip design as well as the experimental conditions have a key influence on both the performance and final sensitivity. The concentrations of the reagents were preliminary optimised to satisfy the following assay criteria: good sensitivity, minimum immunoreagent consumption, and the appearance of the test line with good colour intensity and sharpness (for negative sample). Therefore, checkerboard titration experiments were carried out. Several amounts of NTS-protein conjugate immobilised on the membrane (25–400 ng per strip) against different amounts of primary antibody (0.25–4 μg per strip) were investigated

in the assays of the standard solutions containing NTS at concentrations of 0, 5, and 500 ng/ml. The same experiments were performed for different concentrations of gold-labelled GAR. Preliminary optimised concentrations of reagents were finally specified after the following optimization of other assay conditions. Then, some other factors affecting the strip test performance were also evaluated: the type of membrane and the type of running buffer. Thirteen various types of nitrocellulose membrane were tested. The type of membrane influenced the flow time and sharpness of the detection lines. Also, the composition of the running buffer markedly affected mainly the character of the reactant flow, level of background colour, sharpness, and intensity of the test line. Phosphate buffer and borate buffer of various pH values and ion concentrations were tested. Moreover, the effects of additives such as BSA, PEG, sucrose, surfactants and their combinations were tested to improve the test performance. In all these experiments, two anti-NTS antibodies (No. 102 and No. 120) and two NTS-protein conjugates (NTS-3-OVA and NTS-3-BSA) were used, alternatively. The qualitative evaluation of the lines intensity found visually was quantified using scanner and computer software. The optimised conditions found for the strip test are summarised in Table 3.

The sensitivity of the strip test was determined by testing the NTS standard samples in the concentration range from zero to 2000 ng/ml. The details of the test line intensity on the selected strips are shown in Figure 3. The visual LOD of the assay was defined here as the minimum NTS concentration producing

Table 3. Design parameters of optimised immunochromatographic strip test

Parameter	Chosen as optimal with antibody No. 102 and nortestosterone-3-BSA
Type of nitrocellulose membrane	Prima 85
Amount of nortestosterone-3-BSA conjugate in test line	50 ng
Amount of DAG in control line	200 ng
Amount of primary antibody per strip	1 μg
Amount of GAR-gold conjugate per strip	1 μg
Composition of running buffer	0.1M borate buffer, pH 8.8 (0.1% BSA, 1% PEG, 0.1% Tween)

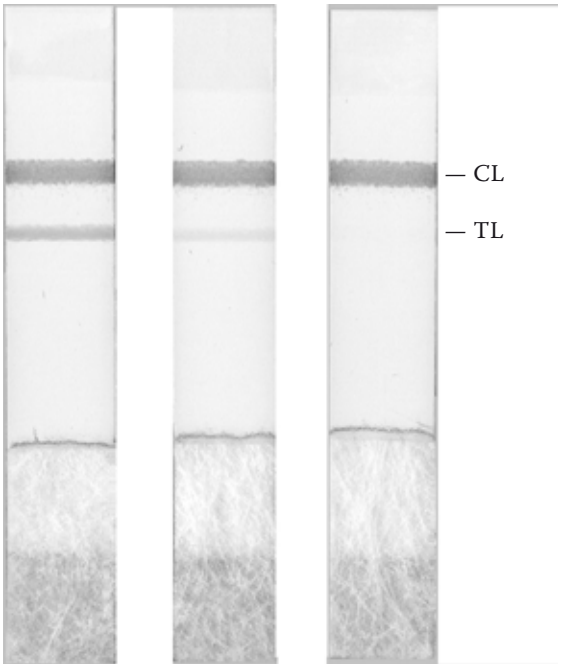

Figure 3. Typical strip tests after assay procedure of standard (Nortestosterone concentrations: (**A**) negative control, (**B**) 5 ng/ml, and (**C**) 500 ng/ml (TL – test line; CL – control line)

the colour density of the test line significantly weaker than that at zero concentration (it means absolute agreement between the result assessments of three repetitions of the same test by five observers). The NTS concentration of 1.0 ng/ml caused a slight but visually distinguishable difference in the test line intensity compared to the negative control. Thus, 1.0 ng/ml of NTS was considered to be the visual LOD for the semi-quantitative test. It is sufficient value for assaying suspended samples of dietary supplements. At concentrations of NTS > 100 ng/ml, the test line was invisible.

CONCLUSION

This study successfully demonstrated the potential of using the rapid immunoassays for sensitive detection of NTS in nutritional supplements. First, the quantitative ELISA assay was designed. Highly specific rabbit polyclonal antibody and the NTS-3-OVA conjugate were used for its construction. Under optimised experimental conditions, the LOD was 0.09 ng/ml. Moreover, the same antibody and conjugate NTS-3-BSA were used also for designing the strip test of optimal parameters that provides visual LOD of 1 ng/ml. Both developed assays are suf-

ficiently sensitive. However, the semi-quantitative strip test is easier to perform, allowing for non-specialised personnel on-site application without requirements for intensive labour and any device for sample analysis. This property makes it useful for incorporation into monitoring programs for the control of food supplements contamination. Additional investigations would be needed to the evaluate this assay as fit-for-purpose.

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Received for publication November 30, 2012
Accepted after corrections April 12, 2013

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Příloha 12:

Jurasek M., Goselová S., Miksatkova P., Holubova B., Vysatova E., Kuchar M., Fukal L., Lapcik O., Drasar P.: Highly sensitive avidin-biotin ELISA for detection of nandrolone and testosterone in dietary supplements. *Drug Test. Anal.* 9(4): 553-560, 2016.

Research article

Drug Testing and Analysis

Received: 13 October 2015 Revised: 10 May 2016 Accepted: 12 May 2016 Published online in Wiley Online Library

(www.drugtestinganalysis.com) DOI 10.1002/dta.2005

Highly sensitive avidin-biotin ELISA for detection of nandrolone and testosterone in dietary supplements

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Avidin-biotin technology was used for the implementation of an enzyme-linked immunosorbent assay (AB-ELISA) as a sensitive method for the detection of anabolic androgenic steroids (AAS) present in dietary supplements. Using click chemistry, novel haptens (linker-optimized biotinylated nandrolone (NT) and testosterone (T) at positions C-3 and C-17, respectively) were designed and synthesized to be then applied as four different immobilized competitors in a proposed set of four indirect competitive AB-ELISAs. Four rabbit polyclonal antibodies of various specificities were prepared using four different immunogens synthesized from C-3 and C-17 carboxymethyloxime and hemisuccinate derivatives of NT and T, respectively. Assembled AB-ELISAs were characterized to establish method parameters such as a half-maximum inhibition concentration (0.18–12.99 ng/mL), limit of detection (0.004–0.032 ng/mL) and linear working range (the best with 0.02–1.38 ng/mL). The stability of the set simulating storage in different conditions was demonstrated. Cross reactivity (CR) was tested for 59 steroids including both endogenous and synthetic analogues in four assembled AB-systems. The focus was placed on the practical use of the method in detection of various AAS in 49 samples of counterfeit dietary supplements. The concordance between ultra high performance liquid chromatography-mass spectrometry (UHPLC-MS) and the CR corrected data from AB-ELISA indicated the potential of this method even to quantification of T propionate, NT phenyl propionate, and NT decanoate in such a complex matter. Copyright © 2016 John Wiley & Sons, Ltd.

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Keywords: anabolics; testosterone; nandrolone; avidin; biotin; ELISA; dietary supplements

Introduction

Anabolic androgenic steroids (AAS) belong to a group of substances that are largely employed considering their tropic effect on muscle tissue of bodybuilders and athletes at all levels of performance. Originally, these substances were designed for the treatment of haematological and post-surgical conditions and substitutive supplementation.[1] Some groups have reported the connection of unwise self-administration and some types of cancer, cardiovascular problems, liver damage, and neurological disorders.[2,3] Nevertheless, there is still an increasing illicit misuse of these steroids, which are often disguised as nutritional supplements.[4,5] Nowadays it is clear that many such supplements are mislabelled and may contain AAS or prohormones that could be metabolized into banned AAS.[6–9] Some of them come from the black market and some are intentionally added to food supplements and sold as protein supplementation, vitamins, minerals, or carbohydrates, for example.[10,11] Therefore, it is important to ensure that commercially available preparations do not contain AAS. Otherwise AAS pose a threat to those people who merely take dietary supplements to maintain performance and vitality.[12,13]

Nandrolone (NT; also known as 19-nortestosterone or 17β-hydroxy-19-nor-4-androsten-3-one) and testosterone (T; 17β-hydroxy-4-androsten-3-one) and derivatives still hold the front line among abused AAS. In most cases, simple 17β-esters[14] are seized (e.g. propionate, phenylpropionate, decanoate, enanthate). Both steroids are banned in sports by the International Olympic Committee.[15] A one-year police investigation led to a successful police raid on the headquarters of an organized crime group responsible for massive imports of counterfeit medical preparations and food supplements from Slovak Republic to the Czech Republic.[16] One hundred forty-eight various types of medical preparations and food supplements for muscle growth, erectile dysfunction treatment, and fat loss pills were found during a police house search. The overall amount of seized material reached over 20 000 tablets and 1000 ampoules. All samples were submitted for forensic analysis to clarify their authenticity. For the purpose of this study, only selected samples of food supplements known to contain AAS were chosen for analysis. Samples without the presence of AAS were used as negative controls.

The main analytical methods for determination and quantification of AAS in human liquids include gas chromatography-mass

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The authors declare no competing financial interest. The university has a legal permission to buy and work with the anabolic steroids.

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spectrometry (GC-MS) and liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) based approaches.[17,18] However, these methods require skilled professionals and relatively high cost for the analysis of samples associated with the operation of devices. This study deals with the development of screening enzyme-linked immunosorbent assay method (ELISA) for detection of nandrolone and testosterone in dietary supplements. Current direct and indirect formats of ELISA utilize the competition between the steroid present in the sample and the hapten conjugate derived from the steroid with carrier protein immobilized on the microtiter plate, membranes, or particles.[19,20] Bovine serum albumin, ovalbumin or other proteins of suitable properties are used as carrier proteins.[21] By conjugation, statistically random steroid occupancy on the protein molecule is achieved. Properties of such constructs from different lots vary and are somewhat heterogeneous, which may adversely affect the set parameters and reproducibility. Moreover, according to our previous experience, the ovalbumin conjugates are sensitive to careless handling and storage, thus possible determination of the length of their use is often difficult. Here we describe the development of stable ELISA set for the detection of NT and T utilizing coating based on avidin-biotin technology (hereinafter AB-ELISA; Figure 1). In this methodology, avidin (or analogues) was coated on microtiter plates and the biotinylated part of the molecule was trapped by avidin. The steroidal part served as a competitor to an analyte in this AB-ELISA method. This methodology appeared to be more advantageous than conventional ELISA, in particular for the sensitivity[22] and stability.[23] To date, only biotinylated 7α-testosterone[24] 17α-ethinylestradiol,[23] and cortisol[25,26] were used as trappers in AB-ELISA. Furthermore, biotinylated steroids, if retaining their pristine biological activity, may be used in molecular biology as proteomic probes of biospecific interactions with their natural targets.[27–29]

To develop the optimal biotinylated structure for ELISA, we synthesized three variants of differently linked biotinylated NT derivatives at the C-3 position. Based on the relevant criteria (I_{50} - the concentration of analyte causing 50% inhibition of the maximum ELISA signal in the competitive standard curve, physicochemical properties, and synthetic accessibility), we selected a derivative with a short linker (PEG_3) as the suitable model for further work. The concept was successively expanded to C-3 biotinylated T and C-17 NT and T derivatives. The methodology utilizing AB-ELISA sets for the mentioned conjugates was established and the optimized method was used to determine the presence and quantity of AAS in real samples. The advantages of our method include, among others, undemanding amount of consumed immunoreagents[30] and the stability of the set towards preservation and temperature fluctuations.

Experimental

Synthesis

Nandrolone was purchased from Sigma-Aldrich (St Louis, MO, USA) and testosterone from Steraloids (Newport, RI, USA). Biotinylated building blocks and PEGs (Figure S1) were purchased from Click chemistry tools (Scottsdale, AZ, USA). For thin-layer chromatography (TLC), aluminum silica gel sheets were used for detection in UV light (TLC Silica gel 60 F_{254}, Merck, Darmstadt, Germany). Diluted solution of sulfuric acid in methanol was used and plates were successively heated for TLC visualization. Silica gel (30-60 μm, SiliTech, MP Biomedicals, Illkirch, France) was used for column chromatography. NMR spectra (^{1}H 300 MHz and ^{13}C 75 MHz) were recorded on a Varian Gemini 300 (Varian, Palo Alto, CA, USA). Chemical shifts are given in δ (ppm). HRMS were measured by LTQ ORBITRAP VELOS with $HESI^+$/$HESI^-$ ionization (Thermo Scientific, Waltham, MA, USA). Fourier transform infrared (FTIR) spectra were measured on Nicolet iS10 (Thermo Scientific, Waltham, MA, USA) using ATR (KBr crystal) technique (symbolism; *s* strong, *vs* very strong, *m* medium, *w* weak, *vw* very weak). Optical rotations were measured with an Autopol VI polarimeter (Rudolph Research Analytical, Hackettstown, NJ, USA). For microwave synthesis, an Initiator Classic 355301 (Biotage, Uppsala, Sweden) was used.

Representative procedure for the synthesis of biotinylated steroid at C-3 position: compound 5

Azido-terminated nandrolone[31] **2** (50 mg, 0.09 mmol) and biotinylated propargylamine (28 mg, 0.1 mmol) were mixed in microwave vial equipped with a stir bar. Then DMF (2 mL), sodium ascorbate (4 mg, 20 mol%), tris((1-benzyl-1*H*-1,2,3-triazolyl)-methyl)amine (TBTA) (10.6 mg, 20 mol%) and $CuSO_4 \cdot 5H_2O$ (2.5 mg, 10 mol%) were added. The mixture was placed onto the microwave reactor and stirred at 55 °C for 60 min. The solvent was removed under the reduced pressure and the product was chromatographed on silica gel ($CHCl_3$-MeOH, grad 25/1 → 5/1). The fractions containing the product were collected and the solvent evaporated. The residue was re-dissolved in AcOEt, silica gel was filtered off and the solvent was evaporated. The product (58 mg, 0.07 mmol) was obtained as a white foamy solid in 79% yield. $R_F = 0.78$ in DCM/MeOH/AcOH, 5/1/0.1%. For spectral characteristics see Supporting Information, Section 1.1.

Representative procedure for the synthesis of biotinylated steroid at C-17 position: compound 10

To a solution of NT (55 mg, 0.2 mmol) and azido-PEG_4-acid (68 mg, 0.23 mmol) in dry benzene (3 mL) were added *N,N´*-dicyclo-

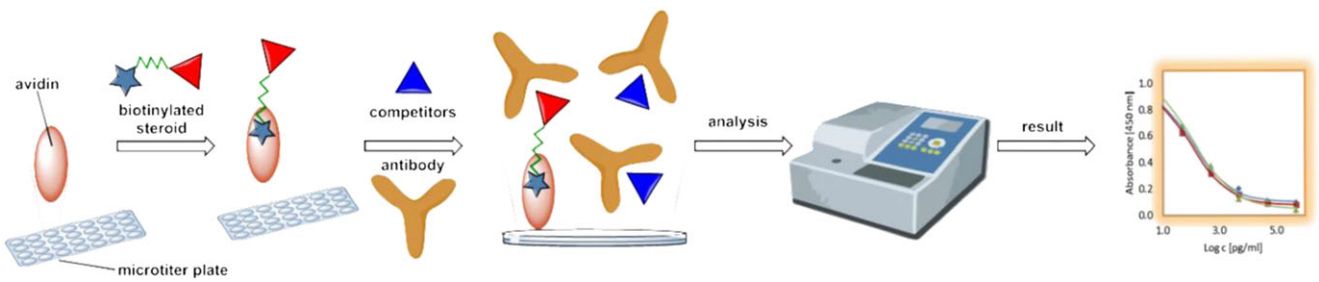


Figure 1. Schematic arrangement of AB-ELISA.

hexylcarbodiimide (DCC, 57 mg, 0.28 mmol) and 4-dimethylaminopyridine (4-DMAP, 34 mg, 0.28 mmol). The mixture was stirred for 4 h at room temperature (RT) after which dicyclohexylurea was filtered off and the solvent was removed under reduced pressure. The residue was chromatographed on silica gel (hexanes-AcOEt, 2/1 → 1/1) to obtain the product **10** (81 mg, 0.15 mmol) as a slightly yellowish gel in 74% yield. $R_F = 0.2$ in hexanes-AcOEt, 1/1, a pink spot after visualization. For spectral characteristics see Supporting Information, Section 1.1.

Compound 12

To a solution of azide derivative **10** (50 mg, 0.091 mmol) and biotinylated propargylamine (28 mg, 0.1 mmol) in dry DMF (3 mL) were added sodium ascorbate (4 mg, 20 mol%), TBTA (5.3 mg, 10 mol%) and $CuSO_4 \cdot 5H_2O$ (2.5 mg, 10 mol%). The mixture was placed onto the microwave reactor and stirred at 60 °C for 60 min. Then the solvent was removed under reduced pressure and the product chromatographed on silica gel ($CHCl_3$-MeOH, grad 15/1 → 5/1). The fractions containing product were collected and the solvents were removed under reduced pressure. The residue was redissolved in AcOEt, silica gel was filtered off and the solvent was evaporated. The product **12** (69 mg, 0.083 mmol) was obtained as a white foamy solid in 91% yield. $R_F = 0.5$ in DCM-MeOH, 20/1. For spectral characteristics see Supporting Information, Section 1.1.

Bioanalytical methods

Preparation of antibodies

Four rabbit polyclonal antibodies (RAbs) were prepared in the certified facility of Meditox s.r.o. (Konárovice, Czech Republic). As immunogens BSA conjugates with various haptens were used (C-3 carboxymethyloxime derivatives of NT and T (compounds **1** and **2**), and respective C-17 hemisuccinates of NT and T). Rabbits were immunized by a standard procedure[32] using four 200 μg doses of the immunogen (BSA) emulsified in a mixture of complete Freund's adjuvant-saline 1:1 (200 μL) in three week-intervals. The sera were collected 10 days after the last boost, lyophilized and stored at -20 °C. Antibodies for interaction with compound **5** were shortly named as RAb-NT3 and with compound **12** as RAb-NT17. Antibodies for interaction with compound **6** were shortly named as RAb-T3, with compound **13** as RAb-T17.

Materials

HRP-Goat-Anti-Rabbit IgG enzyme conjugate (GAR-Px) was purchased from Norvic Immunology (Tilburg, Netherlands). Testosterone and nandrolone standards were purchased from Sigma-Aldrich (St Louis, MO, USA). All other anabolic steroids and hormones used for CR studies (Table 2) were obtained from Sigma-Aldrich (St Louis, MO, USA) or Steraloids (Newport, RI, USA). Other reagents: ammonium fluoride, MeOH for LC-MS, avidin, Tween 20, sulphuric acid, 3,3′,5,5′-tetramethylbenzidine (TMB), hydrogen peroxide (H_2O_2) (30%), DMSO, phosphate-citrate tablets and gelatin were obtained from Sigma-Aldrich (St Louis, MO, USA). 96-Well polystyrene microtiter plates Costar 9018 were obtained from Corning Inc. (Kennebunk, ME, USA).

Used buffers and solutions: (1) buffer for dilution of avidin: carbonate buffer (50 mM, pH 9.6); (2) standard and sample dilution buffer: PBS-phosphate buffered saline (0.01 M PBS, pH 7.4); (3) antibody dilution buffer: PBS (0.01 M PBS, pH 7.4) containing 0.1% gelatin (*w/v*); (4) conjugate buffer and GAR-Px dilution buffer: PBS containing 0.05% Tween 20 (*w/v*); (5) washing buffer: PBS containing 0.05% Tween 20 (*w/v*); (6) substrate solution: 1 mg TMB, 1 mL DMSO, 2 μL H_2O_2 and 9 mL 0.05 M phosphate-citrate buffer (pH 5.0); (7) stop solution: 2 M sulfuric acid.

Preparation and conservation of the stock solutions

The lyophilized material of all reagents was weighed and dissolved in ethanol or in PBS. Stock solutions of antisera were prepared by dissolving 1 mg lyophilisate in 1 mL of PBS buffer and stored at -20 °C. These solutions were diluted in the antibody dilution buffer to working concentration before using in ELISA. The standard stock solutions of steroids and conjugates at a concentration of 1 mg/mL were stored in ethanol at -20 °C until the analysis.

Avidin-biotin ELISA analysis (AB-ELISA)

Nandrolone and testosterone were detected by the indirect competitive ELISA format. The optimized calibration curves were performed in the 0.005–500 ng/mL range. Ninety-six-well plates were coated with avidin (1 μg/mL) in a carbonate buffer (100 μL/well). After 1 h incubation at room temperature (RT) with shaking, the solutions with unbound avidin were removed from the wells (without washing). Subsequently, solutions of biotinylated NT or T were added (100 μL/well) and incubated under the same conditions. Then the plates were washed with washing buffer using an automatic plate washer (4 × 350 μL/well). For the competitive reaction 50 μL/well of standard solution or sample extract and 50 μL/well of antibody solution were added and incubated for 1 h at RT with shaking. After another microplate washing, 100 μL/well of GAR-Px diluted 1:10 000 in appropriate buffer were added and incubated for 1 h at 37 °C with shaking. The plates were washed and 100 L/well of substrate solution was added. Finally, after 10 min incubation at RT with shaking, the reaction was stopped by the addition of 50 μL of stop solution and the absorbance was measured at 450 nm using an ELISA reader (BIO-TEK μQuant operating with Microsoft Excel). To constructed standard calibration curve, absorbance values were fitted to a four-parameter logistic equation.

Specificity

The specificity of the immunoassays was evaluated by determination of the cross-reactivity (CR) considering various anabolic steroids and hormones. The CR studies were carried out in four variants of AB-ELISA using various antibodies. Concentration of free competitors ranged from 0.005 to 500 ng/mL. The CR value was calculated as follows: (I_{50} of standard)/(I_{50} of competitor) × 100.

Real samples and their preparation

Real samples of seized food supplements were supplied by the Police of the Czech Republic. The content of the supplement samples was confirmed by previously optimized and validated methods using two-dimensional gas chromatography with time-of-flight mass spectrometric detection (GC × GC-TOF MS) and liquid chromatography with quadrupole time-of-flight mass spectrometry (LC-QTOF). The individual standard stock solutions and working mixtures were prepared in acetonitrile (ACN) (LC-QTOF) or ethyl acetate (AcOEt) (GC × GC-TOF). Tablets and oil ampoules (sample weight 0.05 g) were extracted with ACN or AcOEt. Before injection, extracts were filtered through micro-filters and diluted in ACN or AcOEt due to expected high content of anabolic steroids. The GC × GC-TOF analysis was performed according to a previously published method.[33] A gas chromatograph Agilent 6890 N (Agilent Technologies, Santa Clara, CA, USA) coupled to a time-of-flight mass spectrometer LECO Pegasus IV (LECO Corporation, USA) with splitless injection technique were used.

Separation was performed using capillary columns DB5-MS (30 m × 0.25 mm × 0.25 mm; J&W Scientific, Folsom, CA, USA) in the first dimension and BPX-50 (2 m × 0.1 mm × 0.1 mm; SGE, Pflugerville, TX, USA) in the second dimension.

An Agilent 1260 Infinity Binary HPLC system coupled to a 6550 Accurate Mass Quadruple time-of-flight (Agilent Technologies, Santa Clara, CA, USA) with ESI ion source in positive mode were used. The sample analysis was performed using LC-QTOF under the following operating conditions: injection volume 3 μL; Kinetex C18 HPLC column (100 × 2.1 mm i.d., 2.6 μm, Phenomenex, Inc., USA) with a pre-column thermostated at 40 °C; mobile phase: water (A) and ACN (B) with 0.1% formic acid; flow rate 0.35 mL/min; gradient elution: 40% B (hold 1 min), from 2 to 13 min the%B were linearly increased to 99% (hold 7 min); total run time 25 min (including equilibration time 5 min); QTOF-MS operated in 4 GHz High Resolution Range mode, 4 spectra/sec, drying gas 15 L/min at 170 °C, sheath gas 12 L/min at 350 °C, collision energy 10–40 eV, capillary voltage 3500 V.

A total of 49 samples were tested. Most of them contained only anabolic steroids, but also, some non-steroidal substances (clenbuterol, phentermine, clomiphene, yohimbin) were included as negative control. Some samples containing NT (22, 20, 41, 46) and T derivatives (26, 27) were subjected to quantitative determination.

For ELISA methods and UHPLC-MS/MS quantification tablets were crushed and dissolved in 10 mL of 96% ethanol. Capsules were dissolved in 10 mL of 96% ethanol. The liquid samples in vials were pipetted (0.25 mL) to the 96% ethanol (2.25 mL) and mixed. The extraction was performed for 30 min at RT (stirring). Supernatants were collected and stored at 4 °C. Before ELISA analysis the samples were 100× diluted in PBS. Thresholds of absorbance were chosen arbitrarily for evaluation of the samples as positive ($A < A_{20\%}$), suspect ($A_{20\%}$ - $A_{80\%}$) and negative ($A > A_{80\%}$) for the incidence of AAS. These limits were selected with regards to the fact that the doping daily dose usually exceeds 10 mg of AAS and as the relevant contents of AAS is thus above 100 μg per tablet. When the test sample contains testosterone or nandrolone, these values correspond to the content of 0.0002 – 0.02 μg per tablet ($A_{20\%}$) and 0.02–2.4 μg per tablet ($A_{80\%}$). At the same time using this threshold the methods should be able to catch physiologically relevant amounts of compounds with CR at least 2.5% (these values correspond to the content of 0.008–0.8 μg per tablet ($A_{20\%}$) and 0.6–96 μg per tablet ($A_{80\%}$)). Absorbance below $A_{20\%}$ corresponds to almost colorless solution, which is easily distinguishable from a negative control even by the eye.

UHPLC-MS/MS quantification of selected AAS

For the UHPLC-MS/MS quantification Agilent 1290 Infinity UHPLC system coupled with Agilent 6460 Triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) was used. Chromatographic separation was performed on Agilent Zorbax Eclipse Plus C18 column (2.1 × 50 mm; 1.8 μm). The mobile phases for gradient elution were ammonium fluoride in water (1 mM) (A) and methanol (B). The flow rate was 0.25 mL/min and column temperature 35 °C. Gradient elution was carried out as follows: 0 min, 80:20 (A:B); 2 min, 15:85; 4 min, 0:100; 5 min, 0:100; 5.2 min, 80:20; 6.5 min, 80:20.

The MS/MS apparatus was operating in positive mode. The applied conditions of electrospray ion source were: drying gas temperature 350 °C; drying gas flow 12 L/min; sheath gas temperature 400 °C; sheath gas flow 12 L/min; nebulizer pressure 50 psi; nozzle voltage 2000 V; capillary voltage was set at 4000 V. Multiple reaction monitoring (MRM) mode was used for the detection. Two transitions of *m/z* per analyte were used: testosterone propionate 345.3 → 109.1, 97.1; nandrolone decanoate 429.3 → 95.2, 71.2; nandrolone phenyl propionate 407.3 → 105.1, 91.1.

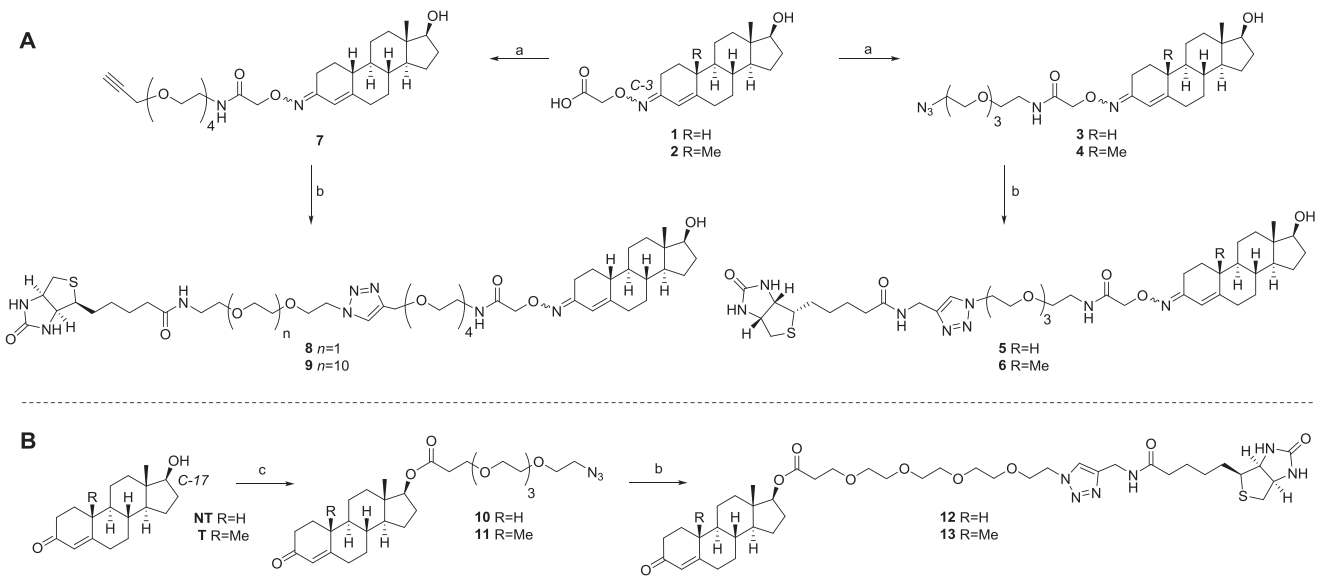
Standard stock solutions were prepared in ethanol at concentration of 1 mg/mL and subsequently diluted in 20% methanol in seven concentration levels ranging from 0.1 to 500 ng/mL. Peak areas of the external standards (at each concentration) were plotted against the corresponding standard concentrations using linear regression to generate standard curve. The stock solution of the real samples in ethanol were 10 000-fold diluted with 20% methanol. Agilent Mass Hunter (Agilent Technologies, Inc. Santa Clara, CA, USA) was used for data acquisition and quantification of samples.

Results and discussion

Chemistry

The synthetic steps leading to biotinylated nandrolone (NT) and testosterone (T) are displayed in Scheme 1. For synthesis of the biotinylated anabolics a copper catalyzed 'click' reaction[34] of a terminal azides and alkynes was used. The synthesis of biotinylated NT and T at C-3 position was carried out from steroid-3-carboxymethyl oximes[20,31,35] (CMO). These starting CMO intermediates were isolated as a mixture of *E/Z* isomers and were not separated. From the intensity of the 4-H signal of NT at δ 5.8 ppm (*E*) and 6.51 ppm (*Z*) the ratio of *E/Z* isomers was 3:2 and in case of T δ 5.75 ppm (*E*) and 6.42 ppm (*Z*) 2:1, respectively.[36] The NT-CMO was elongated by linkers of different length, namely by azidoPEG_3-amine and alkynePEG_4-amine (Supporting Information, Figure S1) using *N*-(3-dimethylaminopropyl)-*N*´-ethylcarbodiimide chemistry (EDCI) to obtain derivatives terminated by azido (Scheme 1, part A, compound **3**) and alkyne moiety (Scheme 1, part A, compound **7**), respectively. Biotin label was attached using standard click chemistry protocol with $CuSO_4 \cdot 5H_2O$, sodium ascorbate and TBTA[37] as catalysts. In one case biotinylated propargylamine[38] (Supporting Information, Section 1.1) was introduced into click reaction with both azido-terminated steroids (**3**, **4**) obtaining the shortest biotinylated conjugates (Scheme 1, part A, compounds **5**, **6**). Other longer linked NT-biotin clickates (Scheme 1, part A, compounds **8**, **9**) were prepared by the cycloaddition of alkyne-terminated NT derivative **7** and commercially available biotin-*link*-azides (namely with biotin-PEG_3-azide, biotin-PEG_{12}-azide; for structures see Supporting Information, Figure S1). We synthesized three differently C-3 linked NT-biotin conjugates and one of T. The C-17 biotinylated steroids were constructed via azidoPEG_4-linker (Scheme 1, part B, compounds **10**, **11**), following the click protocol described above (Scheme 1, part B, compounds **12**, **13**). The yields of the products along the synthetic route to desired products were from good to excellent and are shown in the legend of Scheme 1. The spectral characteristics (see Supporting information for NMR Figures S2-S23, HRMS Figures S24–S33, IR, specific optical rotation) and synthetic details are described in Supporting Information, Section 1.1. As all the compounds contain PEG linkers it was impossible to get crystals, nevertheless after lyophilization we obtained foamy solids in case of compounds **5**, **6**, **12**, **13**. The compounds **8** and **9** remained gel-like.

The polyclonal rabbit antibodies (hereinafter RAb) were prepared accordingly to standardized protocol[32] using CMO derivatives of NT and T (compounds **1** and **2**, respectively, antibodies RAb-NT3, RAb-T3, respectively) and respective C-17 hemisuccinates (synthesis is not shown, antibodies RAb-NT17 and RAb-T17, respectively) as haptens.



Scheme 1. Synthesis of biotinylated steroids. *Reagents and conditions:* Preparation of compounds **1** and **2**; steroid, pyrrolidine, MeOH, 15 min, *O*-(carboxymethyl)hydroxyamine hemihydrochloride, RT, 3 h, yield* 74% for **1** and 78% for **2**; a) azidoPEG$_3$-amine (for **3** and **4**)/aminoPEG$_4$-acetylene (for **7**), EDCI, 4-DMAP, HOBt, DMF, 0 °C → RT, 16 h, yield 83% for **3**, 78% for **4** and 86% for **7**; b) biotin-link-azide/alkyne, $CuSO_4$ $5H_2O$, sodium ascorbate, TBTA, DMF, MW, 90 min, 60 °C, yield 79% for **5** and **6**, 94% for **8**, 84% for **9**, 91% for **12** and 86% for **13**; c) azidoPEG$_4$-acid, DCC, 4-DMAP, benzene, 4 h, RT, yield 74% for **10** and 72% for **11**.*Isolated matter after chromatographic purification.

Selection of a suitable model compound

Synthetized biotinylated conjugates of NT at C-3 position (compounds **5**, **8,** and **9**) were used to assort the most optimal length of the linker. The working calibration curves of the variants based on above mentioned conjugates and RAb-NT3 confirmed the functionality in all versions (Figure 2A). As the test run showed only small differences in the I_{50} value (0.109 ng/mL for **5**, 0.1 ng/mL for **8,** and 0.095 for **9**), representing the concentration of standard that produced 50% inhibitory of antibody binding to the hapten conjugate, we turned our attention to physicochemical appearance of the compounds. Finally, conjugate constructed via PEG$_3$ linker was chosen as the most promising (compound **5**). This compound was obtained as foamy solid after lyophilization (unlike the remaining) and prepared in 79% yield over three steps. Based on the selected length of the linker, we further synthesized C-3 linked biotinylated derivative of T (Scheme 1, compound **6**) and the corresponding biotinylated derivatives of NT and T at C-17 position, bridged via PEG$_4$ linker (Scheme 1, part B, compounds **12**, **13**, respectively).

Assembled method

The AB-ELISA systems for both NT and T were characterized by the distinct analytical parameters (namely I_{50} value, LOD and linear working range) acquired by the construction of the standard calibration curves. The parameters of the systems are summarized in Table 1. The LOD was defined as the lowest concentration of the NT or T that exhibits a signal of 10% inhibition. Under optimized experimental conditions, the LODs for the AB-ELISA based on the compounds **5** (RAb-NT3), **6** (RAb-T3), **12** (RAb-NT17) and **13** (RAb-T17) were 0.014, 0.013, 0.032 and 0.0041 ng/mL, respectively. The linear working range was calculated as the concentrations of the NT or T providing a 20–80% inhibition rate of the maximum signal.

The detection limits and I_{50} values for the methods assembled with **5**, **6** were altogether qualitatively better (shifted by one order of magnitude) than those published previously with classical protein coating.[19,20] The parameters of the sets with **12**, **13** were either comparable or improved compared to those declared on commercial detection kits for T in urine or serum. The quantity of

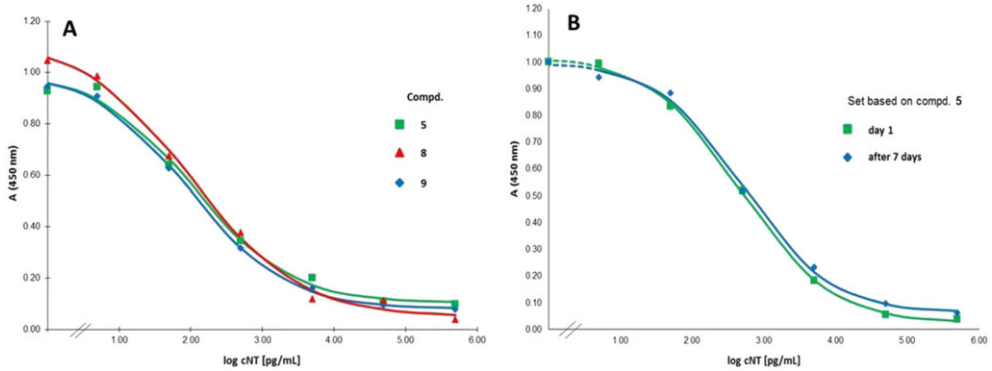


Figure 2. **(A)** Confirmation of the functionality of biotinylated NTs. Calibration curves of indirect competitive ELISA systems using 0.25 ng of compounds **5**, **8** and **9** (haptens) immobilized onto avidin coated microtiter plates and RAb-NT3 antibody. The I_{50} values were 0.109, 0.100, and 0.095 ng/mL for **5**, **8**, and **9**, respectively. **(B)** Calibration curves of AB-ELISA of NT using RAb-NT3 after the exposure of coated plates to non-standard storage conditions after a week.

Table 1. Parameters of the assembled AB-ELISA systems. Standards of the NT and T were diluted to 11 concentrations ranging from 0.005 to 500 ng/mL. Compounds 5, 6, 12 and 13 were used as immobilized haptens on avidin-coated microtiter plates; concentration of antibody is expressed as μg/mL of lyophilized polyclonal antiserum.

Antiserum	RAb-NT3	RAb-T3	RAb-NT17	RAb-T17
Immobilized hapten	**5**	**6**	**6**	**13**
concentration of the compound (ng/mL)	2.5	5	1.25	2.5
concentration of the antiserum (g/mL)	1.25	1.25	2.5	2.5
I_{50} (ng/mL)	0.180 ± 0.005	0.46 ± 0.01	0.53 ± 0.02	12.99 ± 2.45
LOD (ng/mL)	0.032 ± 0.002	0.014 ± 0.001	0.0041 ± 0.0008	0.013 ± 0.001
linear working range (ng/mL)	0.02 - 1.38	0.02 - 15.35	0.02 - 14.88	2.18 - 242.72

AAS in food supplements reached mg/g levels, thus, providing sufficient LOD values when determined in this work.

Specificity of the antibody

The specificity of the antibody was determined by measuring the CR under optimized conditions. Overall 59 standards of anabolic steroids and other steroid hormones (the structures of all cross-reactants are shown as Supporting Information (Figures S34 and S35) were tested in four assembled AB-ELISA systems for the biotinylated conjugates of NT (compounds **5**, **12**) and T (compounds **6**, **13**). Results of the CR study are summarized as Supporting Information (Section 2.1, Table S1). From these data, it is obvious that antibodies against C-3 steroid haptens (RAb-NT3 and RAb-T3) were more specific, since they significantly reacted with fewer standards particularly with steroids comprising identical or closely related substituents at the C-17 position. The significant response in the case of AB-ELISA assembled with RAb-NT3 antibody was determined with boldenone (CR 11.12%), dihydrotestosterone (CR 31.42%), drostanolone (CR 19.65%), testosterone (CR 38.35%), and trenbolone (CR 7.78%). RAb-T3 cross-reacted substantially with the above mentioned steroids, except for trenbolone and further with 17α-methyltestosterone (CR 6.44%), ethisterone (CR 5.71%), methandienone (CR 8.89%), and mesterolone (CR 20.12%). On the other hand, antibodies obtained from the haptens conjugated at C-17 position (RAb-NT17 and RAb-T17) significantly cross-reacted with the steroids and their derivatives having similar features on the A ring of the steroid. Therefore, they could be used as generic methods for high throughput detection of AAS. RAb-NT17 extensively reacted with 11 standards of anabolic steroids particularly NT derivatives CR (4.26 to 39.70%) but also with progesterone (CR 2.42%), trenbolone (CR 24.71%), trenbolone acetate (CR 33.77%). and trenbolone enanthate (CR 2.56%). The CR of other standards of anabolic steroids was not significantly increased. RAb-T17 antibody significantly reacted with 29 standards, especially with those of T and NT (CR ranging from 4.92 to 63.62%) and other 6 samples characterized by moderate CR (ranging from 1.63 to 3.59%). The other standards with considerably different structure at position C-3 showed rather low CR or none.

Analysis of the real samples by four variants of AB-ELISA

The samples of seized food supplements were generously provided by the Police of the Czech Republic. Because these supplements were seized on the black market, the manufacturer was uncertain. For evaluation of AAS presence in real samples, the signal lower than 20% of the maximum absorbance (A20%) was considered as positive, A20%–A80% as suspect, and higher than A80% as negative. The absorbance below A20% may be easily recognized visually, which makes the test suitable for analysis in field conditions. In this experiment, 49 samples of preparations based on AAS or non-steroidal substances abused as doping agents were analyzed. Complete results are provided as Supporting Information (Section 2.2 and Table S2).

By the AB-ELISA method and based on conjugate **5** and Rab-NT3, 21 samples were determined as positive, for example those containing NT esters such as decanoate (CR 0.35%) and phenyl propionate (CR 0.04%), but also derivatives of T, boldenone and methandienone. The CRs of this system were relatively low but still other analytes in addition to NT could be detected. Nineteen samples were identified as suspected to contain AAS and only 9 were negative in this test.

The variant of AB-ELISA set with RAb-T3 and compound **6** positively tested 23 samples. These samples contained e.g. methandienone (CR 8.89%), mesterolone (CR 20.12%), methyltestosterone (CR 6.44%) and esters of T. Furthermore, 15 samples were marked as suspected to contain AAS and 11 were negative in this test.

Solely 12 samples were detected as positive by the AB-ELISA system based on the compound **12** and RAb-NT17. All these samples contained derivatives of NT, for example NT decanoate (CR 6.53%), 2 out of 3 of NT phenylpropionate (CR 4.26%), 2 out of 4 of trenbolone esters (the highest with acetate CR 33.77%) and samples containing esters of T. With this system, 27 samples were indicated as suspected to contain AAS and 10 were negative in this test.

The method was based on RAb-T17 and conjugate **13** marked samples containing T undecanoate, 4 samples containing T propionate, and samples containing T cypionate and enanthate as positive. Another 11 samples were positive containing for example methandienone (CR 17.13%), methyltestosterone (CR 42.97%), boldenone undecylenate (CR 2.31%), trenbolone acetate (CR 0.58%). Moreover, 19 samples were identified as suspected to contain AAS and 11 were negative in this test.

The possibility of quantification of NT and T derivatives in seized preparations by developed ELISA methods was tested on a set of 6 samples (Table 2). For comparison, the values obtained by ELISA were recalculated on the basis of CR values of anabolic steroids and other steroid hormones as follows: Recalculated ELISA/CR (μg/mL) = $M_{(analyte)} \times (100/CR_{analyte})$, where $M_{analyte}$ was the value obtained by ELISA (μg/mL) and $CR_{analyte}$ was the cross-reactivity of the particular compound (in%).

The concordance was calculated as follows: (the CR corrected concentration indicated by AB-ELISA/UHPLC-MS) × 100. All results from this experiment are shown in Table 2. The concordance ranged from 55% to 120% thus indicating the potential of the ELISA

Table 2. Comparison of UHPLC-MS and ELISA measurements in the real samples. *Recalculated concentration of particular steroid derivatives obtained by the ELISA considering the CR in ELISA. #Data coming from ELISA (± standard deviation) using its main analyte as calibrator (i.e. NT for RAb-NT3 and **5**, NT for RAb-NT17 and **12**, T for RAb-T3 and **6**, T for RAb-T17 and **13**). These values are expressed as a concentration of NT and T. For clarity samples **26**, **27** are T propionate, **22** NT phenylpropionate and **20**, **41**, **46** NT decanoate.

RAbs and compounds	Sample	Composition	Results ELISA# (μg/mL)	Recalculated ELISA/CR (mg/mL)*	UHPLC MS/MS (mg/mL)	Concordance (%)
	26	T propionate	2.7 ± 0.1	6.8	6.4	106
	27	T propionate	1.2 ± 0.1	3.0	2.5	120
RAb-NT3	22	NT phenylpropionate	6.6 ± 0.2	16.5	19.1	86
compounds	20	NT decanoate	5.6 ± 0.3	1.6	2.5	64
5	41	NT decanoate	12.6 ± 0.1	3.6	3.8	95
	46	NT decanoate	9.1 ± 0.2	2.6	2.2	118
	26	T propionate	2.9 ± 0.2	4.8	6.4	75
	27	T propionate	1.3 ± 0.1	2.2	2.5	88
RAb-T3	22	NT phenylpropionate	2.1 ± 0.3	10.5	19.1	55
compound	20	NT decanoate	2.7 ± 0.1	2.3	2.5	92
6	41	NT decanoate	4.3 ± 0.1	3.6	3.8	95
	46	NT decanoate	1.6 ± 0.3	1.3	2.2	59
	26	T propionate	9.8 ± 0.2	3.8	6.4	59
	27	T propionate	4.5 ± 0.1	1.7	2.5	68
RAb-NT17	22	NT phenylpropionate	880 ± 12	20.7	19.1	108
compound	20	NT decanoate	156 ± 2.1	2.4	2.5	96
12	41	NT decanoate	268 ± 1.5	4.1	3.8	108
	46	NT decanoate	116 ± 1.9	1.8	2.2	82
	26	T propionate	4010 ± 95	6.3	6.4	98
	27	T propionate	920 ± 47	1.4	2.5	56
RAb-T17	22	NT phenylpropionate	889 ± 24	16.2	19.1	85
compound	20	NT decanoate	125 ± 2	2.5	2.5	100
13	41	NT decanoate	114 ± 10	2.3	3.8	61
	46	NT decanoate	110 ± 1	2.3	2.2	105

methods for the detection of cross-reacting AAS in complex samples. There was a sufficient space for setting up the cut off limits by using proper dilution of the extracts for the detection of levels of AAS that are significant from the doping control point of view in individual types of samples, taking into account the sub-nanogram sensitivity of ELISAs together with the fact that application dosage of AAS substantially exceeds milligram/day amounts. Real preparations often contain more than one AAS (e.g. a mixture of several carboxylic acids esters). It is virtually impossible to develop a universal screening method for quantitative estimation of 'total' AAS, however, the knowledge of the impact of individual cross-reactants on the intensity of ELISA signal might provide a solid basis for a generic semi-quantitative interpretation. In this concept, the numeric value of signal corresponded to the sum of individual AAS contributions. Negative ELISA signal excluded the presence of any cross-reacting AAS at levels above the cut-off limit. A positive signal indicated the presence of at least one such compound. The range of probable cumulative concentrations of contributing compounds could be estimated from the calibration curve as the interval between the corresponding levels of the strongest and the weakest cross-reactant. ELISA enabled parallel analysis of multiple samples in a short time, with a simple procedure and inexpensive instrumentation or even without any instrumentation in field conditions. The demands on qualification of the operators are also substantially lower than for GC-MS or LC-MS, which enables even the non-professionals to practice the method after a short training. ELISAs thus appear to be useful screening tools for efficient preselection of the suspect samples for more demanding instrumental approaches, which nevertheless are irreplaceable in identification and quantification of individual compounds.

Conclusion

In this work, we described the synthesis of biotinylated NT and T using click chemistry, development of ELISA set utilizing the avidin-biotin technology and its application to real samples of dietary supplements. Four AB-ELISA assays characterized by several advantages were designed and developed. Identified features included batch-to-batch reproducibility of immobilized hapten, the stability of the plates, undemanding instrumentation (compared with GC-MS, LC-MS), time of analysis, low consumption of immunoreagents and very good LOD values. The application of AB-ELISAs to seized sample material demonstrated that our methods were capable of detecting a wide range of AAS in such complex samples. Moreover, concordance between UHPLC-MS and the CR corrected data from AB-ELISA indicated the potential of this method for quantification of T propionate, NT phenyl propionate and NT decanoate. The authors intentionally work on the establishment of AB-ELISA sets for the detection of other AAS, as the synthetic approach towards biotinylated AAS is underway.

Acknowledgements

This work was supported by grants VG20112015045 (Ministry of Interior, Czech Republic) and MSMT No 20/2015 (Ministry of Education, Youth and Sports, Czech Republic).

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Příloha 13:

Fojtíková, L., Holubová, B., Kuchař, M. Nové psychoaktivní látky. *Chem Listy*, *111(*4): 234–238, 2017.

Chem. Listy *111*, 234–238 (2017) Referát

NOVÉ PSYCHOAKTIVNÍ LÁTKY

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Došlo 5.1.17, přijato 19.1.17.

Klíčová slova: nové psychoaktivní látky, syntetické drogy, kanabinoidy, fenylethylaminy, kathinony, spice, bath salts

Obsah

1. Úvod

V posledních letech se zvyšuje počet nových psychoaktivních látek na evropské i světové úrovni. Nové psychoaktivní látky (NPS, z angl. New Psychoactive Substances), nebo také nové syntetické drogy, legal highs, designer drugs, případně research chemicals jsou syntetizovány jako strukturní analoga nebo chemické deriváty již zákonem kontrolovaných látek[1,2]. Bývají nabízeny jako sběratelské předměty, vykuřovadla či soli do koupele, tedy jako látky, u nichž není deklarováno použití k vnitřnímu užití. Touto cestou se jejich výrobci vyhýbají právnímu postihu. Současný nárůst NPS je připisován rozvoji jistého druhu tanečních zábavních scén a ochotě zejména mladých lidí vyhledávat a získávat nové psychedelické zkušenosti.

2. Definice nových psychoaktivních látek (NPS) a legislativa na Evropské úrovni

Nová psychoaktivní látka je definována jako: Nová omamná nebo psychotropní látka v čisté formě nebo v přípravku, která nepodléhá kontrole podle Jednotné úmluvy Organizace spojených národů o omamných látkách z roku 1961 nebo podle Úmluvy Organizace spojených národů o psychotropních látkách z roku 1971, ale která může představovat srovnatelnou hrozbu pro veřejné zdraví jako látky uvedené v těchto úmluvách[3].

Problematika NPS je řešena na celoevropské úrovni. Rada Evropské unie přijala v červnu 1997 Společný postup v oblasti nových syntetických drog (Joint Action on New Synthetic Drugs), jehož cílem bylo zajišťovat co nejlepší a nejrychlejší informovanost o syntetických drogách, jež se nově vyskytnou ve státech EU. Dokument se vztahoval na syntetické drogy nepodléhající kontrole podle mezinárodních úmluv, viz výše. Na základě Společného postupu byl vytvořen tzv. systém včasného varování před novými drogami (EWS, z angl. Eearly Warning Systém on New Synthetic Drugs), který je koordinován Evropským monitorovacím střediskem pro drogy a drogovou závislost (EMCDDA, z angl. European Monitoring centre for Drugs and Drug Addiction) a Europolem[4].

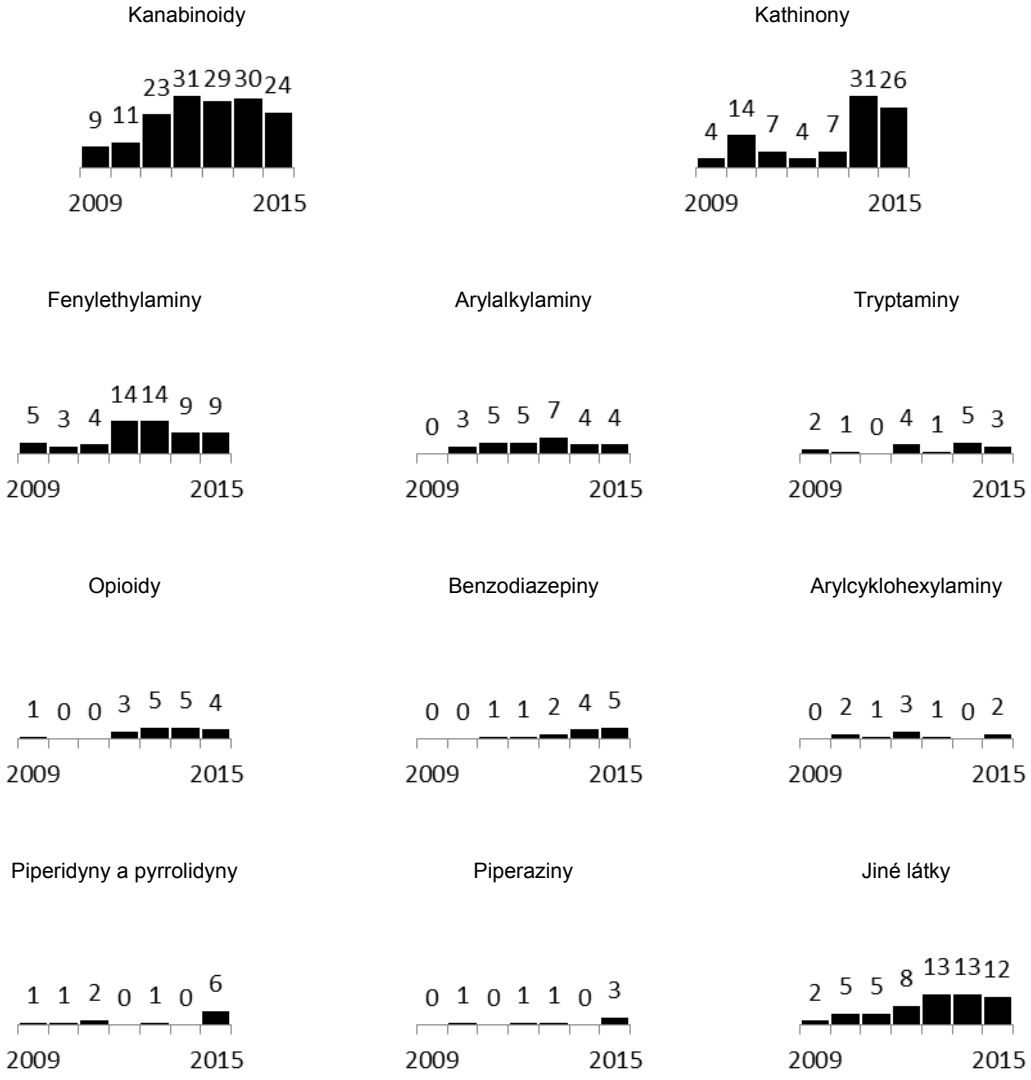
V roce 2005 byl Společný postup proti novým syntetickým drogám nahrazen rozhodnutím Rady o výměně informací, hodnocení rizika a kontrole nových psychoaktivních látek, jenž se vztahuje nejen na nové syntetické látky, které nejsou zahrnuté v mezinárodních úmluvách, ale i na obecně nové omamné a psychotropní látky včetně veterinárních či humánních léčivých přípravků, které nejsou kontrolované mezinárodními úmluvami, a také znovuobjevení se některých starých psychotropních látek a/nebo vysoce rizikových způsobů užívání drog[4].

3. Hlavní skupiny NPS

EMCDDA monitoruje značnou škálu NPS, které dělí podle struktury nebo funkce do jedenácti skupin (obr. 1)[2]. Počet NPS se každým rokem zvyšuje. Pro představu – v r. 2015 bylo do EWS nahlášeno 98 NPS, čímž se jejich počet zvýšil na více než 560, (70 % bylo zjištěno v posledních pěti letech). Nejpočetnějšími skupinami hlášených látek jsou syntetické kanabinoidy, kathinony a fenylethylaminy.

3.1. Syntetické kanabinoidy

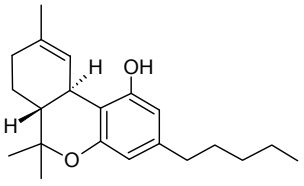
Syntetické kanabinoidy (SK) představují největší procentuální podíl zachycených NPS. Většina těchto látek



Obr. 1. **Počet a kategorie nových psychoaktivních látek poprvé hlášených do systému včasného varování EU, 2009–2015** (převzato z práce[2])

byla původně připravena jako léčiva, proto je jejich název spojen se jmény vědců a institucí kde byly připraveny (př. JWH-200 – Dr. John William Hufman; CP-47,497 – Charles Pfizer; HU-210 – Hebrew University)[5]. Jsou agonisté kanabinoidních receptorů (CB_1 a CB_2), vykazují tedy podobné účinky jako Δ^9-tetrahydrokanabinol (Δ^9-THC, psychoaktivní látka marihuany, obr. 2). Popularitu mezi uživateli získaly v návaznosti na legislativní omezování marihuany. Na trhu se začaly objevovat kolem roku 2008 jako vykuřovadla balená v pestrobarevných sáčcích převážně s názvem *Spice* a popiskou „nevhodné k lidské spotřebě“[6,7].

Z chemického hlediska jsou SK velmi různorodé, nemají řádně prostudovanou farmakologii a toxikologii. Řada z nich však vykazuje vyšší afinitu ke kanabinoidním receptorům než Δ^9-THC, jejich účinky proto mohou být silnější a užívání nebezpečnější ve srovnání s marihuanou.



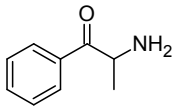
Obr. 2. **Strukturní vzorec Δ^9-tetrahydrokanabinolu**

V souvislosti s užitím SK byla popsána široká škála nežádoucích psychoaktivních účinků, např. úzkostné stavy, psychózy, změna vnímání času a prostoru, poruchy paměti, problémy s koncentrací. Fyziologické účinky SK se značně liší, byly popsány sedativní účinky, ale také neklid, nevolnost, návaly horka, pálení očí, suchost v ústech, třes a bušení srdce. Dlouhodobé užívání *Spice* produktů vedlo k rozvoji tolerance a abstinenčnímu syndromu[8,9].

3.2. Syntetické kathinony

Syntetické kathinony jsou různorodou skupinou derivátů přírodního kathinonu (obr. 3). Kathinon je monoaminový alkaloid obsažený ve stromu kata jedlá (Catha edulis), který je chemicky podobný amfetaminům. Stimuluje centrální nervovou soustavu a způsobuje uvolnění katecholaminů z presynaptických úložišť do oblasti centrální nervové soustavy a periferního nervového systému. Za obměňováním struktury kathinonu byla snaha připravit deriváty se stejným nebo vyšším farmakologickým účinkem. V původní struktuře jsou měněny délky alkylových řetězců, počty alkylů na dusíku, nahrazují se alkyly na aromatickém jádře za halogeny. Další možností je celková záměna benzenu za jiné aromatické jádro (thiofen, naftalen). Tímto způsobem vznikl např. mefedron, methylon, butylon, flefedron a MDPV[10].

Kathinony se prodávají především v krystalické formě jako koupelové soli (bath salt). V internetových obchodech se dají koupit např. pod anglickými názvy *Bliss*, *Magic*, *Meow Meow* nebo *Zoom*, ovšem opět s poznámkou „ne pro lidskou spotřebu“, „rostlinné potraviny“ a „insekticidy“. Takovéto označení brání v zakročení bezpečnostním agenturám a zmíněné látky se stávají snadno dostupnými[11]. Syntetické kathinony mohou být užívány orálně, intranasálně, intravenózně nebo rektálně.



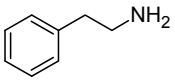
Obr. 3. **Strukturní vzorec kathinonu**

Všechny deriváty kathinonu mají podobné účinky jako kokain a metamfetamin, které stimulují uvolňování a inhibují zpětné vychytávání noradrenalinu, serotoninu a dopaminu. Přesný mechanismus účinku je předmětem výzkumu. Farmakokinetika je vzhledem k proměnlivému složení „koupelových solí“ nepředvídatelná. Důsledkem užívání těchto látek může být halucinace, paranoia, neklid, euforie, sebevražedné myšlenky, ale také poruchy srdečního rytmu, hypertenze nebo špatné prokrvení periferních tkání. Publikovány byly také závažné komplikace, jako je například selhání ledvin, sebevraždy, vraždy a smrt[11,12].

3.3. Fenylethylaminy

Do této skupiny spadají NPS odvozené od strukturního motivu fenylethylaminu (obr. 4). Fenylethylamin je látka vyskytující se nejen v lidském organismu, ale i v řadě přírodních produktů a potravinách. Tento biogenní amin nevykazuje psychoaktivní účinky. V organismu vzniká metabolickou přeměnou z aminokyseliny fenylalaninu a následně podléhá dalším metabolickým přeměnám působením enzymu monoaminooxidasy a dopamin-β-hydroxylasy[12].

Deriváty fenylethylaminu jsou zneužívány na celém světě. Jedná se o širokou skupinu látek, do které spadá přinejmenším 200 různých syntetických derivátů[13]. Velké množství možných modifikací samostatného aromatického jádra, substituentů na aromatickém jádře, postranního řetězce či substituce na atomu dusíku vedla a v budoucnosti povede k možnosti získat stovky až tisíce velmi účinných sloučenin[14].



Obr. 4. **Strukturní vzorec fenylethylaminu**

Nejrozšířenějšími látkami z této skupiny jsou extáze (MDMA) a její analoga 3,4-methylendioxyamfetamin (MDA) a 3,4-methylendioxyethylamfetamin (MDEA), dále pak stimulanty amfetamin a metamfetamin. Poslední dvě jmenované látky byly již za 2. světové války součástí balíčků pro přežití u leteckých jednotek. Díky stimulačním účinkům měly zvýšit bojeschopnost vojáků[15].

V Evropě byl zaznamenán především výskyt látek ze skupiny tzv. 2C’s (2C-B, 2C-D, 2C-E, 2C-I, 2C-B-Fly, 2C-T-7) a substituované deriváty amfetaminu (DOB, DOI, DOC). Řada výše uvedených látek je často vydávána za jinou drogu s velice podobnými účinky. V posledních letech je na drogové scéně vysoký nárůst podvodů, kdy jsou tablety vydávány za extázi, avšak tyto prodávané tablety obsahují jiné psychoaktivní látky[16].

Látky z této skupiny vyvolávají u lidí senzorickopercepční (změněné vnímání barev, zvuků, čichu), psychické (značně proměnné a často protichůdné změny psychiky) a somato-motorické příznaky (stavy slabosti, třes, zvracení)[15]. V některých případech bylo užití fenylethylaminů spojeno se závažnými nežádoucími reakcemi, a některé z nich dokonce se smrtí[13].

4. Metody detekce nových syntetických látek

Vznik stále nových psychoaktivních látek na rekreačním drogovém trhu je neustálou výzvou pro analytické toxikology. Přestože většina nových látek může způsobit vážné toxicity, nejsou NPS rutinně detegovatelné běžně zavedenými analytickými metodami. Jako klíčová se pak

ukazuje identifikace hlavních prokázaných metabolitů v poměrně komplikované matrici[17].

Imunochemické techniky zaměřené na klasické drogy (kokain, opiáty, benzodiazepiny, THC) nedetegují většinu nových psychoaktivních látek. V těchto imunochemických testech, založených na interakci protilátky s antigenem (stanovovaná droga), hraje významnou roli protilátka, která musí vykazovat dostatečnou citlivost a specifitu vůči stanovovanému antigenu[18]. Drobná strukturní změna v molekule antigenu (NPS) může negativně ovlivnit jeho reaktivitu s protilátkou proti původnímu antigenu a tím pádem způsobit negativní výsledek testu. Při porovnání struktury NPS a tradičních drog, lze vidět větší či menší strukturní variabilitu, která vysvětluje, proč jsou testy na klasické drogy nevhodné pro stanovení nových drog.

U sofistikovanějších metod, jako je hmotnostní spektrometrie, nelze NPS identifikovat z důvodu chybějících referenčních hmotnostních spekter v používaných knihovnách. Pokud se tedy objeví neznámá látka, nejprve musí být objasněna její struktura díky kombinaci informací z různých spektroskopických, hmotnostně spektrometrických a chemických metod[19]. Laboratoře pracující v oboru klinické a soudní toxikologie tak získají novou referenční látku a poté mohou buď aktualizovat stávající metody, nebo vyvinout nové, které pokryjí i novou psychoaktivní látku[20].

4.1. Screening syntetických kanabinoidů

Zvyšující se popularita NPS a rizika spojená s jejich užíváním vedla k vývoji rychlých metod pro detekci syntetických kanabinoidů v různých matricích (orální tekutiny, moč, sérum/plazma, vlasy, bylinné směsi). Pro rychlé orientační testování na bázi LFIA (Lateral Flow Immunoassay), využitelné v diagnostice intoxikací ve zdravotnictví či při dopravních kontrolách řidičů, existuje na trhu několik komerčních souprav. Tyto testy poskytují pouze kvalitativní informaci o přítomnosti či nepřítomnosti drogy.

Pro laboratorní podmínky je vhodnější imunoanalytická metoda ELISA (Enzym-Linked Imunosorbent Assay), kterou lze v určitých případech použít i ke kvantifikaci drog. V literatuře je popsáno několik ELISA k detekci vybraných syntetických kanabinoidů v moči či orálních tekutinách[21–24]. Jsou dostupné také komerční ELISA soupravy, jež jsou určeny pro analýzu kanabinoidů z plné krve, séra nebo moči (firma Neogen, Randox). Úskalím všech těchto metod je to, že jsou schopny detegovat pouze vybrané strukturní typy syntetických kanabinoidů a nedetegují nedávno objevená analoga (např. QUPIC nebo CHMINACA).

Pro konfirmaci pozitivních nálezů screeningových testů se využívají chromatografické metody. Identifikace a kvantifikace syntetických kanabinoidů v bylinných směsích se nejčastěji provádí plynovou chromatografií s hmotnostní detekcí (GC-MS). Biologické vzorky jako moč, krev, sérum, sliny nebo vlasy bývají analyzovány technikou kapalinové chromatografie s tandemovou hmotnostní detekcí (LC-MS/MS)[20,25].

4.2. Detekce kathinonů a fenylethylaminů

Existuje několik komerčních LFIA a ELISA souprav (od firem Imunalysis, Neogen, Randox, OraSure) pro stanovení amfetaminu, metamfetaminu – drog natolik rozšířených, že je lze v dnešní době chápat spíše za klasické. V nedávné době firma Randox představila dva ELISA kity pro detekci kathinonů (MDPV a mefedron/metkatinon ELISA kit). Výsledky studie zaměřené na testování specifity komerčních ELISA souprav (16 typů, zahrnujících MDPV a mefedron/metkatinon ELISA) však odhalily, že valná většina kitů, přestože obsahuje polyklonální protilátky, je vysoce specifická pouze na určitý strukturní typ[26].

Nejběžnější instrumentální metodou pro detekci kathinonů a fenylethylaminů je plynová chromatografie (GC) spojená s detekčním systémem k potvrzení struktury (např. hmotnostní spektrometr (MS) nebo infračervený detektor (IRD)). Tato kombinace je důležitá, protože během GC se látky ve směsi oddělí podle velikosti, ale teprve MS/IRD pomůže identifikovat látky podle různých funkčních skupin[27]. K identifikaci a kvantifikaci vzorků z vlasů, krve (i posmrtně), séra, plasmy, mozkomíšního moku nebo moči lze použít nejen metody GC ale také LC-MS/MS[19,20,28].

5. Závěr

Nové psychoaktivní látky představují stále populárnější formu rekreačních návykových látek, zejména u dospívajících osob. Tento fenomén má vzrůstající tendenci a lze očekávat nelegální syntézu stovky dalších látek s těžko odhadnutelnou toxicitou. V poslední době se do popředí dostávají především látky ze skupiny syntetických kanabinoidů, syntetických kathinonů a fenylethylaminů. Tyto syntetické látky představují modifikované struktury ilegálních nebo kontrolovaných substancí, nejsou detegovatelné běžnými screeningovými metodami a velmi často jsou spojeny s různými zdravotními následky, které mohou vést až ke smrti. Včasná detekce NPS je tedy velmi potřebná. Kromě tradičních instrumentálních metod jako je LC/MS nebo GC/MS bylo v souvislosti s analýzou syntetických kanabinoidů, kathinonů a fenylethylaminů vyvinuto několik uživatelsky nenáročných imunochemických metod, které jsou schopny detegovat alespoň některé zástupce těchto skupin.

Tato práce byla podpořena projektem bezpečnostního výzkumu Ministerstva vnitra (projekt VI20172020056). Financováno z účelové podpory na specifický vysokoškolský výzkum (MŠMT No 20-SVV/2016).

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The presented review demonstrates that new psychoactive substances represent an increasingly popular form of recreational drugs. In recent years, primarily substances from the group of synthetic cannabinoids, synthetic cathinone and phenethylamines come to the fore. These synthetic substances pose a significant health risk for users and their early detection is desirable. Besides traditional instrumental methods, such as LC/MS or GC/MS, several user-friendly immunochemical methods were developed in connection with the analysis of synthetic cannabinoids, cathinones and phenethylamines, which can detect at least some members of these groups.

Příloha 14:

Kuchař M., Šuláková A., Fojtíková L., Lapčík O., Holubová B. Derivát syntetického kanabinoidu, způsob jeho přípravy a použití. CZ Patentový spis 306547, 2017.

PATENTOVÝ SPIS

(11) Číslo dokumentu:
306 547
(13) Druh dokumentu: **B6**
(51) Int. Cl.:

C07D 209/08	(2006.01)
C07D 209/12	(2006.01)
C07D 209/14	(2006.01)
C07D 295/08	(2006.01)
C07D 413/06	(2006.01)
C07D 471/04	(2006.01)
C07K 16/16	(2006.01)

(19) ČESKÁ REPUBLIKA

ÚŘAD PRŮMYSLOVÉHO VLASTNICTVÍ

(21) Číslo přihlášky: **2015-657**
(22) Přihlášeno: **31.12.2014**
(40) Zveřejněno: **09.03.2016**
(Věstník č. 10/2016)
(47) Uděleno: **18.01.2017**
(24) Oznámení o udělení ve věstníku: **01.03.2017**
(Věstník č. 9/2017)

(56) Relevantní dokumenty:
Yamada, K., et al: J. Med. Chem. (1996) 39, 1967 – 1974; Huffman, J. W., et al: Bioorg. Med. Chem. (2003) 11, 539 – 549.
US 2013/0 066 053 A1; US 6 013 648; US 2013/0 196 354.

(73) Majitel patentu:
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(54) Název vynálezu:
Derivát syntetického kanabinoidu, způsob jeho přípravy a použití

(57) Anotace:
Vynález se týká nově připravených derivátů syntetických kanabinoidů, součástí jejichž struktury je krátký spojovací můstek nesoucí karboxylovou funkční skupinu, a chemických postupů vedoucích k přípravě takovýchto sloučenin. Tyto deriváty syntetických kanabinoidů lze s výhodou využít pro konjugaci s fluorescentní značkou, která by následně umožnila vizualizaci interakcí takto značených ligandů s kanabinoidními receptory, či pro přípravu imunogenů konjugací s nosným proteinem. Polyklonální králičí protilátky získané z imunizovaných laboratorních zvířat lze uplatnit při vývoji rychlých, uživatelsky příjemných imunochemických testů formátu LFIA (Lateral-Flow Immunochromatographic Assay).

CZ 306547 B6

Derivát syntetického kanabinoidu, způsob jeho přípravy a použití

Oblast techniky

Vynález se týká nově připravených derivátů syntetických kanabinoidů, součástí jejichž struktury je krátký spojovací můstek nesoucí karboxylovou funkční skupinu, a chemických postupů vedoucích k přípravě takovýchto sloučenin. Tyto deriváty syntetických kanabinoidů lze s výhodou využít pro konjugaci s fluorescentní značkou, která by následně umožnila vizualizaci interakcí takto značených ligandů s kanabinoidními receptory, či pro přípravu imunogenů konjugací s nosným proteinem. Polyklonální králičí protilátky získané z imunizovaných laboratorních zvířat lze uplatnit při vývoji rychlých, uživatelsky příjemných imunochemických testů formátu LFIA (Lateral-Flow Immunochromatographic Assay).

Dosavadní stav techniky

Na drogové scéně se v posledních letech kromě tradičních drog (kokain, opiáty, amfetaminy, kanabinoidy) objevují také nové syntetické drogy (NSD). Důvodem je snaha výrobců a distributorů obejít stávající legislativní normy, v nichž jsou omamné a psychotropní látky vymezeny obvykle taxativně. Na ilegální trh se tak dostávají analoga známých látek s psychotropním potenciálem, která dosud nejsou uvedena na seznamu ilegálních látek, nebo jejichž prekurzory nejsou monitorovanými substancemi. Hlavní nebezpečí spojené s užíváním těchto nových syntetických drog (NSD) tkví v nedostatku informací o jejich farmakokinetickém a toxikologickém chování, neboť tyto látky neprošly žádnými klinickými testy.

Početně nejbohatší a ze strukturního hlediska značně rozmanitou skupinou NSD jsou syntetické kanabinoidy. Tyto látky se váží na kanabinoidní receptory, což jsou s G-proteinem spřažené transmembránové receptory. Podtyp receptoru CB1 se nachází především v centrální nervové soustavě, podtyp CB2 je exprimován zejména v buňkách imunitního systému. Endokanabinoidní systém dodnes nebyl zcela pochopen. Je však známo, že endogenní kanabinoidy, jejichž nejznámějším zástupcem je anandamid, ovlivňují cítění bolesti, náladu, paměť, ale i chuť k jídlu. Ke kanabinoidním receptorům se váže též celá řada exogenních sloučenin, k nejvýznamnějším patří aktivní složky konopí Δ9-tetrahydrokanabinol (THC) a kanabidiol (CBD), které jsou parciálními agonisty obou zmíněných podtypů receptorů. Syntetické kanabinoidy jsou plní agonisté kanabinoidních receptorů s mnohem dramatičtějšími psychickými účinky. Zaznamenána byla celá řada intoxikací, které měly v některých případech i fatální důsledky. Popsány jsou i případy indukované psychózy. (Banister SD, Wilkinson SM, Longworth M, Stuart J, Apetz N, English K, Brooker L, Goebel C, Hibbs DE, Glass M, Connor M, McGregor IS, Kassiou M. The Synthesis and Pharmacological Evaluation of Adamantane-Derived Indoles: Cannabimimetic Drugs of Abuse. Chem. Neurosci. 2013;4:1081–92.)

Tradiční drogy lze detekovat pomocí komerčních imunochemických testů založených na selektivní reakci protilátky a antigenu, kterým je v tomto případě hledaná omamná či psychotropní látka. K detekci NSD však tyto testy použít nelze. (Páleníček T, Kuchař M. Je možná detekce a identifikace nových syntetických drog (NSD) pomocí orientačních testů? Adiktologie 2011;11:208–14.) Odhalit intoxikaci osob novými syntetickými drogami je možné pomocí metod klinické biochemie, a to zejména analýzou pomocí plynové chromatografie s hmotnostním detektorem (GC-MS), což je poměrně náročné jak na přístroje, tak na odbornost obsluhy. Sestavení přístrojově nenáročných, jednoduchých, uživatelsky příjemných imunochemických testů na principu LFIA (Lateral Flow Immunochromatographic Assay) by umožňovalo daleko rychlejší a levnější orientační detekci látek v biologickém materiálu ve zdravotnictví nebo při dopravních kontrolách řidičů.

Přestože existuje mnoho evaluovaných imunochemických testů pro stanovení tradičních kanabinoidů, možnosti imunoanalýzy syntetických kanabinoidů jsou omezené. V literatuře jsou popsány příklady metod pro ELISA (Enzyme-Linked ImmunoSorbent Assay) stanovení JWH-018 a JWH-250 v krvi, krevním séru a moči (Arntson A, Ofsa B, Lancaster D, Simon JR, McMullin M, Logan B. Validation of a Novel Immunoassay for the Detection of Synthetic Cannabinoids and Metabolites in Urine Specimens. J. Anal. Toxicol. 2013; 37:284-90.), JWH-200 ve slinách (Rodrigues WC, Catbagan P, Rana S, Wang G, Moore C. Detection of Synthetic Cannabinoids in Oral Fluid Using ELISA and LC–MS-MS. J. Anal. Toxicol. 2013; 37:526-33.) a HEIA (Homogenous Enzyme ImmunoAssay) stanovení JWH-018 *N*-pentanové kyseliny, což je významný metabolit JWH-018, v moči (Barnes AJ, Young S, Spinelli E, Martin TM, Klette KL, Huestis MA. Evaluation of a homogenous enzyme immunoassay for the detection of synthetic cannabinoids in urine. Forensic Sci Int. 2014;241:27-34.).

Jako nosičové proteiny se používají hovězí sérový albumin (BSA), hovězí thyroglobumin (BTG), popř. další proteiny vhodných vlastností. Konjugáty se připravují reakcí aktivované formy haptenu (reaktivní anhydridy či estery) s ε-aminoskupinami lysinových zbytků proteinu za vytvoření amidových vazeb. Konjugací se dosáhne statisticky náhodného obsazení lysylů přítomných v proteinu.

Podstata vynálezu

Podstatou vynálezu jsou nové deriváty syntetických kanabinoidů. Tyto deriváty nesou krátký spojovací můstek s karboxylovou skupinou. Spojovací můstek je využitelný pro připojení fluorescentní značky, která následně umožní vizualizaci interakcí kanabinoidů s kanabinoidními receptory, či je karboxylová skupina spojovacího můstku využitelná pro přípravu imunogenů konjugací s nosným proteinem. Z laboratorních zvířat imunizovaných těmito imunogeny lze získat polyklonální králičí protilátky a uplatnit je při vývoji imunoanalytických metod stanovení syntetických kanabinoidů.

Spojovací můstek je připojen přes kyslík v poloze 4 benzenového jádra 1-alkyl-3-benzoylindolů nebo v poloze 4 naftalenu u 1-alkyl-3-naftoylindolů či přímo na dusíku 1-alkyl-*1H*-indol-3-karboxamidů.

Sloučeniny obecného vzorce II a IV se připraví tak, že se benzylem chráněná 4-hydroxyaren-1-karboxylová kyselina nejprve převede na příslušný chlorid. Ten slouží jako acylační činidlo při Friedelově-Craftsově reakci s *N*-substituovaným indolem v přítomnosti Lewisovy kyseliny. Jako Lewisova kyselina je s výhodou použit zirkonium tetrachlorid.

Hydroxylová skupina je odchráněna debenzylací na palladiovém katalyzátoru. *O*-alkylací ethylesterem ω-bromalkanové kyseliny a následnou bazickou hydrolýzou je do molekuly zaveden spojovací můstek nesoucí karboxylovou funkční skupinu.

Připravené deriváty syntetických kanabinoidů lze s výhodou využít pro konjugaci s fluorescentní značkou, která by následně umožnila vizualizaci interakcí takto značených ligandů s kanabinoidními receptory, či pro přípravu imunogenů konjugací s nosným proteinem. Polyklonální králičí protilátky získané z imunizovaných laboratorních zvířat lze uplatnit při vývoji rychlých, uživatelsky příjemných imunochemických testů formátu LFIA (Lateral-Flow Immunochromatographic Assay).

Pro získání protilátek proti těmto syntetickým kanabinoidům, tedy nízkomolekulárním látkám (haptenům), bylo nutné připojit je k nosnému proteinu. Pro tyto účely byl jako vhodný nosný protein vybrán hovězí sérový albumin (BSA). Při tvorbě konjugátů byla využita metoda aktivovaného esteru, při níž je hapten reakcí s *N,N'*-dicyklohexylkarbodiimidem (DCC) a *N*-hydroxysukcinimidem (NHS) nejprve převeden na nestabilní derivát *O*-acylisomočoviny, a poté na aktivovaný

ester. Vlastní konjugační reakce, při níž dochází k tvorbě amidové vazby mezi karboxylovou skupinou haptenu a primární aminoskupinou aminokyseliny lysinu v BSA, pak probíhala v reverzním micelárním prostředí anionaktivního tenzidu.

Způsob přípravy derivátů syntetických kanabinoidů

Sloučeniny obecného vzorce II a IV se připraví tak, že se benzylem chráněná 4-hydroxyaren-1-karboxylová kyselina nejprve převede na příslušný chlorid. Ten slouží jako acylační činidlo při Friedelově-Craftsově reakci s *N*-substituovaným indolem v přítomnosti zirkonium tetrachloridu jako Lewisovy kyseliny. Hydroxylová skupina je odchráněna debenzylací na palladiovém katalyzátoru. *O*-alkylací ethyl-esterem ω-bromalkanové kyseliny a následnou bazickou hydrolýzou je do molekuly zaveden spojovací můstek nesoucí karboxylovou funkční skupinu. Produkty, získané ve vysokém výtěžku, je proto možné konjugovat s ε-aminoskupinami lysinových zbytků proteinu za vytvoření amidových vazeb.

Zatímco 4-benzyloxybenzoová kyselina byla připravena standardním, v literatuře popsaným způsobem, pro přípravu 4-benzyloxynaftalen-1*karboxylové kyseliny je představena inovativní syntetická cesta. V molekule 1-benzyloxy-4-bromnaftalenu je brom nejprve nahrazen nitrilovou funkční skupinou při Rosenmundově – von Braunově reakci. Redukce získaného nitrilu diisobutylaluminium hydridem a zpětná oxidace aldehydu oxidem stříbrným, připravovaným in situ z dusičnanu stříbrného a hydroxidu sodného, umožní zavést do molekuly karboxylovou funkční skupinu s vyšším výtěžkem, než publikovaný postup založený na lithiaci a následné reakci organokovového species s oxidem uhličitým, přestože je reakční sekvence delší.

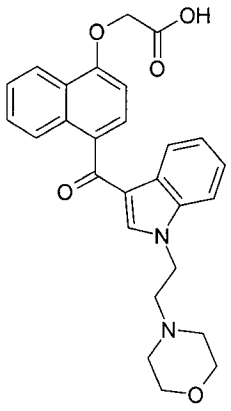
Metodou enzymové imunoanalýzy byly testovány interakce polyklonálních králičích protilátek připravených proti příslušným syntetickým kanabinoidům. S protilátkami vykazujícími nejlepší charakteristiky byly pro imunochemické stanovení optimalizovány a charakterizovány varianty ELISA v nepřímém kompetitivním uspořádání. Byl určen významný analytický parametr tzv. 50% intercept, (I_{50}, viz tabulka). I_{50} představuje koncentraci analytu, potřebnou k vyvázání 50 % protilátek přítomných v reakčním roztoku, která je nezbytná pro kvalitativní stanovení syntetického kanabinoidu v neznámém vzorku.

Při imunoanalýze v nepřímém kompetitivním uspořádání soutěží antigen zakotvený na pevném nosiči (imobilizační konjugát) se stanovovaným antigenem ve vzorku o omezený počet vazebných míst na molekulách protilátky. Čím více antigenu obsahuje analyzovaný vzorek, tím méně protilátky se naváže na zakotvený antigen. Nenavázané složky se odstraní a přidá se enzymem značená sekundární protilátka proti navázané protilátce. Detekce je uskutečněna enzymovou reakcí, kdy vzniká barevný produkt, jehož intenzita zbarvení je měřena spektrofotometricky.

Byly připraveny tyto hapteny:

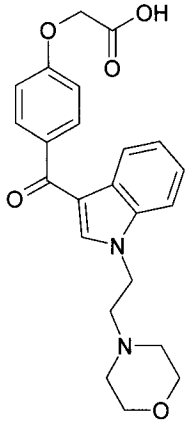
Hapten II:

o názvu 2-{4-[1-(2-(4-morfolino)ethyl)-*1H*-indol-3-ylkarbonyl]naftalen-1-yloxy}octové kyseliny a struktuře II:



a hapten IV:

o názvu 2-{4-[1-(2-(4-morfolino)ethyl)-*1H*-indol-3-ylkarbonyl]benzyloxy}octové kyseliny a struktuře IV:



Objasnění výkresů

Obr.1: Schéma syntézy haptenu II

Obr.2: Schéma syntézy haptenu IV

Obr.3: Schéma provedené konjugace hapten s BSA

Obr.4: MALDI spektrum prokazující strukturu připraveného konjugátu haptenu II s BSA, detekující, že 20 molekul haptenu II se průměrně navázalo na nosný protein – BSA

Obr.5: MALDI spektrum prokazující strukturu připraveného konjugátu haptenu IV s BSA, detekující, že 20 molekul haptenu IV se průměrně navázalo na nosný protein – BSA

Obr.6: Schématický průběh nepřímé kompetitivní ELISA; **A** imobilizace konjugátu na stěny jamek mikrotitrační destičky; **B** aplikace kompetujících složek; **C** aplikace sekundární protilátky značené enzymem (peroxidasou); **D** aplikace substrátu pro peroxidasu; **E** enzymová reakce peroxidasy se substrátem; **F** zastavení enzymové reakce kyselinou sírovou ● imobilizační konjugát ◎ cílový analyt ve vzorku; Y primární protilátka; sekundární protilátka značená enzymem ◎ substrát pro peroxidasu.

Obr.7: Přehled použitých konjugátů a protilátek při vývoji metod ELISA; BSA: hovězí sérový albumin z ang. bovine serum albumin; specifická protilátka: protilátka proti imunogenu příslušného kanabinoidu s BSA

Obr.8: Chemická struktura haptenu II

Obr.9: Chemická struktura haptenu IV

Příklady provedení vynálezu

Příklad 1

Syntéza haptenu II

Výchozí naft-1-ol (1442 mg, 10,00 mmol) byl rozpuštěn v acetonitrilu (40 mL), k roztoku byl přisypán bezvodý uhličitan draselný (2764 mg, 20.00 mmol). Po 30 minutách byl přidán benzylbromid (1784 µL, 15,00 mmol), reakční směs byla zahřáta k varu a míchána 16 hodin. Po zchladnutí reakční směsi na laboratorní teplotu byl přidán ethyl-acetát, organická fáze byla postupně promyta 2 mol/l kyselinou chlorovodíkovou, vodou a nasyceným roztokem chloridu sodného, poté byla vysušena pomocí síranu sodného. Rozpouštědla byla odpařena a produkt přečištěn sloupcovou chromatografií (hexan-dichlormethan 19/1). Byl izolován produkt 1-benzyloxynaftalen (2239 mg, 9,56 mmol) ve výtěžku 96 %.

1-Benzyloxynaftalen (1531 mg, 6,53 mmol) byl rozpuštěn v acetonitrilu (35 mL), roztok byl pomocí ledové lázně ochlazen na 0 °C a v několika podílech byl přidán *N*-bromsukcinimid (1163 mg, 6,53 mmol). Reakční směs byla 3 hodiny míchána při 0 °C. Po ohřátí na laboratorní teplotu byl přidán ethyl-acetát a organická fáze byla třikrát promyta vodou. Vodná fáze byla zpětně extrahována dvěma podíly ethyl-acetátu a spojené organické fáze byly sušeny pomocí síranu hořečnatého. Rozpouštědla byla odpařena a produkt přečištěn sloupcovou chromatografií (hexan-dichlormethan 19/1). Byl izolován produkt 1-benzyloxy-4-bromnaftalen (1885 mg, 6,02 mmol) ve výtěžku 92 %.

1-Benzyloxy-4-bromnaftalen (1404 mg, 4,48 mmol) byl rozpuštěn v *N*,*N*-dimethylformamidu (150 mL) a k roztoku byl přidán kyanid měďný (605 mg, 6,72 mmol). Reakční směs byla po dobu 24 hodin zahřívána k varu na elektrickém topném hnízdě. Poté byl přisypán další podíl kyanidu měďného (121 mg, 1,35 mmol) a reakční směs byla zahřívána k varu dalších 21 hodin. Rozpouštědlo bylo odpařeno a odparek extrahován mezi ethyl-acetát a vodu. Nerozpuštěný podíl byl odfiltrován a suspendován ve vodném roztoku amoniaku. Amoniakální roztok byl protřepán s dvěma podíly ethyl-acetátu, spojené organické fáze byly promyty 1 mol/l kyselinou chlorovodíkovou a vodou a poté vysušeny pomocí síranu hořečnatého. Ethyl-acetát byl odpařen a produkt přečištěn sloupcovou chromatografií (hexan-ethyl-acetát 20/1). Byl izolován produkt 4-benzyloxynaftalen-1-karbonitril (1013 mg, 3,91 mmol) ve výtěžku 87 %.

4-Benzyloxynaftalen-1-karbonitril (1000 mg, 3,86 mmol) byl rozpuštěn v suchém tetrahydrofuranu (60 mL), roztok byl pomocí ledové lázně ochlazen na 0 °C a stříkačkou byl přikapán 1 mol/l roztoku diisobutylaluminium hydridu v hexanu (7,7 mL, 7,70 mmol). Reakční směs byla ponechána míchat přes noc za laboratorní teploty. Přebytečný diisobutylaluminium hydrid byl rozložen vodou. Vodná fáze byla okyselena 2 mol/l kyselinou chlorovodíkovou až na pH 1 a promyta dvěma podíly dichlormethanu. Organické fáze byly spojeny a vysušeny pomocí síranu hořečnatého. Rozpouštědla byla odpařena a produkt přečištěn sloupcovou chromatografií (hexan-ethyl-acetát 9/1). Byl izolován produkt 4-benzyloxynaftalen-1-karbaldehyd (762 mg, 2,91 mmol) ve výtěžku 75 %.

4-Benzyloxynaftalen-1-karbaldehyd (1544 mg, 5,89 mmol) byl rozpuštěn v ethanolu (80 mL) při 60 °C, k roztoku byl přidán dusičnan stříbrný (4002 mg, 23,56 mmol) a stříkačkou byl postupně přikapáván roztok hydroxidu sodného (942 mg, 23,56 mmol) ve směsi ethanolu s vodou. Po přidání veškerého hydroxidu byla reakční směs míchána a zahřívána po dobu 24 hodin. Přebytečný oxid stříbrný i vyloučené stříbro byly odfiltrovány a promyty větším množstvím vody. Filtrát byl okyselen 2 mol/l kyseliny chlorovodíkové až na pH 1 a promyt dvěma podíly diethyletheru. Organické fáze byly spojeny a vysušeny pomocí síranu hořečnatého. Rozpouštědla byla odpařena a získaný surový produkt byl triturován chloroformem. Žádaný produkt 4-benzyloxynaftalen-1-karboxylová kyselina (1409 mg, 5,07 mmol) byl izolován ve výtěžku 86 %.

4-Benzyloxynaftalen-1-karboxylová kyselina (1200 mg, 4,30 mmol) byla rozpuštěna v suchém dichlormethanu (20 mL), k roztoku bylo přidáno několik kapek *N,N*-dimethylformamidu a stříkačkou byl přikapán oxalylchlorid (482 µL, 5,60 mmol). Reakce probíhala 3 hodiny při laboratorní teplotě. Byl přidán toluen (10 mL), přebytečný oxalylchlorid a rozpouštědla byly odpařeny. Surový 4-benzyloxynaftalen-1-karbonylchlorid (382 mg, 1,29 mmol) byl rozpuštěn v suchém dichlormethanu (25 mL) a stříkačkou byl přikapán roztok 1-[2-(morfolin-4-yl)ethyl]-*1H*-indolu (385 mg, 1,67 mmol) v suchém dichlormethanu (5 mL) při teplotě -10 °C. K reakční směsi byl přidán zirkonium tetrachlorid (450 mg, 1,93 mmol). Teplota byla 1 hodinu udržována na -10 °C, poté 3 hodiny na 0 °C. Reakce dále probíhala přes noc za laboratorní teploty, ukončena byla přidáním vody. Vodná fáze byla promyta dvěma podíly ethyl-acetátu, spojené organické fáze byly zpětně extrahovány vodou a vysušeny pomocí síranu hořečnatého. Rozpouštědla byla odpařena a produkt přečištěn sloupcovou chromatografií (hexanaceton 3/1). Byl izolován produkt 1-[2-(4-morfolino)ethyl]-3-(4-benzyloxy-1-naftoyl)-*1H*-indol (444 mg, 0,91 mmol) ve výtěžku 70 %.

1-[2-(4-morfolino)ethyl]-3-(4-benzyloxy-1-naftoyl)-*1H*-indol (144 mg, 0,29 mmol) byl rozpuštěn v bezvodém ethanolu (35 mL), k roztoku byl přidán formiát amonný (93 mg, 1,47 mmol) a katalytické množství palladia na uhlíku. Reakce byla míchána 1 hodinu za laboratorní teploty. Palladiový katalyzátor byl odfiltrován, filtrát byl zahuštěn částečným odpařením ethanolu a následně naředěn ethyl-acetátem. Organická fáze byla promyta roztokem uhličitanu draselného, a dvěma podíly roztoku chloridu sodného, poté byla vysušena pomocí síranu hořečnatého. Rozpouštědla byla odpařena a produkt byl přečištěn sloupcovou chromatografií (hexan/aceton 9/4). Byl izolován produkt 1-[2-(4-morfolino)ethyl]-3-(4-hydroxy-1-naftoyl)-*1H*-indol (116 mg, 0,29 mmol) ve výtěžku 99 %.

1-[2-(4-morfolino)ethyl]-3-(4-hydroxy-1-naftoyl)-*1H*-indol (110 mg, 0,28 mmol) byl rozpuštěn v suchém acetonu (30 mL) při 50 °C, k roztoku byl přisypán bezvodý uhličitan draselný (57 mg, 0,41 mmol). Po 30 minutách byl přidán ethyl-2-bromacetát (36 µL, 0,32 mmol) a reakční směs byla zahřívána k varu. Reakce probíhala 3 hodiny. Po zchladnutí reakční směsi na laboratorní teplotu byl přidán ethyl-acetát, organická fáze byla postupně promyta dvěma podíly vody a nasyceným roztokem chloridu sodného, poté byla vysušena pomocí síranu hořečnatého. Rozpouštědla byla odpařena a produkt přečištěn sloupcovou chromatografií (hexan/aceton 3/1). Byl izolován produkt ethyl-2-{4-[1-(2-(4-morfolino)ethyl)-*1H*-indol-3-ylkarbonyl]naftalen-1-yloxy}acetát (111 mg, 0,23 mmol) ve výtěžku 83 %.

Ethyl-2-{4-[1-(2-(4-morfolino)ethyl)-*1H*-indol-3-ylkarbonyl]naftalen-1-yloxy}acetát (105 mg, 0,22 mmol) byl suspendován v ethanolu (15 mL), k roztoku byl stříkačkou přikapán 1M roztok hydroxidu sodného ve vodě (3 mL). Reakce probíhala 1 hodinu při teplotě 50 °C. Ethanol byl odpařen, vodná fáze byla okyselena na pH 1, poté byla odpařena i voda. Vyloučený chlorid sodný byl odstraněn triturací odparku vodou. Produkt byl přečištěn chromatografií na reverzní fázi (voda/methanol, gradient 10/1→1/1). Byl izolován produkt ve formě hydrochloridu 2-{4-[1-(2-(4-morfolino)ethyl)-*1H*-indol-3-ylkarbonyl]naftalen-1-yloxy}octové kyseliny (73 mg, 0,15 mmol) s výtěžkem 68 %.

1H NMR (300 MHz, $CDCl_3$) δ ppm: 2,52 (t, J=4,50 Hz, 4 H, CH_2NCH_2), 2,78 (t, J=6,60 Hz, 2 H, NCH_2), 3,62 (t, J=4,50 Hz, 4 H, CH_2OCH_2), 3,83 (br s, 1 H, NH), 4,24 (t, J=6,90 Hz, 2 H, NCH_2), 4,66 (s, 2 H, OCH_2), 6,60 (d, J=7,80 Hz, 1 H, ArH), 7,30 - 7,46 (m, 7 H, ArH), 8,19 - 8,22 (m, 1 H, ArH), 8,32 - 8,35 (m, 1 H, ArH), 8,41 - 8,44 (m, 1 H, ArH). 13C NMR (75 MHz, CDCl3) δ ppm: 42,75, 52,86, 56,44, 65,42, 66,86, 103,33, 109,84, 118,07, 122,57, 122,96, 123,10, 124,04, 125,62, 125,79, 125,92, 127,12, 127,48, 127,67, 131,70, 132,15, 136,87, 138,34, 155,58, 172,61, 191,69. IČ ($CHCl_3$): 3107, 3075, 3053, 2957, 2927, 2859, 2816, 1920, 1727,1622, 1605, 1578, 1520, 1509, 1485, 1463, 1425, 1394, 1372, 1322, 1258, 1230, 1196, 1159, 1134, 1098, 1071, 1032, 1014, 986, 910, 870, 820, 794, 774, 749, 715, 669, 626, 561, 425. HRMS-ESI: monoisotopická hmota: 458,18417 Da, nalezeno (m/z) 459,19109 (vypočteno 459,19145) odpovídá iontu [M+H]+ připravené látky.

Příklad 2

Syntéza haptenu IV

Výchozí 4-hydroxybenzoová kyselina (8287 mg, 60,00 mmol) byla rozpuštěna v acetonitrilu (140 mL) při 60 °C, k roztoku byl přisypán bezvodý uhličitan draselný (19073 mg, 138,00 mmol). Po 30 minutách byl přidán benzylbromid (14 986 µL, 126,00 mmol), reakční směs byla zahřáta k varu a míchána 16 hodin. Po zchladnutí reakční směsi na laboratorní teplotu byl přidán ethyl-acetát a voda, organická fáze byla oddělena a vodná promyta dalšíma dvěma podíly ethyl-acetátu. Organické extrakty byly spojeny a vysušeny pomocí síranu hořečnatého. Rozpouštědla byla odpařena a produkt přečištěn sloupcovou chromatografií (hexan/dichlormethan, gradient 4/1→1/1). Byl izolován produkt benzyl-4-benzyloxybenzoát (18714 mg, 58,83 mmol) ve výtěžku 98 %.

Benzyl-4-benzyloxybenzoát (18593 mg, 58,44 mmol) byl suspendován ve směsi ethanolu (300 mL) s vodou (100 mL). Byl přidán hydroxid sodný (12 000 mg, 300,02 mmol) a reakční směs byla zahřáta k varu. Reakce probíhala 1 hodinu. Ethanol byl odpařen a vodná fáze byla po naředění promyta dvěma podíly diethyletheru. Poté byla okyselena na pH 1 a protřepána postupně s třemi podíly diethyletheru a ethyl-acetátem. Organické fáze byly spojeny a vysušeny pomocí síranu hořečnatého. Rozpouštědla byla odpařena a produkt přečištěn sloupcovou chromatografií (dichlormethan/methanol 97/3). Byl izolován produkt 4-benzyloxybenzoová kyselina (12 658 mg, 55,50 mmol) ve výtěžku 95 %.

4-Benzyloxybenzoová kyselina (1460 mg, 6,40 mmol) byla rozpuštěna v suchém dichlormethanu (35 mL), k roztoku bylo přidáno několik kapek *N,N*-dimethylformamidu a stříkačkou byl přikapán oxalylchlorid (715 µL, 8,32 mmol). Reakce probíhala 3 hodiny při laboratorní teplotě. Byl přidán toluen (10 mL), přebytečný oxalylchlorid a rozpouštědla byly odpařeny, vznikl 4-benzyloxybenzoylchlorid.

Surový 4-benzyloxybenzoylchlorid (1081 mg, 4,38 mmol) byl rozpuštěn v suchém dichlormethanu (55 mL) a stříkačkou byl přikapán roztok 1-[2-(morfolin-4-yl)ethyl]-*1H*-indolu (1313 mg, 5,70 mmol) připravený v suchém dichlormethanu (5 mL) při teplotě -30 °C. K reakční směsi byl přidán zirkonium tetrachlorid (1533 mg, 6,58 mmol). Teplota byla 90 minut udržována na -30 °C, 90 minut na -20 °C, poté 90 minut na -10 °C a nakonec 3 hodiny na teplotě 0 °C. Reakce dále probíhala přes noc za laboratorní teploty, ukončena byla přidáním vody. Vodná fáze byla promyta dvěma podíly ethyl-acetátu, spojené organické fáze byly zpětně extrahovány vodou a vysušeny pomocí síranu hořečnatého. Rozpouštědla byla odpařena a produkt přečištěn sloupcovou chromatografií (hexan/aceton, gradient 16/3→3/1). Byl izolován produkt 1-[2-(4-morfolino)-ethyl]-3-(4-benzyloxybenzoyl)-*1H*-indol (827 mg, 1,88 mmol) ve výtěžku 43 %.

1-[2-(4-morfolino)ethyl]-3-(4-benzyloxybenzoyl)-*1H*-indol (839 mg, 1,90 mmol) byl rozpuštěn v bezvodém ethanolu (65 mL), k roztoku byl přidán formiát amonný (600 mg, 9,51 mmol) a kata-

lytické množství palladia na uhlíku. Reakce byla míchána 3 hodiny za laboratorní teploty. Palladiový katalyzátor byl odfiltrován, filtrát byl zahuštěn částečným odpařením ethanolu a následně naředěn ethyl-acetátem. Organická fáze byla postupně promyta roztokem uhličitanu draselného, roztokem chloridu sodného a vodou, poté byla vysušena pomocí síranu hořečnatého. Rozpouštědla byla odpařena a produkt byl přečištěn sloupcovou chromatografií (hexan-aceton, gradient 3/1→2/1). Byl izolován produkt 1-[2-(4-morfolino)ethyl]-3-(4-hydroxybenzoyl)-*1H*-indol (551 mg, 1,57 mmol) ve výtěžku 83 %.

1-[2-(4-morfolino)ethyl]-3-(4-hydroxybenzoyl)-*1H*-indol (480 mg, 1,37 mmol) byl rozpuštěn v suchém acetonu (35 mL) při 50 °C, k roztoku byl přisypán bezvodý uhličitan draselný (285 mg, 2,05 mmol). Po 30 minutách byl přidán ethyl-2-bromacetát (183 µL, 1,64 mmol) a reakční směs byla zahřívána k varu. Reakce probíhala 3 hodiny. Po zchladnutí reakční směsi na laboratorní teplotu byl přidán ethyl-acetát, organická fáze byla postupně promyta dvěma podíly vody a nasyceným roztokem chloridu sodného, poté byla vysušena pomocí síranu hořečnatého. Rozpouštědla byla odpařena a produkt přečištěn sloupcovou chromatografií (hexan-aceton 5/2). Byl izolován produkt ethyl-2-{4-[1-(2-(4-morfolino)ethyl)-*1H*-indol-3-ylkarbonyl]benzyloxy}acetát (549 mg, 1,26 mmol) ve výtěžku 92 %.

Ethyl-2-{4-[1-(2-(4-morfolino)ethyl)-*1H*-indol-3-ylkarbonyl]benzyloxy}acetát (63 mg, 0,14 mmol) byl rozpuštěn v ethanolu (15 mL), k roztoku byl stříkačkou přikapán 1M roztok hydroxidu sodného (8 mg, 0,20 mmol) ve vodě. Reakce probíhala 3 hodiny při teplotě 60 °C. Ethanol byl odpařen, vodná fáze byla okyselena na pH 1, poté byla odpařena i voda. Surový produkt byl zbaven vyloučeného chloridu sodného a přečištěn chromatografií na reverzní fázi (voda/methanol, gradient 10/1→1/1). Byl izolován produkt ve formě hydrochloridu 2-{4-[1-(2-(4-morfolino)ethyl)-*1H*-indol-3-ylkarbonyl]benzyloxy}octové kyseliny (36 mg, 0,08 mmol) s výtěžkem 56 %.

1H NMR (300 MHz, CD_3OD) δ ppm: 2,94 (t, J=4,50 Hz, 4 H, CH_2NCH_2), 3,23 (t, J=6,15 Hz, 2 H, NCH_2), 4,07 (t, J=4,50 Hz, 4 H, CH_2OCH_2), 4,82 (t, J=6,50 Hz, 2 H, NCH_2), 5,03 (s, 2 H, OCH_2), 7,49 (d, J=7,50 Hz, 2 H, ArH), 7,69 - 7,75 (m, 2 H, ArH), 7,97 (d, J=7,50 Hz, 1 H, ArH), 8,24 (d, J=7,50 Hz, 2 H, ArH), 8,35 (s, 1 H, ArH), 8,69 (d, J=7,80 Hz, 1 H, ArH). IČ ($CHCl_3$): 3606, 3403, 3326, 3218, 3147, 3058, 3043, 2999, 2965, 2943, 2897, 2883, 2868, 2824, 2796, 1672, 115, 1598, 1573, 1523, 1470, 1452, 1425, 1380, 1304, 1261, 1242, 1209, 1176, 1131, 1114, 1098, 1048, 1028, 991, 904, 879, 871, 843, 818, 762, 751, 716, 702, 659, 637, 621, 568, 493, 477, 449. HRMS-ESI: monoisotopická hmota: 408,16852 Da, nalezeno (m/z) 409,17600 (vypočteno 409,17580) odpovídá iontu [M+H]+ připravené látky.

Příklad 3

Konjugace haptenu II s BSA

Hydrochlorid 2-{4-[1-(2-(4-morfolino)ethyl)-*1H*-indol-3-ylkarbonyl]naftalen-1-yloxy}octové kyseliny (15 mg, 30.3 µmol) byl rozpuštěn v *N,N*-dimethylformamidu (150 µl), k roztoku byl přidán roztok *N,N'*-dicyklohexylkarbodiimidu (8,1 mg, 39,4 µmol) v *N,N*-dimethylformamidu (85 µl) a roztok N-hydroxysukcinimidu (4,5 mg, 39,4 µmol) v *N,N*-dimethylformamidu (45 µl). Reakční směs byla ponechána stát přes noc v mikrozkumavce při laboratorní teplotě. Po vyloučení krystalků derivátu močoviny, bylo pomocí TLC analýzy ověřeno, že veškerá výchozí kyselina byla převedena na aktivovaný ester.

K roztoku hovězího sérového albuminu (24,2 mg, 0,4 µmol) v bikarbonátovém pufru (1,9 ml) byl přidán roztok dioktylsulfosukcinátu sodného v oktanu (12,4 ml). Do micelárního prostředí byl pipetou opatrně přenesen aktivovaný ester v *N,N*-dimethylformamidu tak, aby došlo k oddělení krystalků derivátu močoviny. Reakční směs byla ponechána míchat přes noc při laboratorní teplotě. Poté byl modifikovaný protein vysrážen chladným acetonem (-30 °C) z reakční směsi. Pro-

dukt byl oddělen centrifugací a promyt dalším podílem chladného acetonu(-30 °C). Po centrifugaci a odlití promývacího acetonu byl modifikovaný protein ponechán 30 minut na vzduchu, aby došlo k odpaření zbylého acetonu. Poté byl produkt rozpuštěn ve vodě a lyofilizován. Analýza pomocí MS-MALDI ukázala, že k jedné molekule hovězího sérového albuminu je navázáno průměrně 20 molekul haptenu II.

Příklad 4

Konjugace haptenu IV s BSA

Hydrochlorid 2-{4-[1-(2-(4-morfolino)ethyl)-*1H*-indol-3-ylkarbonyl]benzyloxy}octové kyseliny (12 mg, 27,0 μmol) byl rozpuštěn v *N,N*-dimethylformamidu (120 μl), k roztoku byl přidán roztok *N,N'*-dicyklohexylkarbodiimidu (7,2 mg, 35,1 μmol) v *N,N*-dimethylformamidu (75 μl) a roztok *N*-hydroxysukcinimidu (4,0 mg, 351 μmol) v *N,N*-dimethylformamidu (40 μl). Reakční směs byla ponechána stát přes noc v mikrozkumavce při laboratorní teplotě. Po vyloučení krystalků derivátu močoviny, bylo pomocí TLC analýzy ověřeno, že veškerá výchozí kyselina byla převedena na aktivovaný ester.

K roztoku hovězího sérového albuminu (21,6 mg, 0,4 μmol) v bikarbonátovém pufru (1,7 ml) byl přidán roztok dioktylsulfosukcinátu sodného v oktanu (11,1 ml). Do micelárního prostředí byl pipetou opatrně přenesen aktivovaný ester v *N,N*-dimethylformamidu tak, aby došlo k oddělení krystalků derivátu močoviny. Reakční směs byla ponechána míchat přes noc při laboratorní teplotě. Poté byl modifikovaný protein vysrážen chladným acetonem (-30 °C) z reakční směsi. Produkt byl oddělen centrifugací a promyt dalším podílem chladného acetonu (-30 °C). Po centrifugaci a odlití promývacího acetonu byl modifikovaný protein ponechán 30 minut na vzduchu, aby došlo k odpaření zbylého acetonu. Poté byl produkt rozpuštěn ve vodě a lyofilizován. Analýza pomocí MS-MALDI ukázala, že k jedné molekule hovězího sérového albuminu je navázáno průměrně 20 molekul haptenu IV.

Příklad 5

Provedení nepřímé nekompetitivní ELISY

Imobilizace konjugátu na stěny jamek mikrotitrační destičky

Zásobní roztok imobilizačního konjugátu (I-BSA – VI-BSA) byl vhodně naředěn v 0,05 mol/l karbonát-bikarbonátovém pufru, pH 9,6 a pipetován do jamek mikrotitrační destičky v množství 100 μl na jamku. Imobilizace probíhala přes noc při laboratorní teplotě. Nenavázaný imobilizační konjugát byl následující den odstraněn pomocí automatické promývačky 4x 300 μl 0,01 mol/l PBS-Tw (0.01M PBS, pH 7,4 obohacený 0,05 % Tweenem).

Aplikace kompetujících složek

Po promytí nenavázaného konjugátu byly do jamek mikrotitrační destičky pipetovány kompetující složky v pořadí 50 μl roztoku antigenu (kalibračního standardu nebo vzorku) ředěného v PBS a 50 μl roztoku polyklonální králičí protilátky ředěné v příslušném pufru (různé pufry pro různé systémy). Inkubace probíhala za mírného třepání při laboratorní teplotě, po dobu 1 hodiny. Nezreagované imunoreaktanty byly odstraněny opakovaným promytím jamek 0,01 mol/l PBS-Tw (4x 300 μl, promývačka).

Aplikace sekundární protilátky

Ke kvantifikaci navázaných králičích protilátek na imobilizační konjugát bylo využito tzv. sekundární protilátky GAR-Po (z angl. Goat Anti-Rabbit - kozí protilátky proti králičím protilátkám značené peroxidasou). Sekundární protilátka byla ředěna 1:10 000 v příslušném pufru (v závislosti na systému) a pipetována v množství 100 µl na jamku. Inkubace probíhala za mírného třepání při laboratorní teplotě, po dobu 1 hodiny. Nenavázaná protilátka byla poté odstraněna opakovaným promytím jamek 0,01M PBS-Tw (4x 300 µl, promývačka).

Aplikace substrátu pro peroxidasu

Do každé jamky bylo následně přidáno 100 µl čerstvě připraveného roztoku substrátu pro peroxidasu Enzymová reakce probíhala za mírného třepání při laboratorní teplotě deset minut.

K zastavení enzymové reakce byl použit přídavek 2 mol/l kyseliny sírové v množství 50 µl na jamku. Absorbance reakční směsi byla měřena v jamkách mikrotitrační destičky při vlnové délce 450 nm.

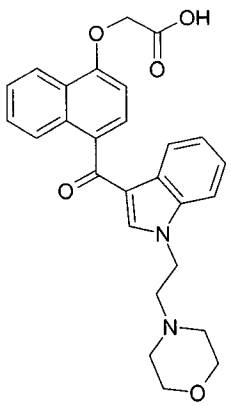
Průmyslová využitelnost

Aplikace v imunoanalytických metodách stanovení syntetických kanabinoidů ve formátech nepřímé ELISA, LFIA, FIA a dalších, které využívají kompetice mezi haptenem – některým z připravených derivátů syntetických kanabinoidů, a analytem – psychoaktivní látkou přítomnou ve vzorku, o vazebná místa protilátky.

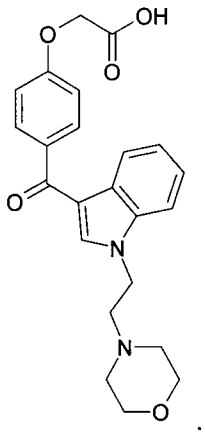
PATENTOVÉ NÁROKY

1. Derivát syntetického kanabinoidu o struktuře 2-{4-[1-(2-(4-morfolino)ethyl)-*1H*-indol-3-ylkarbonyl]R_1oxy}octové kyseliny, přičemž R_1 je naftalen-1-yl nebo benzyl.

2. Derivát syntetického kanabinoidu podle nároku 1 o názvu 2-{4-[1-(2-(4-morfolino)ethyl)-*1H*-indol-3-ylkarbonyl]naftalen-1-yloxy}octové kyseliny o struktuře II:



3. Derivát syntetického kanabinoidu podle nároku 1 o názvu 2-{4-[1-(2-(4-morfolino)ethyl)-*1H*-indol-3-ylkarbonyl]benzyloxy}octové kyseliny o struktuře IV:

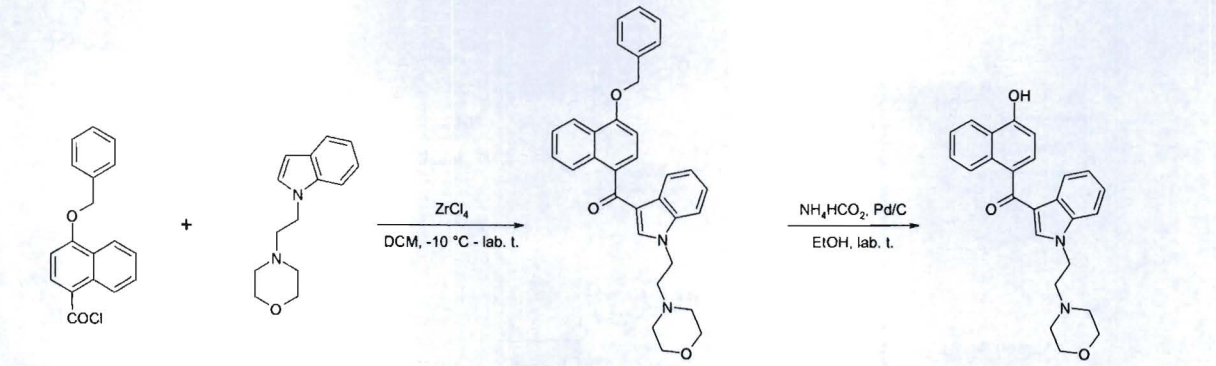


4. Způsob přípravy derivátu syntetického kanabinoidu podle nároků 1 až 3, **v y z n a č u j í c í s e t í m**, že výchozí 4-hydroxyaren-1-karboxylová kyselina se podrobí reakci s benzylbromidem, ke vzniklé 4-benzyloxyarenkarboxylové kyselině se přikape oxalylchlorid, ke vzniklému chloridu 4-benzyloxyarenkarboxylové kyseliny se přikape *N*-substituovaný indol, k reakční směsi Friedel-Craftsově reakci je přidán zirkonium tetrachlorid, vzniklý produkt je debenzylován na paladiovém katalyzátoru, debenzylovaný produkt se izoluje, provede se *O*-alkylace, kdy se přidá ethyl-ester ω- bromalkanové kyseliny a vzniklý ester nesený na spojovacím můstku se v zásaditém prostředí hydrolyzuje na karboxylovou skupinu.

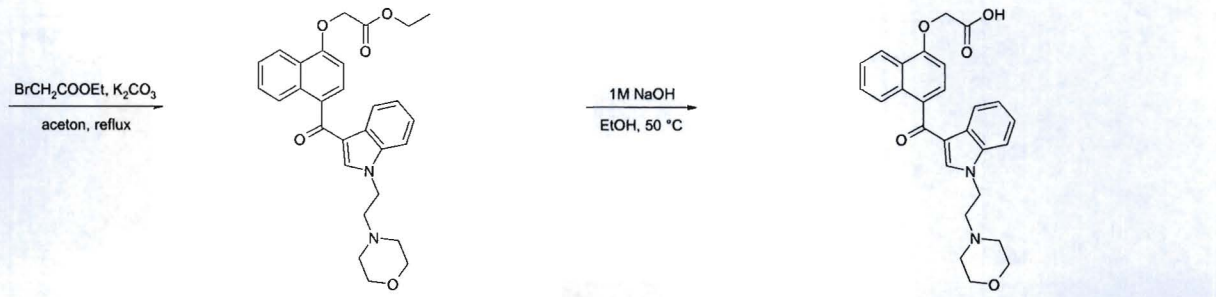
5. Použití derivátu syntetického kanabinoidu podle nároků 1 až 3 jako prostředku pro vizualizaci interakcí s kanabinoidními receptory.

6. Použití derivátu syntetického kanabinoidu podle nároků 1 až 3 jako prostředku pro přípravu imunogenů konjugací s nosným proteinem, pro získání protilátky pro analýzu drog interagujících s kanabinoidními receptory.

4 výkresy

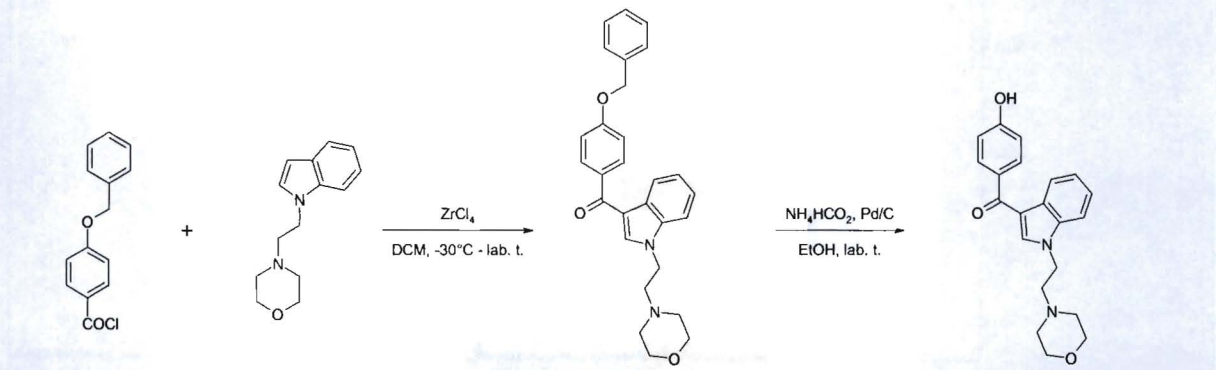


4-benzyloxynaftoylchlorid 1-[2-(morfolin-4-yl)ethyl]-1*H*-indol 1-[2-(4-morfolino)ethyl]-3-(4-benzyloxy-1-naftoyl)-1*H*-indol 1-[2-(4-morfolino)ethyl]-3-(4-hydroxy-1-naftoyl)-1*H*-indol

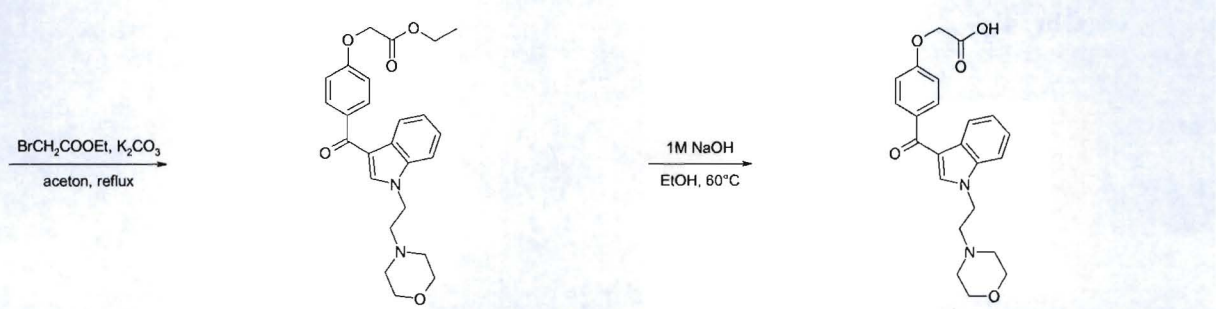


ethyl-2-{4-[1-(2-(4-morfolino)ethyl)-1*H*-indol-3-ylkarbonyl]naftalen-1-yloxy}acetát 2-{4-[1-(2-(4-morfolino)ethyl)-1*H*-indol-3-ylkarbonyl]naftalen-1-yloxy}octová kyselina

Obr. 1

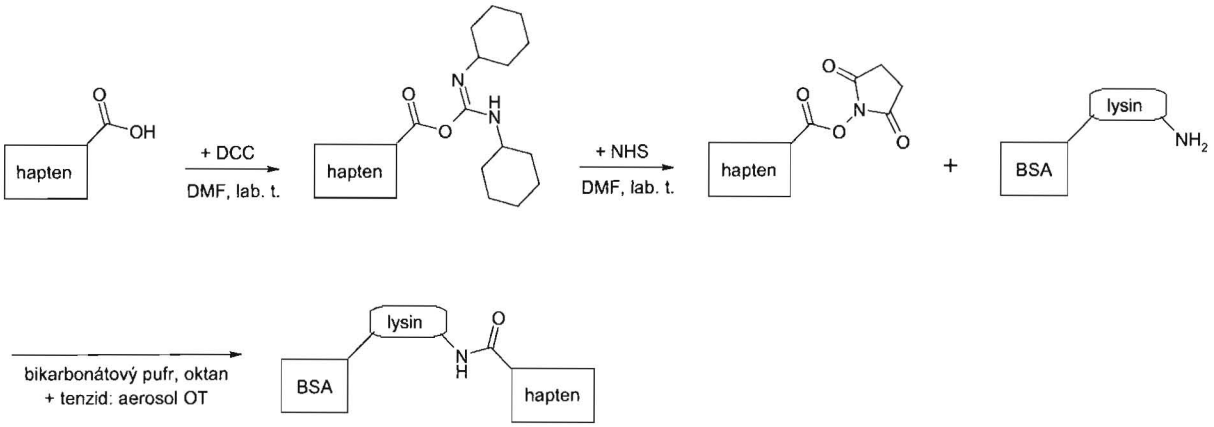


4-benzyloxybenzoylchlorid 1-[2-(morfolin-4-yl)ethyl]-1*H*-indol 1-[2-(4-morfolino)ethyl]-3-(4-benzyloxybenzoyl)-1*H*-indol 1-[2-(4-morfolino)ethyl]-3-(4-hydroxybenzoyl)-1*H*-indol

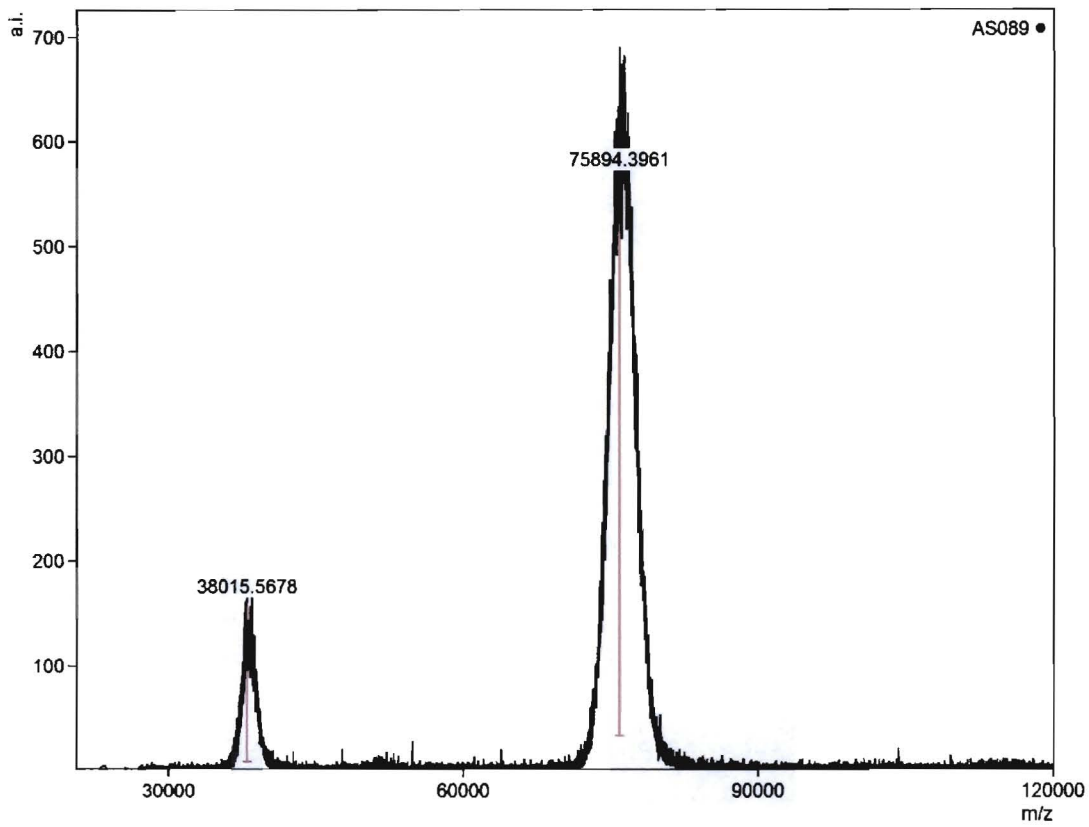


ethyl-2-{4-[1-(2-(4-morfolino)ethyl)-1*H*-indol-3-ylkarbonyl]benzyloxy}acetát 2-{4-[1-(2-(4-morfolino)ethyl)-1*H*-indol-3-ylkarbonyl]benzyloxy}octová kyselina

Obr. 2


hapten
OH
+ DCC
DMF, lab. t.
hapten
+ NHS
DMF, lab. t.
hapten
+
BSA
lysin
NH2
bikarbonátový pufr, oktan
+ tenzid: aerosol OT
BSA
lysin
hapten

Obr. 3


a.i.
AS089
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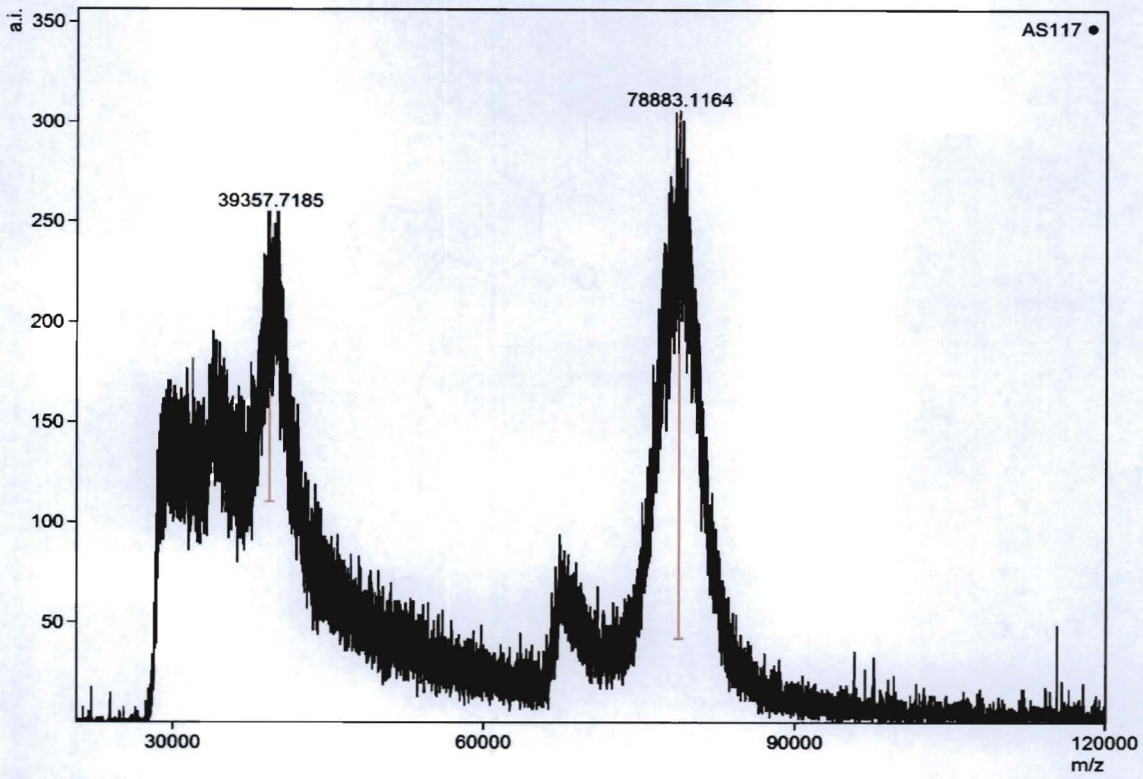
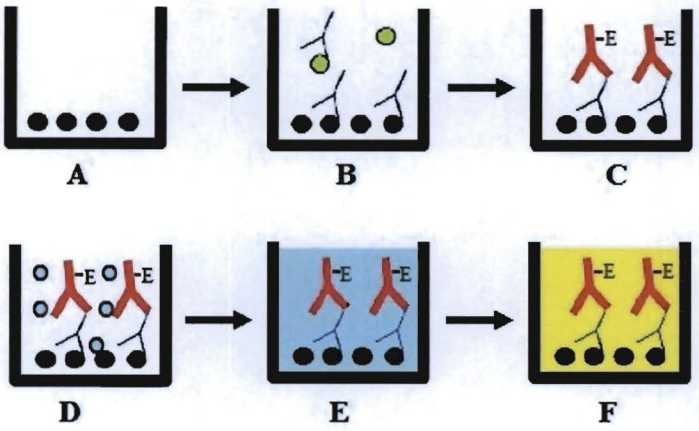
m/z

Obr. 4



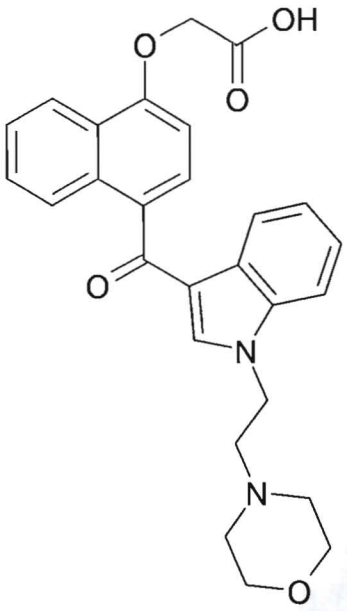
Obr. 5



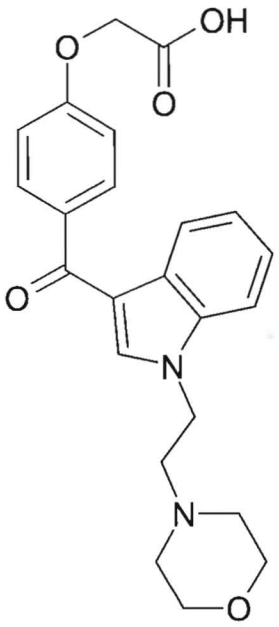
Obr. 6

konjugát kanabinoidu s BSA (označení)	koncentrace imunoreagencií (μg.ml^{-1})		I_{50} (ng.ml^{-1})
	imobilizační konjugát	specifická protilátka	
I-BSA	0,3125	20	12,5
II-BSA	0,0125	1,25	0,4
IV-BSA	0,025	2,5	1,3

Obr. 7



Obr. 8



Obr. 9

Konec dokumentu

Příloha 15:

Fojtíková, L., Šuláková, A., Blažková, M., Holubová, B., Kuchař, M., Mikšátková, P., Lapčík, O., Fukal, L. Lateral Flow Immunoassay and Enzyme Linked Immunosorbent Assay as Effective Immunomethods for the Detection of Synthetic Cannabinoid JWH-200 Based on the Newly Synthesized Hapten. *Toxicol. Reports 5*: 65–75, 2018.

Toxicology Reports 5 (2018) 65–75

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Lateral flow immunoassay and enzyme linked immunosorbent assay as effective immunomethods for the detection of synthetic cannabinoid JWH-200 based on the newly synthesized hapten

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ARTICLE INFO

Keywords:
Hapten synthesis
Synthetic cannabinoid
JWH-200
Immunomethods
ELISA
LFIA

ABSTRACT

In recent years, the use of synthetic cannabinoids (SCs) as drugs of abuse has greatly increased. SCs are associated with a risk of severe poisoning or even death. Therefore, more rapid, cost effective and reliable methods are needed, especially for the screening of drivers after traffic accidents and for detailed toxicological analysis in forensic laboratories. In this study, we developed a lateral flow immunoassay (LFIA) and an enzyme linked immunosorbent assay (ELISA) for the detection of JWH-200 in oral fluids. For this purpose a new hapten was prepared using a ten-step synthetic route. The developed immuno methods are based on antibodies obtained from rabbit immunized with synthesized hapten conjugated to carrier protein. The proposed methods are highly sensitive ($LOD_{LFIA} = 0.08 \pm 0.04\,ng\,mL^{-1}$; $LOD_{ELISA} = 0.04 \pm 0.02\,ng\,mL^{-1}$). They were applied to the quantification of JHW-200 in spiked oral fluids. The recoveries ranged from 82 to 134% for both methods. The results correlated excellently with results obtained using UHPLC–MS/MS ($R^2_{LFIA} = 0.99$; $R^2_{ELISA} = 0.99$). Our developed methods could be an important tool for analyses of JWH-200 in human oral fluids. The one-step LFIA is particularly suitable for roadside and on-site monitoring due to the rapid qualitative results it delivers, while the ELISA is especially useful for laboratory quantitative analyses of positive samples captured by LFIA.

1. Introduction

The frequent appearance of new psychoactive substances (NPS) as drugs of abuse is a matter of concern with the public. Synthetic cannabinoids (SCs) are the largest group of NPS monitored in Europe by the Early Warning System. These substances – commonly called 'Spice' are sold as 'legal' alternative to cannabis and may be marketed as 'herbal incense blends' or 'herbal mixtures' and usually labelled 'not for human consumption' in order to circumvent consumer protection and the law [1]. Their easy accessibility (especially via online shops), and impossible detectability using routine screening tests for cannabis contribute to an expansion in their abuse.

There have been numerous reports that abuse of SCs can cause a wide range of serious harms to human health (acute ischemic stroke, kidney damage, pulmonary and cardiovascular effects, and psychiatric symptoms) [1–6]. Therefore, development of simple methods that could be used for rapid determination of SCs is needed.

JWH-200, systematically named 1-[2-(morpholin-4-yl)ethyl]-3-(naphthalene-1-carbonyl)-1H-indole, is considered to be one of the most widely known SCs. A seizure of Spice adulterated with JWH-200 was first reported in 2009 by Europol but the drug soon spread out throughout Europe, North America and Japan [5,7]. JWH-200 became one of few SCs added to the list of controlled substances. That resulted in the great interest of state authorities to develop an effective analysis of this substance intended for use in the field.

Current methods used for the analysis of SCs in human fluids are

Abbreviations: BSA, bovine serum albumin; DCC, *N,N'*-dicyclohexylcarbodiimide; DIBAH, diisobutylaluminium hydride; DMF, *N,N*-dimethylformamide; ELISA, enzyme-linked immunosorbent assay; GAR, goat anti-rabbit antibody; GAR-Po, peroxidase labelled goat anti-rabbit antibody; LFIA, lateral flow immunoassay; LOD, limit of detection; NBS, *N*-bromosuccinimide; NHS, *N*-hydroxysuccinimide; NPS, new psychoactive substances; PEG, polyethylene glycol; RSA, rabbit serum albumin; RSD, relative standard deviation; SCs, synthetic cannabinoids; THC, thin layer chromatography

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https://doi.org/10.1016/j.toxrep.2017.12.004
Received 28 June 2017; Received in revised form 23 November 2017; Accepted 4 December 2017
Available online 06 December 2017

mainly based on high performance liquid chromatography or gas chromatography coupled to various selective detectors [8,9]. However, these techniques are relatively demanding with respect to costs, sample preparation, analysis times, and highly trained personnel and are unsuitable for screening analysis. On the other side, immunoassays provide an attractive alternative for rapid screening of samples. These days, enzyme-linked immunosorbent assay (ELISA) carried out in a microtiter plate is the most common technique used for immunoassays. The possibility of analysing liquid samples without any purification is one of the most outstanding advantages the immunoassays have over commonly used instrumental methods. ELISA has been successfully applied for the analysis of selected SCs mainly in urine [9–14]. Its main advantages are the possibility to analyse multiple samples simultaneously, sensitivity and the relative simplicity. However, the performance of the necessary operations including repeated incubation and washing steps and enzyme reaction for final signal generation is laborious for laboratories that are not specialized for this process. Lateral flow immunoassay (LFIA) is considered to be one of the simplest methods, which fits perfectly for on-site and roadside monitoring [15]. It combines several benefits including primarily rapidity, user-friendly format and cost-effectiveness [16,17]. On the other hand, LFIA gives only preliminary results, so it could be useful to have both, rapid and simple LFIA and also a sensitive method for quantification such as ELISA.

Herein we report the synthesis of a new hapten structurally derived from JWH-200 itself which will serve for the development of immunomethods. To the best of our knowledge, this is the first hapten bearing 1-[2-(morpholin-4-yl)ethyl]-1*H*-indole moiety used as an immunogen precursor in the detection of synthetic cannabinoids. The aims of the study are to provide the sensitive LFIA that would be as simple as possible to be applied by the state authorities for rapid roadside and on-site monitoring of JWH-200 in oral fluids and ELISA for toxicological quantitative analyses of positive samples captured by LFIA.

2. Material and methods

2.1. Material and reagents

Bovine serum albumin (BSA), rabbit serum albumin (RSA), Tween 20, polyethylene glycol (PEG), Triton X-100, *N*-hydroxysuccinimide (NHS), indole, naphthalen-1-ol, 4-(2-hydroxyethyl) morpholine, benzyl bromide, methanesulfonyl chloride, *N*-bromosuccinimide (NBS), ammonium formate, 10% palladium on carbon, sodium hydride, copper cyanide, silver nitrate and zirconium tetrachloride were purchased from Sigma-Aldrich Inc., USA. Ethyl 2-bromoacetate was obtained from Merck and *N*,*N*'-dicyclohexylcarbodiimide (DCC) was obtained from Fluka. Diisobutylaluminium hydride (DIBAH) solution in hexane, oxalyl chloride and *N*,*N*-dimethylformamide (DMF) extra dry were purchased from Acros. All the other solvents were obtained from Penta. Thin layer chromatography (TLC) was performed on Merck aluminium backed sheets coated with 60F 254 silica gel. Artificial saliva (1700-0305) was purchased from Pickering Laboratories, USA. Gold colloid nanoparticles (an average diameter of 40 nm) were purchased from BB international, UK. Goat anti-rabbit antibody (GAR) and peroxidase labelled goat anti-rabbit antibody (GAR-Po) were obtained from Nordic Immunological Laboratories, Netherlands. Nitrocellulose membranes (PRIMA 85; AE 99; AE 100) were supplied from Whatman GmbH, Germany. Other nitrocellulose membranes (HiFlow Plus HF135; HiFlow Plus HFB180), laminated card (HF000MC100), glass fiber conjugate pad (CFCP03000), cellulose fiber sample pad (CFSP173000) and absorbent pad (CFSP) were purchased from Millipore Corp., USA.

JWH-200 standard and all of the other drug standards used for cross-reactivity studies (Table 2) were obtained from Alfarma s.r.o., Czech Republic or Cayman Pharma, Czech Republic. Individual stock standard solutions containing 1 mg mL^{-1} of each compound were prepared by dissolving accurately weighted amounts in 96% ethanol and stored at −20 °C.

96-well polystyrene microtiter plates Costar 9018 were purchased from Corning Inc., USA.

2.2. Instrumentation

NMR spectra were recorded on a Varian Gemini 300 (300 MHz for ^{1}H; 75 MHz for ^{13}C) or Agilent 400-MR DD2 (400 MHz for ^{1}H; 100 MHz for ^{13}C) spectrometers. High resolution mass spectra were measured on a LTQ Orbitrap XL (Thermo Fischer Scientific) spectrometer using ESI ionization technique. Mass spectra of hapten-protein conjugates were measured on a Bruker Autoflex Speed MALDI-TOF/TOF spectrometer. Automated reverse phase chromatography was carried out using a CombiFlash Rf 200 apparatus (Teledyne ISCO) with prepacked Redisep Rf Gold C18 columns (packed with C18-reverse phase silica gel).

Microplate reader uQuant BIO-TEK was from Inc. Winooski, USA. Linomat V (Camag AG, Switzerland) and a strip cutter (Economic Cutter ZQ2000, Shanghai Kinbio Tech Co., China) were used for the preparation of immunostrips.

2.3. Buffers and solutions

2.3.1. LFIA buffers

Coating buffer (0.01 M carbonate/bicarbonate buffer pH 9.6); assay buffer (0.1 M borate buffer pH 8.8 containing Triton X-100 (1% v/v); conjugate pad buffer (0.2 M borate buffer pH 8.8 containing BSA (1% w/v); sucrose (1% w/v) and Tween 20 (1%)).

2.3.2. ELISA buffers

Coating buffer (0.01 M carbonate/bicarbonate buffer pH 9.6); assay buffer (0.01 M phosphate buffered saline (PBS), pH 7.4); wash buffer (0.01 M PBS pH 7.4 containing Tween 20 (0.05% v/v)); substrate solution for enzyme (9 mL 0.05 M citrate/phosphate buffer pH 5.0, 1 mg of TMB, 1 mL of DMSO, and 2 µL of 30% H_2O_2 (v/v)); stopping solution (2 M sulphuric acid in distilled water).

2.4. Synthesis of hapten (derivative of JWH-200)

The structure of the hapten was derived from JWH-200. The synthesis of the hapten bearing the linker with carboxylic functional group in the position 4 of the naphthalene ring was proposed and carried out (Fig. 1). Spectral data of intermediates and the final product are provided in Table 1.

2.5. 1-(Benzyloxy)naphthalene (**2**) [18]

Naphthalen-1-ol (**1**) (1442 mg, 10 mmol) was dissolved in acetonitrile (40 mL) and anhydrous potassium carbonate (2764 mg, 20 mmol) was added to the solution. After 30 min, benzyl bromide (1784 µL, 15 mmol) was gradually added. The reaction mixture was heated to reflux and stirred for 16 h. After cooling to room temperature, the mixture was diluted with ethyl acetate and washed twice with 2 M hydrochloric acid, once with water and brine. The organic layer was dried over sodium sulfate and concentrated to dryness in vacuo. The crude product was purified by column chromatography (hexane/dichloromethane 19:1) to afford 1-(benzyloxy)naphthalene (**2**) (2239 mg, 96%) as a colorless crystalline solid.

2.6. 1-(Benzyloxy)-4-bromonaphthalene (**3**) [19,20]

1-(Benzyloxy)naphthalene (**2**) (1531 mg, 6.53 mmol) was dissolved in acetonitrile (35 mL), the solution was cooled in an ice bath and NBS (1163 mg, 6.53 mmol) was added portionwise over 20 min. The reaction mixture was stirred for 3 h at 0 °C. After warming to ambient

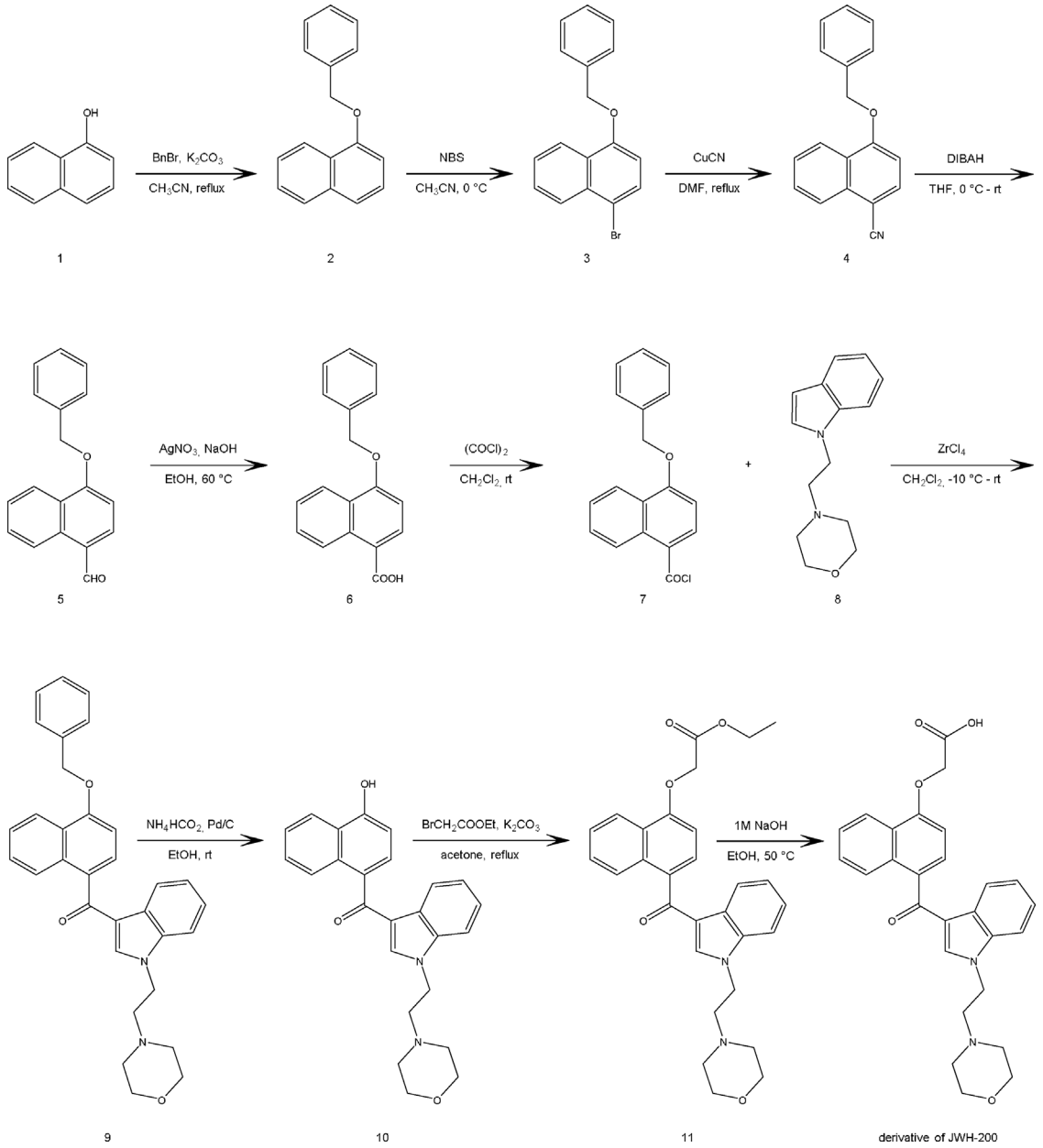


Fig. 1. Synthetic route leading to the derivative of JWH-200.
NBS: *N*-bromosuccinimide, DMF: *N*,*N*-dimethylformamide, DIBAH: diisobutylaluminium hydride, THF: tetrahydrofuran.

temperature, the mixture was diluted with ethyl acetate and washed with water three times. The collected aqueous layers were extracted with ethyl acetate twice. The organic extracts were combined, dried over magnesium sulfate and concentrated under reduced pressure. The residue was purified by column chromatography (hexane/dichloromethane 19:1) to afford 1-(benzyloxy)-4-bromonaphthalene (**3**) (1885 mg, 92%) as a colorless crystalline solid.

2.7. 4-(Benzyloxy)naphthalene-1-carbonitrile (*4*) [21,22]

1-(Benzyloxy)-4-bromonaphthalene (**3**) (1404 mg, 4.48 mmol) was dissolved in DMF (150 mL) and copper cyanide (605 mg, 6.72 mmol) was added to the solution. The flask was placed in heating mantle and the reaction mixture was heated to reflux for 24 h. Another portion of copper cyanide (121 mg, 1.35 mmol) was added and the reaction mixture was boiled for additional 21 h. The solvent was evaporated and the residue was suspended in water and extracted with ethyl acetate. Insoluble part was dissolved in aqueous solution of ammonia, which was then extracted with ethyl acetate two times. The organic extracts were combined, washed with 1 M hydrochloric acid and water and dried over magnesium sulfate. Ethyl acetate was evaporated and the crude product was purified by column chromatography (hexane/dichloromethane 20:1) to afford 4-(benzyloxy)naphthalene-1-carbonitrile (**4**) (1013 mg, 87%) as a yellowish crystalline solid.

2.8. 4-(Benzyloxy)naphthalene-1-carbaldehyde (*5*) [23]

4-(Benzyloxy)naphthalene-1-carbonitrile (**4**) (1000 mg, 3.86 mmol) was dissolved in dry tetrahydrofuran (60 mL), the solution was cooled to 0 °C in an ice bath and 1 M solution of DIBAH in hexane (7.7 mL, 7.70 mmol) was added dropwise. The reaction mixture was stirred at room temperature overnight. Excess DIBAH was decomposed by the addition of water. The aqueous phase was acidified to pH 1 with 2 M hydrochloric acid and extracted with dichloromethane twice. The

Table 1
Spectral data of synthesized compounds.

Compound	Spectral data
2	^{1}H NMR (300 MHz, $CDCl_3$): δ 8.34–8.38 (m, 1H, Ar**H**), 7.80 − 7.83 (m, 1H, Ar**H**), 7.35–7.56 (m, 9H, Ar**H**), 6.90 (d, J = 7.6 Hz, 1H, Ar**H**), 5.27 (s, 2H, C**H$_2$**). ^{13}C NMR (75 MHz, $CDCl_3$): δ 154.61, 137.28, 134.67, 128.72, 128.05, 127.59, 127.50, 126.57, 125.96, 125.88, 125.37, 122.33, 120.61, 105.28, 70.18.
3	^{1}H NMR (300 MHz, $CDCl_3$): δ 8.36 (d, J = 8.1 Hz, 1H, Ar**H**), 8.18 (d, J = 8.1 Hz, 1H, Ar**H**), 7.36–7.66 (m, 8H, Ar**H**), 6.76 (d, J = 8.1 Hz, 1H, Ar**H**), 5.24 (s, 2H, C**H$_2$**). ^{13}C NMR (75 MHz, $CDCl_3$): δ 154.40, 136.81, 132.68, 129.55, 128.81, 128.24, 127.96, 127.52, 127.14, 127.04, 126.17, 122.77, 113.69, 106.08, 70.46.
4	^{1}H NMR (300 MHz, $CDCl_3$): δ 8.40 (dd, J = 8.5, 1.2 Hz, 1H, Ar**H**), 8.18 (dd, J = 8.7, 1.2 Hz, 1H, Ar**H**), 7.85 (d, J = 8.1 Hz, 1H, Ar**H**), 7.71 (td, J = 7.6, 1.2 Hz, 1H, Ar**H**), 7.60 (td, J = 7.6, 1.2 Hz, 1H, Ar**H**), 7.39–7.53 (m, 5H, Ph-**H**), 6.91 (d, J = 8.1 Hz, 1H, Ar**H**), 5.32 (s, 2H, C**H$_2$**). ^{13}C NMR (75 MHz, $CDCl_3$): δ 158.48, 135.89, 134.10, 133.67, 129.13, 128.92, 128.55, 127.57, 126.91, 125.46, 125.06, 123.06, 118.54, 104.71, 102.21, 70.72. MS (ESI) m/z: 260.1 [M + H$^+$], 282.1 [M + Na$^+$]. HRMS (ESI) m/z: calculated for $C_{18}H_{13}ON + H^+$: 260.10699 [M + H$^+$]; found: 260.10705.
5	^{1}H NMR (300 MHz, $CDCl_3$): δ 10.21 (s, 1H, CO**H**), 9.32 (d, J = 8.7 Hz, 1H, Ar**H**), 8.42 (d, J = 8.4 Hz, 1H, Ar**H**), 7.90 (d, J = 8.1 Hz, 1H, Ar**H**), 7.71 (t, J = 7.8 Hz, 1H, Ar**H**), 7.51–7.61 (m, 3H, Ar**H**), 7.39–7.48 (m, 3H, Ar**H**), 6.98 (d, J = 8.1 Hz, 1H, Ar**H**), 5.34 (s, 2H, C**H$_2$**). ^{13}C NMR (75 MHz, $CDCl_3$): δ 192.36, 159.94, 139.60, 136.07, 132.11, 129.71, 128.91, 128.51, 127.58, 126.59, 125.81, 125.30, 125.02, 122.68, 104.30, 70.70.
6	^{1}H NMR (300 MHz, CD_3OD): δ 9.04 (d, J = 8.2 Hz, 1H, Ar**H**), 8.36 (d, J = 8.5 Hz, 1H, Ar**H**), 8.26 (d, J = 8.4 Hz, 1H, Ar**H**), 7.48–7.60 (m, 5H, Ar**H**), 7.35–7.45 (m, 3H, Ar**H**), 7.04 (d, J = 8.4 Hz, 1H, Ar**H**), 5.37 (s, 2H, C**H$_2$**). ^{13}C NMR (100 MHz, DMSO-d_6): δ 168.27, 157.37, 136.55, 132.41, 132.28, 128.59, 128.13, 128.06, 127.65, 125.68, 125.64, 125.14, 122.01, 119.17, 104.68, 69.84. MS (ESI negative) m/z: 186.0 [M-C$_7$H$_7^-$], 233.1 [M-COOH$^-$], 277.0 [M-H$^-$], 577.1 [2M-2H + Na$^-$]. HRMS (ESI negative) m/z: calculated for $C_{18}H_{14}O_3-H^-$: 277.08702 [M-H$^-$]; found: 277.08693.
8	^{1}H NMR (300 MHz, $CDCl_3$): δ 7.63 (d, J = 7.5 Hz, 1H, Ar**H**), 7.36 (dd, J = 8.6, 0.8 Hz, 1H, Ar**H**), 7.19–7.26 (m, 1H, Ar**H**), 7.15 (d, J = 3.2 Hz, 1H, Ar**H**), 7.08–7.13 (m, 1H, Ar**H**), 6.50 (dd, J = 3.2, 0.9 Hz, 1H, Ar**H**), 4.26 (t, J = 6.9 Hz, 2H, N-C**H$_2$**), 3.71 (dd, J = 4.7, 4.7 Hz, 4H, C**H$_2$**-O-C**H$_2$**), 2.76 (t, J = 6.9 Hz, 2H, N-CH$_2$-C**H$_2$**), 2.50 (dd, J = 4.7, 4.7 Hz, 4H, C**H$_2$**-N-C**H$_2$**). ^{13}C NMR (75 MHz, $CDCl_3$): δ 136.00, 128.65, 128.11, 121.55, 121.09, 119.45, 109.26, 101.37, 67.02, 58.26, 53.95, 44.04. MS (ESI) m/z: 231.1 [M + H$^+$].
9	^{1}H NMR (300 MHz, $CDCl_3$): δ 8.47–8.50 (m, 1H, Ar**H**), 8.42 − 8.45 (m, 1H, Ar**H**), 8.28–8.31 (m, 1H, Ar**H**), 7.65 (d, J = 8.1 Hz, 1H, Ar**H**), 7.34–7.58 (m, 11H, Ar**H**), 6.91 (d, J = 8.1 Hz, 1H, Ar**H**), 5.34 (s, 2H, O-C**H$_2$**), 4.19 (t, J = 6.5 Hz, 2H, N-C**H$_2$**), 3.59 (dd, J = 4.5, 4.5 Hz, 4H, C**H$_2$**-O-C**H$_2$**), 2.72 (t, J = 6.5 Hz, 2H, N-CH$_2$-C**H$_2$**), 2.42 (dd, J = 4.5, 4.5 Hz, 4H, C**H$_2$**-N-C**H$_2$**). ^{13}C NMR (75 MHz, $CDCl_3$): δ 191.85, 156.22, 138.36, 137.12, 136.87, 132.43, 131.91, 128.83, 128.26, 127.72, 127.58, 127.51, 127.25, 126.08, 126.00, 125.89, 123.67, 123.12, 122.89, 122.43, 118.07, 109.73, 103.61, 70.43, 66.99, 57.69, 53.79, 44.32. MS (ESI) m/z: 261.2 [BnO-C$_{10}$H$_6$-CO$^+$], 491.4 [M + H$^+$], 513.4 [M + Na$^+$]. HRMS (ESI) m/z: calculated for $C_{32}H_{30}O_3N_2 + H^+$: 491.23309 [M + H$^+$]; found: 491.23292.
10	^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 8.48 − 8.51 (m, 1H, Ar**H**), 8.21 − 8.29 (m, 2H, Ar**H**), 7.46 − 7.51 (m, 4H, Ar**H**), 7.34 − 7.38 (m, 3H, Ar**H**), 6.72 (d, J = 7.8 Hz, 1H, Ar**H**), 4.19 (t, J = 6.5 Hz, 2H, N-C**H$_2$**), 3.61 (dd, J = 4.8, 4.8 Hz, 4H, C**H$_2$**-O-C**H$_2$**), 2.73 (t, J = 6.5 Hz, 2H, N-CH$_2$-C**H$_2$**), 2.43 (dd, J = 4.8, 4.8 Hz, 4H, C**H$_2$**-N−C**H$_2$**). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 192.92, 154.78, 139.10, 137.06, 132.53, 130.54, 128.19, 127.41, 127.15, 125.71, 125.43, 125.11, 123.80, 123.07, 123.02, 122.42, 117.89, 109.83, 107.02, 66.76, 57.51, 53.61, 44.12. MS (ESI) m/z: 401.4 [M + H$^+$], 423.4 [M + Na$^+$], 823.9 [2M + Na$^+$]. HRMS (ESI) m/z: calculated for $C_{25}H_{24}O_3N_2 + H^+$: 401.18597 [M + H$^+$]; found: 401.18596.
11	^{1}H NMR (300 MHz, $CDCl_3$): δ 8.43 − 8.50 (m, 2H, Ar**H**), 8.23 − 8.26 (m, 1H, Ar**H**), 7.61 (d, J = 8.0 Hz, 1H, Ar**H**), 7.51 − 7.55 (m, 2H, Ar**H**), 7.48 (s, 1H, Ar**H**), 7.34–7.39 (m, 3H, Ar**H**), 6.73 (d, J = 8.0 Hz, 1H, Ar**H**), 4.88 (s, 2H, O-C**H$_2$**), 4.37 (q, J = 7.2 Hz, 2H, O-C**H$_2$**-CH$_3$), 4.18 (t, J = 6.5 Hz, 2H, N−C**H$_2$**), 3.59 (dd, J = 4.7, 4.7 Hz, 4H, C**H$_2$**-O-C**H$_2$**), 2.72 (t, J = 6.5 Hz, 2H, N-CH$_2$-C**H$_2$**), 2.42 (dd, J = 4.7, 4.7 Hz, 4H, C**H$_2$**-N-C**H$_2$**), 1.34 (t, J = 7.2 Hz, 3H, O-CH$_2$-C**H$_3$**). ^{13}C NMR (100 MHz, CDCl3): δ 191.72, 168.62, 155.24, 138.55, 137.06, 132.72, 132.35, 127.70, 127.11, 127.03, 126.10, 125.86, 125.82, 123.68, 123.04, 122.91, 122.41, 117.89, 109.75, 103.30, 66.95, 65.77, 61.65, 57.63, 53.73, 44.27, 14.32. MS (ESI) m/z: 487.5 [M + H$^+$], 509.5 [M + Na$^+$], 974.0 [2M + H$^+$], 996.0 [2M + Na$^+$]. HRMS (ESI) m/z: calculated for $C_{29}H_{30}O_5N_2 + H^+$: 487.22275 [M + H$^+$]; found: 487.22285.
derivative of JWH-200	^{1}H NMR (300 MHz, $CDCl_3$): δ 8.41–8.44 (m, 1H, Ar**H**), 8.32–8.35 (m, 1H, Ar**H**), 8.19–8.22 (m, 1H, Ar**H**), 7.30–7.46 (m, 7H, Ar**H**), 6.60 (d, J = 7.8 Hz, 1H, Ar**H**), 4.66 (s, 2H, O-C**H$_2$**), 4.24 (t, J = 6.8 Hz, 2H, N-C**H$_2$**), 3.83 (br s, 1H, N**H**), 3.62 (dd, J = 4.5, 4.5 Hz, 4H, C**H$_2$**-O-C**H$_2$**), 2.78 (t, J = 6.8 Hz, 2H, N-CH$_2$-C**H$_2$**), 2.52 (dd, J = 4.5, 4.5 Hz, 4H, C**H$_2$**-N-C**H$_2$**). ^{13}C NMR (75 MHz, $CDCl_3$): δ 191.69, 172.61, 155.58, 138.34, 136.87, 132.15, 131.70, 127.67, 127.48, 127.12, 125.92, 125.79, 125.62, 124.04, 123.10, 122.96, 122.57, 118.07, 109.84, 103.33, 66.86, 65.42, 56.44, 52.86, 42.75. MS (ESI) m/z: 459.2 [M + H$^+$], 481.1 [M + Na$^+$]. HRMS (ESI) m/z: calculated for $C_{27}H_{26}O_5N_2 + H^+$: 459.19145 [M + H$^+$]; found: 459.19109.

organic phases were collected and dried over magnesium sulfate. The solvents were evaporated and the crude product was purified by column chromatography (hexane/ethyl acetate 9:1). 4-(Benzyloxy) naphthalene-1-carbaldehyde (**5**) (762 mg, 75%) was isolated as a white solid.

2.9. 4-(Benzyloxy)naphthalene-1-carboxylic acid (6) [24]

4-(Benzyloxy)naphthalene-1-carbaldehyde (**5**) (1544 mg, 5.89 mmol) was dissolved in ethanol (80 mL), the solution was heated to 60 °C and silver nitrate (4002 mg, 23.56 mmol) was added. Sodium hydroxide (942 mg, 23.56 mmol) was dissolved in ethanol-water mixture and the solution was gradually added to the reaction flask. After the addition was complete, the reaction mixture was stirred and heated for another 24 h. Excess silver oxide and the silver formed in the reaction were filtered off and washed with hot water. The filtrate was acidified to pH 1 with 2 M hydrochloric acid and extracted with diethyl ether twice. The organic phases were combined and dried over magnesium sulfate. The solvents were evaporated and the crude product was triturated with chloroform. Desired 4-(benzyloxy)naphthalene-1-carboxylic acid (**6**) (1409 mg, 86%) was isolated as a colorless crystalline solid.

2.10. 1-[2-(Morpholin-4-yl)ethyl]-1H-indole (8)

1-[2-(Morpholin-4-yl)ethyl]-1*H*-indole (**8**) was synthesized according to the procedure previously described [25]. The title compound was obtained as light yellow oil.

2.11. 3-[4-(Benzyloxy)naphthalene-1-carbonyl]-1-[2-(morpholin-4-yl) ethyl]-1H-indole (9) [26]

4-(Benzyloxy)naphthalen-1-carboxylic acid (**6**) (1200 mg, 4.30 mmol) was dissolved in dry dichloromethane (20 mL) and a few drops of DMF were added to the solution. Oxalyl chloride (482 μL, 5.60 mmol) was added gradually until the evolution of gas stopped. After stirring for another hour, the volatiles were evaporated in vacuo. The crude product **7** was azeotropically dried with toluene and used in the subsequent reaction without further purification. 4-(Benzyloxy) naphthalene-1-carbonyl chloride (**7**) (382 mg, 1.29 mmol) was dissolved in dry dichloromethane (25 mL) and the solution was cooled to −10 °C. A solution of 1-[2-(morpholin-4-yl)ethyl]-1*H*-indole (**8**) (385 mg, 1.67 mmol) in dry dichloromethane (5 mL) was added to the reaction mixture, followed by zirconium tetrachloride (450 mg, 1.93 mmol). The reaction was stirred for 1 h at −10 °C. Subsequently, the temperature was maintained at 0 °C for 3 h, before the reaction mixture was allowed to warm to room temperature. After stirring overnight, the reaction was stopped by addition of water. The aqueous phase was extracted with ethyl acetate twice. The organic extracts were combined, washed with water and dried over magnesium sulfate. The solvents were evaporated and the crude product was purified by column chromatography (hexan/aceton 3:1). 3-[4-(Benzyloxy)naphthalene-1-carbonyl]-1-[2-(morpholin-4-yl)ethyl]-1*H*-indole (**9**) (444 mg , 70%) was obtained as a white foam.

2.12. 3-(4-Hydroxynaphthalene-1-carbonyl)-1-[2-(morpholin-4-yl)ethyl]-1H-indole (10) [19]

3-[4-(Benzyloxy)naphthalene-1-carbonyl]-1-[2-(morpholin-4-yl) ethyl]-1*H*-indole (**9**) (144 mg, 0.29 mmol) was dissolved in dry ethanol (35 mL). Ammonium formate (93 mg, 1.47 mmol) was added to the solution, followed by a catalytic amount of palladium on carbon. The reaction mixture was stirred at room temperature for 1 h. The palladium catalyst was filtered off and the filtrate was concentrated in vacuo. The residue was diluted with ethyl acetate. The organic phase was washed with potassium carbonate solution and then with brine twice. The organic layer was dried over magnesium sulfate. The solvents were evaporated and the crude product was purified by column chromatography (hexan/aceton 9:4) to afford 3-(4-hydroxynaphthalene-1-carbonyl)-1-[2-(morpholin-4-yl)ethyl]-1*H*-indole (**10**) (116 mg, 99%) as a white solid.

2.13. Ethyl 2-[(4-{1-[2-(morpholin-4-yl)ethyl]-1H-indole-3-carbonyl} naphthalen-1-yl)oxy]acetate (11)

3-(4-Hydroxynaphthalene-1-carbonyl)-1-[2-(morpholin-4-yl)ethyl]-1*H*-indole (**10**) (110 mg, 0.28 mmol) was dissolved in dry acetone (30 mL), freshly annealed potassium carbonate (57 mg, 0.41 mmol) was added to the solution and the temperature was raised to 50 °C. After stirring for 30 min, ethyl 2-bromoacetate (36 μL, 0.32 mmol) was added. The reaction mixture was heated to reflux for 3 h. After cooling to room temperature, ethyl acetate was added and the organic phase was washed with water twice and once with brine. The organic layer was dried over sodium sulfate and concentrated to dryness in vacuo. The crude product was purified by column chromatography (hexane/acetone 3:1) to afford ethyl 2-[(4-{1-[2-(morpholin-4-yl)ethyl]-1*H*-indole-3-carbonyl}naphthalen-1-yl)oxy]acetate (**11**) (111 mg, 83%) as a white solid.

2.14. 2-[(4-{1-[2-(morpholin-4-yl)ethyl]-1H-indole-3-carbonyl} naphthalen-1-yl)oxy]acetic acid (derivative of JWH-200)

Ethyl 2-[(4-{1-[2-(morpholin-4-yl)ethyl]-1*H*-indole-3-carbonyl} naphthalen-1-yl)oxy]acetate (**11**) (105 mg, 0.22 mmol) was suspended in ethanol (15 mL) and 1 M solution of sodium hydroxide in water (3 mL) was added to the reaction mixture. The reaction was stirred at 50 °C for 1 h. Subsequently, ethanol was evaporated. The residual aqueous phase was acidified to pH 1 with hydrochloric acid and concentrated to dryness in vacuo. The formed sodium chloride was removed by trituration of the residue with water. The obtained crude product was purified by reverse phase chromatography (water/methanol, gradient elution 10:1 → 1:1). The title compound was isolated in the form of 2-[(4-{1-[2-(morpholin-4-yl)ethyl]-1*H*-indole-3-carbonyl}naphthalen-1-yl)oxy]acetic acid hydrochloride (73 mg, 68%).

2.15. Preparation of hapten-protein conjugates

The synthesized hapten was conjugated to BSA and to RSA to form an immunogen and a coating antigen, respectively. Conjugates were prepared by activated ester method, the conjugation step being carried out in reversed micellar solution [27].

Derivative of JWH-200 (1 eq) was dissolved in the exact volume of DMF to form a solution of concentration of 100 mg mL^{-1}. DCC (1.3 eq) and NHS (1.3 eq) were added to the micro test tube, both in the form of solution in DMF (100 mg mL^{-1}). The reaction mixture was left to stand at room temperature until the by-product *N,N*'-dicyclohexylurea spontaneously crystalized from the solution. After the reaction was complete (confirmed by TLC), the crystals were separated and the solution with an activated ester was directly used in subsequent reaction. A solution of BSA or RSA (0.013 eq) in bicarbonate buffer (13 μg mL^{-1}) was mixed with a 0.3 M solution of AOT in octane to form a reversed micellar solution. The activated ester of the hapten was added to these solutions with stirring. The reaction mixture was stirred vigorously at room temperature overnight. The modified BSA or RSA was precipitated by addition of cooled acetone and the suspension was centrifuged (4 °C; 30 min; 1 500*g*). The supernatant was then removed and the collected precipitate was washed with cooled acetone one more time. The suspension was again centrifuged (4 °C; 20 min). After the removal of the supernatant, the crude product was air dried, dissolved in water and lyophilised to afford the conjugate in a form suitable for immunization of laboratory animals.

2.16. Immunogen (derivative of JWH-200-BSA)

The derivative of JWH-200 (10.1 mg, 20.4 μmol) was converted into the activated ester by the reaction with DCC (5.5 mg, 26.5 μmol) and NHS (3.1 mg, 26.5 μmol). Subsequent conjugation of the activated ester with BSA (18.1 mg, 0.27 μmol) afforded the immunogen (24.2 mg).

2.17. Coating antigen (derivative of JWH-200-RSA)

The derivative of JWH-200 (14.5 mg, 29.3 μmol) was converted into the activated ester by the reaction with DCC (7.8 mg, 38.1 μmol) and NHS (4.4 mg, 38.1 μmol). The conjugation of the ester with RSA (25.9 mg, 0.39 μmol) afforded the coating antigen (31.0 mg).

2.18. Antiserum against JWH-200 (Anti-JWH-200) and its specificity

Rabbits were immunized with the immunogen and an antiserum against JWH-200 was collected by using a standard procedure described previously [28]. A stock solution of the Anti-JWH-200 was prepared by dissolving 1 mg of lyophilisate in 1 mL of the PBS and stored at −20 °C.

Anti-JWH-200 specificity was investigated by cross-reactivity experiments (CR%) performed by ELISA. The calibration curves for each of the tested cannabinoids (14synthetic; 6 phytocannabinoids) were constructed. The CR% was calculated as: (IC_{50} of JWH-200)/IC_{50} of tested compound) × 100.

2.19. Gold labelled antibodies (Anti-JWH-200-Au)

The gold labelled antibodies were prepared according to the procedure described for carbon nanoparticles with minor modification [29]. To a gold colloid nanoparticles suspension (gold colloid nanoparticles 0.5 mg mL^{-1} of 5 mM borate buffer, pH 8.8) Anti-JWH-200 (1 mg mL^{-1} in PBS) were added. The mixture was stirred gently at 37 °C for 2 h. Afterwards, the suspension was centrifuged (10 °C; 60 min; 6 000*g*). The sediment was washed three times in a 5 mM borate buffer, pH 8.8 containing BSA (1% w/v) and NaN_3 (0.02% w/v) using centrifugation (10 °C; 15 min; 13 640*g*). The final sediment was resuspended in 0.1 M borate buffer, pH 8.8 containing BSA (1% w/v) and NaN_3 (0.02% w/v). Prepared stock suspension of the Anti-JWH-200-Au conjugate (200 µg mL^{-1}) was stored at 4 °C in the dark.

2.20. Immunochemical methods

2.20.1. Preparation of LFIA strip and LFIA procedure

The NC membrane AE 100 was stuck to the laminated card to increase the robustness of the membrane. Subsequently, the membrane was coated with GAR at the control line and with derivative of JWH-200-RSA at the test line using the Linomat V (conc. 100 µg mL^{-1} in coating buffer; 0.1 µL mm^{-1}). The conjugate pad (pre-treated with conjugate pad buffer, 5 min soaking, then drying at 37 °C for 1 h) was dispensed with the mixture of Anti-JWH-200-Au (200 µg mL^{-1}; 1.25 µL mm^{-1}). Than the NC membrane and the conjugate pad were dried at 37 °C overnight. On the following day, the conjugate pad, the sample pad and the absorbent pad were stuck to the laminated card with proper overlap. The membrane was cut into LFIA strips (4 mm) using a strip cutter. The strip was assembled according to the scheme shown in Fig. 2. Prepared strips were put into LFIA cassettes and were stored in sealed bags under dry conditions at laboratory temperature until used.

The JWH-200 standard solution (the concentration range 0–1000 ng mL^{-1} in the artificial saliva) or the oral fluid sample was mixed with the assay buffer (1:3) and the mixture was added into the well of the LFIA cassette (100 µL/strip).

In the diffusion flow of reactants, the JWH-200 in a sample interacted with Anti-JWH-200-Au (dried on the conjugate pad) and migrated because of capillary effects along the membrane. Samples free of JWH-200 resulted in just free Anti-JWH-200-Au captured by derivative of JWH-200-RSA dispensed on the test line and formed a red zone of maximum intensity. Otherwise, JWH-200 positive samples inhibited the interaction of the Anti-JWH-200-Au with the derivative of JWH-200-RSA resulting in decrease of red zone intensity. Therefore, the intensity of the zone (test line) inversely correlated with the JWH-200 concentration in the sample.

If the sample produced the test line appearance of the same intensity as a negative control (blank artificial saliva), it was considered to be negative (−). If the colour intensity was weaker than that of the negative control, the result was evaluated as weakly positive (±). The sample was assessed as positive (+) when the test line was absent or extremely weak. Dried strips were scanned and test line intensities converted to pixel grey volumes using TotalLab in order to obtain quantitative results.

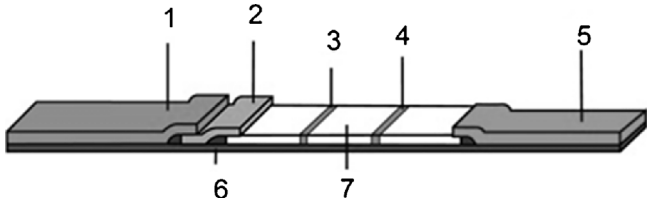


Fig. 2. Construction of LFIA strip.
1–sample pad; 2–conjugate pad; 3–test line; 4–control line; 5–absorbent pad; 6–laminated card; 7–nitrocellulose membrane.

2.20.2. ELISA procedure

ELISA was performed as an indirect competitive format. Each well of the microtiter plates was coated with the coating antigen (50 ng mL^{-1} in the coating buffer; 100 µL/well) and incubated at 4 °C overnight. Afterwards, the unbound antigen was removed with the wash buffer (4 times, 350 µL/well). The standards of JWH-200 (the concentration range of 0–250 ng mL^{-1} in the PBS) or oral fluid samples (diluted 30 times in the PBS) were added to microtiter plates (50 µL/well) followed by the corresponding solution of Anti-JWH-200 (diluted 1:40 000 in the PBS-0.1% BSA (w/v); 50 µL/well) and incubated at room temperature for 45 min. Microtiter plates were washed again (4 times, 350 µL/well) and GAR-Po (diluted 1:10 000 in the PBS; 100 µL/well) was added and incubated at room temperature for 1 h. After the washing step, the substrate solution for the enzyme was added (100 µL/well) and incubated at room temperature for 10 min. The enzyme reaction was stopped by adding the stopping solution (50 µL/well) and the absorbance was measured at 450 nm.

2.20.3. Calibration curves and interpretation of results

Sigmoid calibration standard curves were obtained by plotting the mean values of absorbance (ELISA) or pixel grey volumes (LFIA) against the logarithm of JWH-200 concentrations through a four-parameter logistic equation as we described previously [30]. The limit of detection (LOD) was defined as the concentration of an analyte corresponding to the maximum assay signal minus 3 x standard deviation (SD) in accordance with the calibration curve (the blank was calculated from 3 parallel determinations with the absence of an analyte). The IC_{50} corresponded to the concentration of analyte giving 50% inhibition of the asymptotic maximum. The linear working range corresponded to the analyte concentration causing the 20–80% inhibition of the maximum assay signal.

2.20.4. Oral fluid samples

Human oral fluids were obtained from five laboratory volunteers. Freshly collected oral fluids were spiked with JWH-200 in order to obtain the following concentrations: 0; 10; 50; 100; 500; 1000 ng mL^{-1}. The samples were analysed immediately by our immunomethods and were stored at −20 °C until next analyses. Two sets of prepared samples were used to evaluate the correlation with UHPLC–MS/MS.

2.20.5. UHPLC–MS/MS analysis

For the UHPLC–MS/MS analysis Agilent 1290 Infinity UHPLC system coupled with Agilent 6460 Triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) were used. Chromatographic separation was performed on Agilent Zorbax Eclipse Plus C18 column (2.1 × 50 mm; 1.8 µm). The mobile phases for gradient elution were 10 mM formic acid (A) and methanol (B). The flow rate was 0.25 mL/min and column temperature 35 °C. Gradient elution was carried out as follows: 0 min, 80:20 (A:B); 2 min, 0:100; 3.5 min, 0:100; 3.7 min, 80:20; 6 min, 80:20. Retention time of JWH 200 was 2.54 min.

The MS/MS apparatus was operating in positive mode. The applied conditions of electrospray ion source were: drying gas temperature 340 °C; drying gas flow 4 L/min; sheath gas temperature 200 °C; sheath gas flow 12 L/min; nebulizer pressure 30 psi; nozzle voltage 300 V; capillary voltage was set at 2300 V. Multiple reaction monitoring (MRM) mode was used for the detection. Three transitions of *m/z* were used (collision energies (eV) are given in brackets): 385.19 → 114.1 (25); 127.1 (69) and 155 (21).

Agilent Mass Hunter (Agilent Technologies, Inc.) was used for data acquisition and quantification of samples.

Simple dilution of oral fluid was used. 100 µL of oral fluid with 900 µL of 20% methanol were vortexed, centrifuged (10 min, 13 000*g*) and supernatant was used for the analysis.

For calibration blank oral fluid sample was spiked with different concentrations of JWH 200 and prepared the same way as described.

Six final concentrations of JWH 200 ranging between 0.1 and 500 ng mL^{-1} were used. Peak areas of the external standard (at each concentration) were plotted against the corresponding standard concentrations using linear regression to generate standard curve.

3. Results and discussion

3.1. Hapten design and synthesis

Hapten design plays a key role in the development of immunoassays targeted on small molecules such as synthetic cannabinoids. As the structural similarity between the target compound and the designed hapten should be maximized, the hapten was derived directly from JWH-200. The most active part of a haptenic determinant is distal to the point of binding with the carrier protein, thus the linker with carboxylic functional group was placed in the position 4 of the naphthalene ring, so the most active part is the morpholine moiety characteristic for JWH-200. The aforementioned linker serves only to join the hapten to the carrier protein and it should not become an epitope, once the immunogen is applied to laboratory animals [31,32].

The synthesis of the hapten was based on Friedel – Crafts acylation of *N*-substituted indole **8** by 4-(benzyloxy)naphthalene-1-carbonyl chloride (**7**) in the presence of zirconium tetrachloride as a Lewis acid [26]. The hydroxyl group was then deprotected by catalytic hydrogenolysis of benzyl protecting group using palladium on activated charcoal as a catalyst [19]. *O*-alkylation by ethyl 2-bromoacetate and subsequent base catalysed hydrolysis afforded the desired hapten bearing the linker with carboxylic functional group.

It is also worth noticing that an innovative synthetic route was employed for the preparation of 4-(benzyloxy)naphthalene-1-carboxylic acid (**6**). In the molecule of 4-(benzyloxy)-1-bromonaphtalene (**3**), the bromine atom was first replaced by nitrile functional group in Rosenmund – von Braun reaction [21,22]. By the reduction of formed nitrile **4** with diisobutylaluminium hydride [23] and subsequent reoxidation of the intermediate aldehyde **5** with silver oxide, formed *in situ* from silver nitrite and sodium hydroxide [24], carboxylic functional group was introduced into the molecule. This three-step sequence afforded the desired acid **6** with higher overall yield than the published procedure based on lithiation and subsequent reaction of organolithium intermediate with carbon dioxide [19].

The hapten was prepared using a ten-step synthetic sequence from commercially available naphthalen-1-ol (**1**) with moderate to high yields in each step. The synthesized hapten and all the intermediates were characterized by NMR and ESI–MS (Table 1).

3.2. Preparation of hapten-protein conjugates

The synthesized hapten was coupled to BSA to form the immunogen or to RSA to form the coating antigen. The conjugate formation was confirmed by a MALDI-TOF analysis. The binding ratio of the hapten to the carrier protein was determined to be approximately 20:1 and 31:1 for the immunogen and the coating antigen, respectively. The average number of haptens bound to BSA was considered sufficient to illicit a specific immune response in immunized animals.

3.3. Optimisation of LFIA conditions and characterisation of assay

The assay was optimized using artificial saliva considering the enormous influence of the matrix (oral fluid) on the test lines appearance. The line intensities were reduced if the oral fluid samples were added compared to the intensities obtained from tests carried out in the assay buffer (data not shown). During the optimization, checkboard titration experiments were performed. Several concentrations of the derivative of JWH-200-RSA dispensed on the membrane (50–200 μg mL^{-1} in coating buffer; 0.1 μL mm^{-1}) against different volume of Anti-JWH-200-Au (200 μg mL^{-1}; 1–1.75 μL mm^{-1}) were investigated. The amount of the immunoreagents should be kept low enough to achieve good sensitivity, but must be sufficient to provide an acceptable signal [33]. Several other factors affecting LFIA strip appearance were evaluated. Five types of NC membrane were tested (PRIMA 85, AE 99, AE 100, HiFlow Plus HF 135, HiFlow Plus HFB180). The type of membrane influenced flow time and sharpness of tested lines. Faster-flowing membranes reached endpoint more quickly but caused a loss in signal intensity and decrease of test sensitivity [17,34]. As shown on Fig. 3, the best performance was observed when membrane AE100 was used. The composition of the assay buffer also influence test sensitivity [17]. In this experiment, 0.01 M PBS, pH 9.6 and 0.1 M borate buffer, pH 8.8 were tested. Additives such as BSA (0.1–2% w/v); PEG (0.1–2% v/v) and sucrose (0.1–5% w/v) and their combinations with and without surfactants Tween 20 (0.1–1% v/v) or Triton X-100 (0.1–1% v/v) were tested to further improve the LFIA sensitivity. The complete specifications of the optimized conditions are included in section *Preparation of LFIA strip and LFIA procedure*.

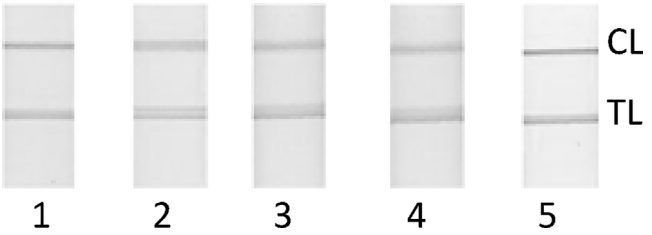


Fig. 3. Appearance of test and control lines on different type of nitrocellulose membrane in LFIA.
CL – control line; TL – test line; 1–AE98; 2–Prima 85; 3–HF135 UB; 4–HF180; 5–AE100.

The sensitivity of the LFIA was determined from the JWH-200 calibration curve (concentration range 0–1000 ng mL^{-1}created in the artificial saliva). The assay was carried out in triplicate under optimized conditions. The colour intensity of the test line was evaluated visually. As shown in Fig. 4, the JWH-200 concentration of 1 ng mL^{-1} caused a slight but visually distinguishable difference in the test line intensity compared to the negative control. Thus, 1 ng mL^{-1} of JWH-200 was considered to be a visual detection limit. The value of the visual LOD suggests enough sensitivity for the intended use. Published SCs concentrations in oral fluids are in the range of units to tens ng mL^{-1} [35]. To obtain the quantitative results, colour intensities of the test lines were converted to pixel grey volumes. The mean signal values were fitted to a sigmoid standard curve. The LOD was 0.08 ± 0.04 ng mL^{-1} with the linear working range of 0.3–42 ng mL^{-1} and the IC_{50} value 3.4 ± 0.6 ng mL^{-1}.

To evaluate the precision of the LFIA, three standard samples with JWH-200 concentrations in the range of 10–50 ng mL^{-1} in human oral fluid were assayed. For the intra-assay precision study, one run of analyses (n = 3) was performed with each sample on the same day. Similarly, one run of analyses was carried out with each sample daily on three non-consecutive days for the inter-assay precision study. The intra- and inter-assay relative standard deviations (RSD) were calculated in the range 2.4–9.2% and 3.1–16.7%, respectively, indicating the acceptable precision.

3.4. ELISA characterisation and antiserum specificity

The assay was developed as an indirect competitive format using polyclonal antibody targeted at JWH-200. Checkerboard titrations were performed and suitable immunoreagent concentrations were determined when the maximum absorbance ranged from 1.2 to 1.9 and the calibration curve reached the lowest IC_{50} values. To enhance the sensitivity of the ELISA, several other conditions (time and temperature of incubations, composition of dilution buffers) were evaluated. The optimized conditions are listed in the section *ELISA procedure*. Based on the JWH-200 standard curve (concentration range 0–250 ng mL^{-1} in

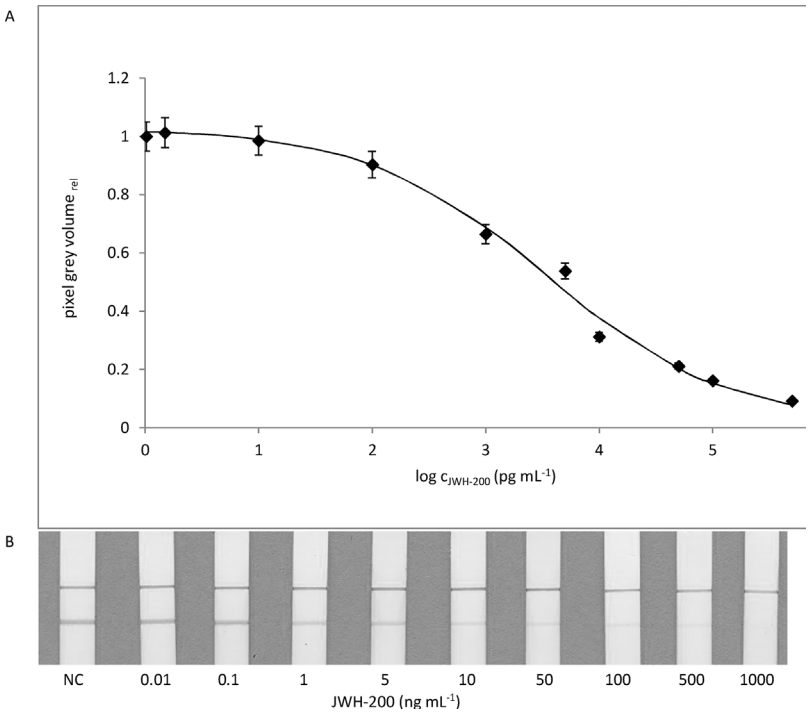


Fig. 4. Typical LFIA standard curve using the optimized assay protocol (mean value, n = 3).
(A) Quantitative evaluation LOD = 0.08 ± 0.04 ng mL^{-1}; IC_{50} = 3.4 ± 0.6 ng mL^{-1}; linear working range = 0.3–42 ng mL^{-1} (B) Visual evaluation.

PBS), the LOD was 0.04 ± 0.02 ng mL^{-1} with the IC_{50} value of 0.42 ± 0.09 ng mL^{-1} and the linear working range of 0.16–1.80 ng mL^{-1}. The parameters characterising our ELISA are comparable with the parameters published previously [9].

The influence of the human oral fluid on the assay was tested by comparing JWH-200 standard curves (concentration range 0–250 ng mL^{-1}) obtained in the PBS and in the presence of various proportion of oral fluid (0; 10; 20; and 30-times diluted in the PBS). As can be seen in Fig. 5, undiluted oral fluid sample significantly affect obtained signal as well as IC_{50} values. However, 30-fold diluted oral fluid does not affect assay parameters, implying oral fluid samples could be directly applied to the immunoassay without difficult clean-up procedure (only diluted).

The specificity of obtained antibody was evaluated with 20 cannabinoids. Cross-reactivity values are summarized in Table 2. Substances structurally similar to the target analyte (e.g.AM-2233; AM-1220) showed medium cross-reactivity (5 %), while SCs with a different group than naphthoyl indole group interacted weakly (< 0.2 %), except the pravadoline (> 2 %).

For the precision study, the same experiments were used as for LFIA. The intra- and inter-assay RSD were calculated in the range of 1.5–8.8% and 2.0–9.7%, respectively.

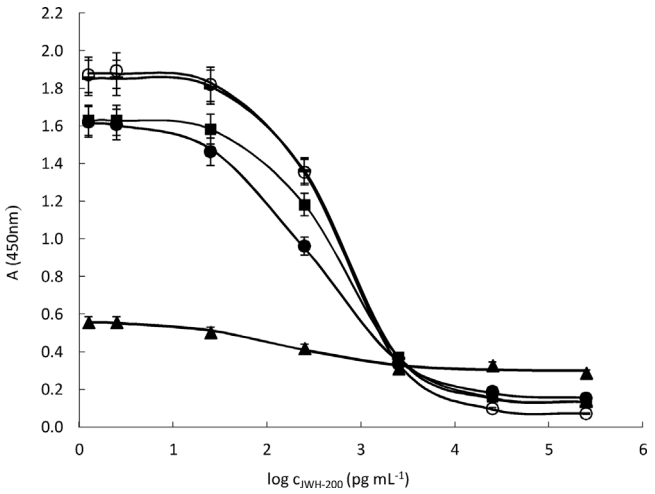


Fig. 5. The comparison of ELISA curves obtained from standard prepared in assay buffer and in the presence of various proportion of oral fluid.
(○) assay buffer; (▲) oral fluid; (●) 10 x diluted oral fluid; (■) 20 x diluted oral fluid (−)30 x diluted oral fluid.

3.5. Analysis of spiked oral fluid samples

The accuracy of the developed immunomethods was evaluated through recovery study with samples of human oral fluid spiked with JWH-200 at levels from 0 to 1000 ng mL^{-1}. In the visually assessed LFIA, the negative results were obtained for non-spiked samples. The weakly positive results were obtained for samples spiked at concentrations 10 ng mL^{-1}, while the samples spiked at level >50 ng mL^{-1}of the JWH-200 were assessed as positive. Using the quantitative LFIA the recoveries ranged from 86 to 134% with RSD 2.4–16.8% and for ELISA from 82 to 131 %, with RSD 0.4–7.8 % (Table 3).

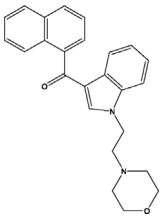
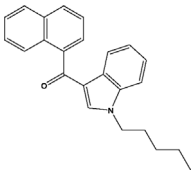
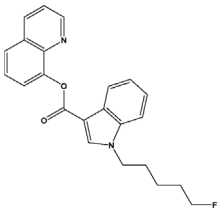
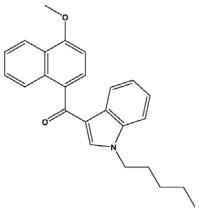
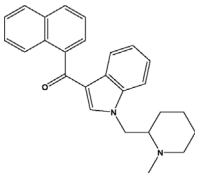
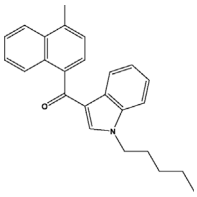
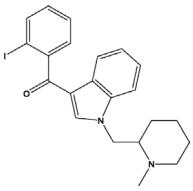
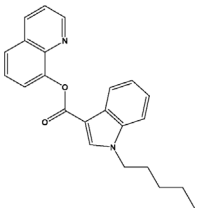
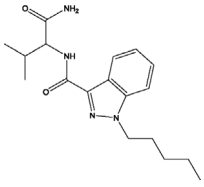
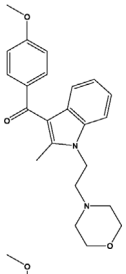
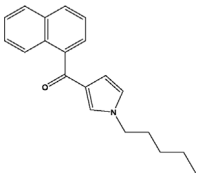
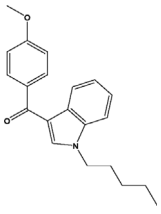
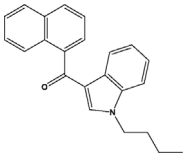
3.6. Correlation of immunomethods with the UHPLC–MS/MS

Twelve spiked human oral fluid samples (concentration of JWH-200 from 0 to 1000 ng mL^{-1}) were assayed three times by the newly developed ELISA and LFIA and the results were compared with those obtained using UHPLC–MS/MS (Table 4). The excellent correlations between developed immunomethods and UHPLC–MS/MS were obtained. The linear equation is y = 0.99 x (R^2 $_{ELISA}$ = 0.99) and y = 0.79 x (R^2 $_{LFIA}$ = 0.99). Our results demonstrate a good potential of the proposed immunomethods for using in routine analyses of JWH-200 in oral fluids.

4. Conclusions

We have successfully synthesized the new hapten structurally derived from the synthetic cannabinoid JWH-200. The hapten was used to prepare the immunogen and the coating antigen. The antibodies obtained from rabbits immunized with hapten conjugated to BSA served

Table 2
Cross-reactivity data for Anti-JWH-200 used in ELISA.

Compound	Structure	CR (%)	Compound	Structure	CR (%)
JWH-200		100.0	JWH-018		0.7
5F-PB-22		0.2	JWH-081		0.9
AM-1220		4.7	JWH-122		1.0
AM-2233		5.6	PB-22		0.1
AB-PINACA		0.1	pravadoline		2.6
JWH-030		0.4	RCS-4		0.2
JWH-073		1.5			

Other tested cannabinoids (AB-FUBINACA, cannabidiol, cannabidivarin, canabigerol, cannabinol, dihydrocannabidiol, tetrahydrocannabinol) – all with cross-reactivity < 0.01% in developed ELISA.

for the development of two immunomethods for the JWH-200 detection in human oral fluids. Our developed one-step LFIA can provide a visual evidence of JWH-200 presence in oral fluids within 15 min. This assay is primarily suitable for the on-site and roadside monitoring due to the rapid qualitative results it delivers. For greater accuracy, the analyte presence should be verified by more sophisticated method such as ELISA. The ELISA proposed in this study is highly sensitive and has the excellent correlation with the UHPLC–MS/MS.

Table 3
Results from spiked oral fluids obtained using LFIA, ELISA (n = 3).

Saliva	Spiked concentration (ng mL^{-1})	LFIA[a]				ELISA[b]		
		Visual detection[c]	Mean (ng mL^{-1})	Recovery (%)	RSD[d] (%)	Mean (ng mL^{-1})	Recovery (%)	RSD[d] (%)
1.	0	− − −	< LOD[e]	–	–	< LOD[e]	–	–
	10	± ± ±	11.2	112.0	9.3	13.1	131.0	6.6
	50	± ± ±	48.1	96.2	10.4	56.2	112.4	5.2
	100	+ + +	94.2	94.2	9.6	94.6	94.6	2.3
	500	+ + +	526.4	105.3	11.7	535.8	107.2	0.4
	1000	+ + +	1274.9	127.5	4.7	1063.6	106.4	2.5
2.	0	− − −	< LOD[e]	–	–	< LOD[e]	–	–
	10	± ± ±	8.6	86.0	7.6	8.2	82.0	3.8
	50	± ± ±	46.4	92.8	3.7	54.3	108.6	5.4
	100	+ + +	89.7	89.7	8.4	97.7	97.7	6.4
	500	+ + +	534.6	106.9	4.2	582.2	116.4	2.1
	1000	+ + +	1206.7	120.7	2.4	1070.7	107.1	1.3
3.	0	− − −	< LOD[e]	–	–	< LOD[e]	–	–
	10	± ± ±	9.3	93.0	7.7	9.4	94.0	4.2
	50	± ± ±	56.6	113.2	16.8	49.8	99.6	3.9
	100	+ + +	92.7	92.7	9.6	96.2	96.2	2.5
	500	+ + +	668.2	133.6	4.6	523.0	104.6	7.8
	1000	+ + +	1278.4	127.8	12.2	1027.7	102.8	2.6

[a] Before the LFIA, samples were appropriately diluted with the synthetic saliva to fall into the linear working range.
[b] Before the ELISA, samples were appropriately diluted with the assay buffer to fall into the linear working range.
[c] Visual assessment of the test line; (–) negative result; (±) weakly positive result (the JWH-200 concentration in the range of 10–50 ng mL^{-1};); (+) positive result (JWH-200 concentration > 100 ng mL^{-1}).
[d] RSD, relative standard deviation.
[e] LOD, limit of detection.

Table 4
Recovery obtained using the LFIA, ELISA and UHPLC–MS/MS (n = 3).

Oral fluid	Spiked concentration (ng mL^{-1})	LFIA[a]			ELISA[b]			UHPLC–MS/MS		
		Mean (ng mL^{-1})	Recovery (%)	RSD[c] (%)	Mean (ng mL^{-1})	Recovery (%)	RSD[c] (%)	Mean (ng mL^{-1})	Recovery (%)	RSD[c] (%)
1.	0	< LOD[d]	–	–	< LOD[d]	–	–	< LOD[d]	–	–
	10	8.4	84.1	1.3	13.4	134.0	10.5	10.0	99.8	0.03
	50	46.0	92.0	6.1	56.5	113.0	1.3	50.8	101.6	0.09
	100	88.7	88.7	12.8	121.8	121.8	14.5	104.5	104.5	0.32
	500	615.8	123.2	9.7	468.8	93.8	2.7	518.2	103.6	2.02
	1000	1297.5	129.7	4.3	975.3	97.5	8.9	1042.8	104.3	2.56
2.	0	< LOD[d]	–	–	< LOD[d]	–	–	< LOD[d]	–	–
	10	8.3	83.5	3.5	9.4	94.2	1.3	94.0	94.2	0.03
	50	44.5	89.0	2.4	49.8	99.5	0.7	49.8	99.7	0.02
	100	95.1	95.1	4.9	96.2	96.2	0.6	99.1	99.1	0.11
	500	633.8	126.8	2.0	523.0	104.6	3.2	487.2	97.4	1.29
	1000	1232.4	123.2	3.6	1027.7	102.8	2.8	954.4	95.4	3.53

[a]Before the LFIA, samples were appropriately diluted with the artificial saliva to fall into the linear working range.
[b] Before the ELISA, samples were appropriately diluted with the assay buffer to fall into the linear working range.
[c] RSD, relative standard deviation.
[d] LOD, limit of detection.

Conflict of interest

All authors declare no conflict of interest.

Acknowledgements

Financial support from specific university research (MSMT No 20-SVV/2016) and project MICR VI20172020056.

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Příloha 16:

Maryska M., Fojtikova L., Jurok R., Holubova B., Lapcik O., Kuchar M. Use of novel haptens in the production of antibodies for the detection of tryptamines. *RSC Adv* 8(29): 16243-16250, 2018.

RSC Advances

PAPER

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Cite this: *RSC Adv.*, 2018, **8**, 16243

Use of novel haptens in the production of antibodies for the detection of tryptamines†

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Tryptamines are a group of hallucinogenic drugs whose detection in body fluids could be simplified by immunochemical assay kits. Antibodies for these assays are obtained by the immunization of laboratory animals with conjugates of a hapten similar to the target analyte and a suitable protein. Therefore we synthesized novel haptens derived from tryptamine-based drugs, with *N*,*N*-dimethyltryptamine (DMT), 5-methoxy-*N*,*N*-dimethyltryptamine (5-MeO-DMT) and *N*,*N*-diisopropyltryptamine (DiPT) selected as the target analytes. Their structures were modified with a short linker ended with a carboxylic group. The haptens were conjugated with bovine serum albumin (BSA) and rabbits were immunized with the conjugates. The obtained polyclonal antibodies showed good reactivity and the LOD of the constructed ELISAs was in the range 0.006–0.254 ng mL^{-1}. Thus, they are suitable for the development of immunochemical assay kits.

Received 22nd March 2018
Accepted 24th April 2018

DOI: 10.1039/c8ra02528b

rsc.li/rsc-advances

1. Introduction

Tryptamines, a group of hallucinogenic drugs derived from tryptamine, include both natural and synthetic compounds. The best known tryptamine is dimethyltryptamine (DMT), the active compound of ayahuasca, the ritual beverage, which has traditionally been used in several South American indigenous cultures.[1] Other traditional natural tryptamines are psilocybin and psilocin, active compounds of the *Psilocybe* mushrooms also known as 'magic mushrooms'. Over the past few decades, lots of synthetic derivatives of tryptamine have been prepared. Their rise in popularity has been attributed to Alexander Shulgin, whose book TiHKAL (Tryptamines I have known and loved) described the synthesis and effects of many of them.[2] These synthetic tryptamines belong to the group of novel psychoactive substances (NPS), which are produced to give 'legal highs' and bypass legal restrictions.[3] Although there is widespread use of NPS on the drug scene, options for their detection, including for the detection of tryptamines, remain rather limited.

There are currently only two methods for the detection of tryptamines. The first involves the use of specific colour tests, which are based on the reaction of specific reagents forming coloured products with indole-derived compounds. But, with so many different NPS now on the drug scene, colour tests are not reliable because of their low specificity.[4] The more commonly used second method involves the analysis of tryptamines by liquid chromatography coupled with mass spectrometry (LC-MS).[5,6] This method is precise, capable of determining multiple targets in one run, offers low detection limits and can be used for various matrices, especially body fluids. However, it is also demanding with respect to cost, sample preparation and analysis time. Furthermore, LC-MS-based techniques cannot be used in the field.

Because of the above-mentioned disadvantages, attention has focused on immunochemical methods, such as ELISA (enzyme-linked immunosorbent assay) and LFIA (lateral flow immunosorbent assay). Antibodies for immunochemical detection are usually produced by the immunization of laboratory animals. Because tryptamines are haptens, *i.e.* molecules too small to be immunogenic on their own, they must be conjugated to a carrier protein prior to immunization. To do this, molecules of the target analytes are modified with short linkers containing a suitable functional group. Using bufotenine and serotonin as haptens, Skerritt *et al.* reported the development of an ELISA kit for the detection of DMT and 5-MeO-DMT in *Phalaris* plants.[7] However, this assay is limited only to these natural tryptamines and is not selective between them. Yamaguchi *et al.* prepared monoclonal antibodies against psilocin for identification of magic mushrooms.[8] These antibodies showed cross reactivity with DMT, but their limit of

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c8ra02528b

detection was relatively high. Thus far, no other immunochemical assay has been reported that targets either natural or synthetic tryptamines.

The development of LFIA kits for the detection of synthetic and other tryptamines is needed to screen potential users. To provide such a kit, we first synthesized novel haptens carrying a short linker with a carboxyl group for the production of antibodies selective to different tryptamines. Rabbits were immunized with conjugates of these haptens with bovine serum albumin (BSA). The antibodies obtained from the immunization showed expedient sensitivity and selectivity for individual tryptamines, and, thus, appear to provide a viable basis for LFIA development.

2. Materials and methods

Ethyl 2-bromoacetate was obtained from Merck and *N*,*N*′-dicyclohexylcarbodiimide (DCC) was obtained from Fluka. Diisobutylaluminium hydride (DIBAL-H) solution in hexane, oxalyl chloride and dry DMF were purchased from Acros. Other reagents were purchased from Sigma-Aldrich. Dry THF, dichloromethane and diethyl ether were dried with molecular sieves. All reactions were carried out under argon atmosphere. Thin layer chromatography (TLC) was performed on aluminium backed sheets coated with 60F 254 silica gel from Merck. Column chromatography was performed on silica (0.045–0.200 μm) from Merck. Reverse phase chromatography was carried out using a CombiFlash Rf 200 apparatus (Teledyne ISCO) with prepacked Redisep Rf Gold C18 columns (packed with C18-reverse phase silica gel). NMR spectra were recorded on a Varian Gemini 300 (300 MHz for ^{1}H; 75 MHz for ^{13}C) or Agilent 400-MR DD2 (400 MHz for ^{1}H; 100 MHz for ^{13}C) spectrometers. High resolution mass spectra were measured on a LTQ Orbitrap XL (Thermo Fischer Scientific) spectrometer using ESI ionization technique. Mass spectra of hapten–protein conjugates were measured on a Bruker Autoflex Speed MALDI-TOF/TOF spectrometer. Microplate reader uQuant BIO-TEK was from Inc. Winooski, USA and 96-well polystyrene microtiter plates Costar 9018 were purchased from Corning Inc., USA. Peroxidase labelled goat anti-rabbit antibody (GAR-Po) was obtained from Nordic Immunological Laboratories, Netherlands. Analytical standards psilocin and psilocybin were obtained from THC Pharm GmbH The Health Concept, Germany. Analytical standards of DMT, 5-MeO-DMT, DiPT and 5-MeO-DiPT were prepared according to literature[9] in purity ≥98% (LC). Serotonin was purchased from Sigma-Aldrich. Other tryptamines 5-MeO-DALT, 4-HO-MET, acetylpsilocin, αMT and 5-MeO-DIPT were generously donated by the Institute of Criminalistics Prague.

2.1 Preparation of conjugates

Following the procedure we described previously,[10] the prepared haptens I–IV were conjugated to BSA using activated ester method. Obtained conjugates I–IV were analyzed by MALDI-TOF to determine the number of hapten molecules bound to the protein. The average values were calculated from the peak with highest intensity.

2.2 Immunisation and antiserum preparation

Four different immunisation conjugates (conjugate I–IV) were used. Antisera were produced in rabbits and obtained according to the procedure described previously.[11] Stock solutions of antisera were prepared by dissolving 1 mg of lyophilisate in 1 ml of PBS and were stored at −20 °C.

2.3 Indirect competitive ELISA

ELISA microtiter plates were coated with the coating conjugates (appropriately diluted in the carbonate–bicarbonate buffer; 100 μl per well) and incubated at 4 °C overnight. The following day, the plates were washed with the PBS-Tw (4 times, 350 μl per well). The suitable standard (the concentration range of 0–500 ng mL^{-1} in the PBS; 50 μl per well) was added into microtiter plates followed by the solution of appropriate antiserum (diluted in the PBS-0.1% BSA (w/v); 50 μl per well) and incubated at room temperature for 1 hour. Microtiter plates were washed again (4 times, 350 μl per well) and GAR-Po (diluted 1 : 10 000 in the PBS-Tw; 100 μl per well) was added and incubated at room temperature for 1 h. After the washing step, the TMB substrate solution was added (100 μl per well) and incubated at room temperature for 10 min. The enzyme reaction was stopped by addition of 2 mol L^{-1} H_2SO_4 (50 μl per well) and the absorbance was measured at 450 nm.

2.4 Calibration standard curve

Sigmoid calibration standard curves were obtained by plotting the mean values of absorbance against the logarithm of standard concentrations through a four-parameter logistic equation as described previously.[11] The limit of detection (LOD) was defined as the concentration of an analyte corresponding to the maximum assay signal minus 3× standard deviation (SD) in accordance with the calibration curve (the blank was calculated from 3 parallel determinations with the absence of an analyte). The IC_{50} corresponded to the concentration of analyte giving 50% inhibition of the asymptotic maximum. The linear working range corresponded to the analyte concentration causing the 20–80% inhibition of the maximum assay signal.

2.5 Specificity of antibodies

To verify the ability of the antibodies to react with similar epitopes on different tryptamines, the cross-reactivity (CR) tests were performed. The percent CR (CR (%)) was calculated from IC_{50} obtained from the calibration curves: (IC_{50} of target drug)/(IC_{50} of tested compound) × 100.

3. Results and discussion

To design the structure of haptens, DMT, 5-MeO-DMT and DiPT were selected as target analytes and their structures were modified with a short alkyl chain ended with a carboxylic group. Four different haptens were prepared, differing in a position of

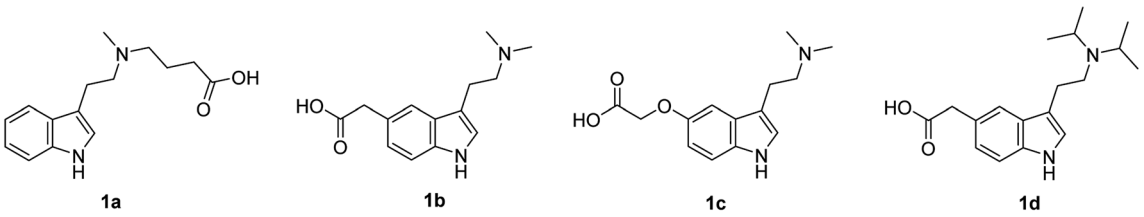


Fig. 1 Designed haptens I–IV.

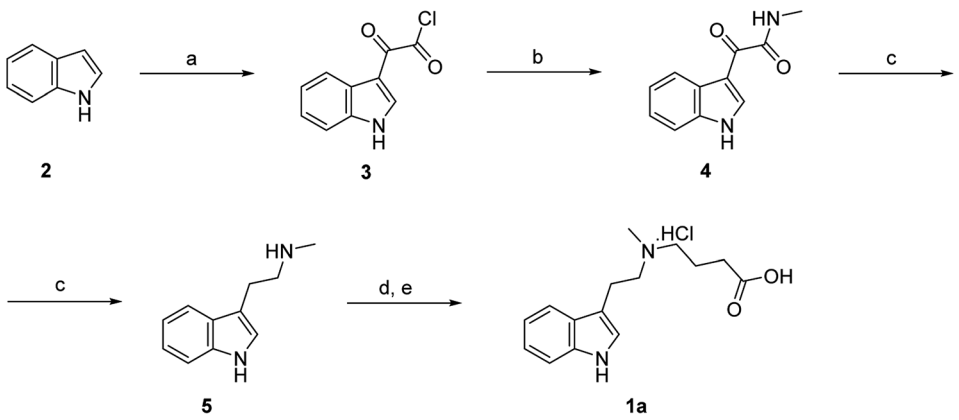
the linker (Fig. 1). Hapten I (**1a**) with the linker on the amino group was derived from DMT. Haptens II–IV (**1b–d**) with the linker in position 5 of indole ring were derived from 5-MeO-DMT (haptens II and III) and DiPT, respectively (hapten IV).

Synthesis of hapten I (**1a**) started from indole (**2**) and used a sequence of reactions developed by Speeter and Anthony to obtain *N*-methyltryptamine (**5**).[9,12] Alkylation of **5** with ethyl-4-bromobutyrate and subsequent hydrolysis of ester **6** yielded hapten I (**1a**) (Fig. 2).

We used an approach based on the Fischer reaction for the synthesis of haptens II–IV (**1b–d**). Haptens were obtained directly from suitable arylhydrazines and amino acetals (Fig. 3). Arylhydrazinium chlorides **7a,b** were prepared in high yields from aniline derivatives **8a,b** by diazotation and subsequent reduction of diazonium salts with $SnCl_2 \cdot 2H_2O$.[13]

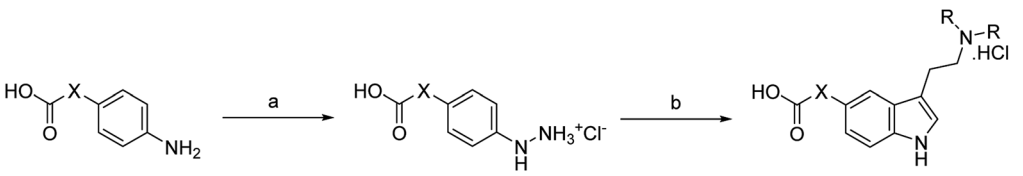
As an acetal component of the Fischer reaction, we used *N*,*N*-dimethylamino-1,1-dimethoxybutane (**9**) and *N*,*N*-diisopropylamino-1,1-dimethoxybutane (**10**). Compound **9** was prepared in two steps from ethyl-4-chlorobutyrate (**11**) (Fig. 4).

Because the same sequence of reactions could not be used in the synthesis of acetal **10**, we developed a different approach starting from succinic anhydride (**13**) (Fig. 5). Reaction of **13** with diisopropylamine and subsequent reduction of acid **14** with $LiAlH_4$ led to the amino alcohol **15**. Swern conditions were



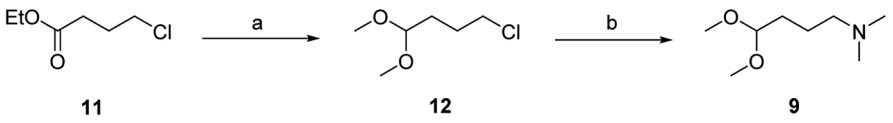
Reagents and conditions: (a) $(COCl)_2$, THF, 0 °C (93 %); **(b)** CH_3NH_2, H_2O, r.t. (81 %); **(c)** $LiAlH_4$, THF, reflux (42 %); **(d)** DIPEA, ethyl-4-bromobutyrate, NaI, *i*PrOH, 60 °C (65 %); **(e)** (i) NaOH, H_2O, r.t., (ii) HCl, H_2O, r.t. (63 %)

Fig. 2 Synthesis of hapten I.



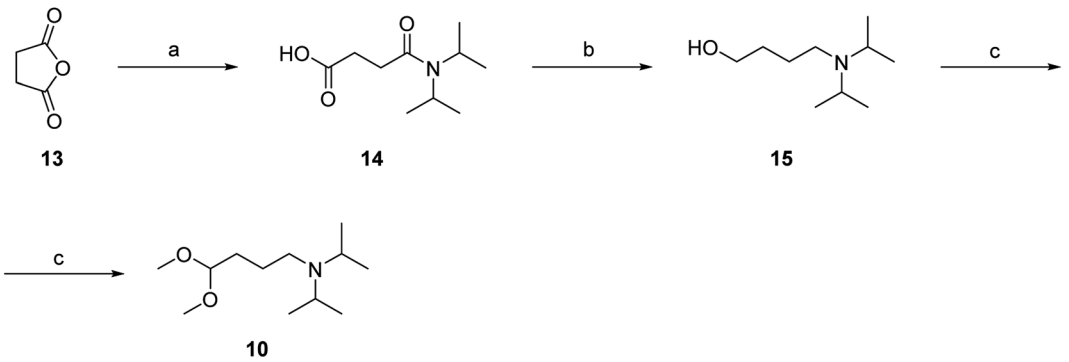
Reagents and conditions: (a) (i) $NaNO_2$, conc. HCl, -10 °C; (ii) $SnCl_2.2H_2O$, conc. HCl, -20 °C; **(b)** (i) **9 or 10**, 4% H_2SO_4, 80 °C; (ii) neutralization, then 1M HCl

Fig. 3 Direct synthesis of haptens II–IV using Fischer reaction.



Reagents and conditions: (a) (i) DIBAL-H, CH_2Cl_2, -78 °C; (ii) CH_3OH, cat. H_2SO_4, rt. (78 %); **(b)** 40 wt.% aqueous dimethylamine, r.t. (84 %)

Fig. 4 Preparation of *N,N*-dimethylamino-1,1-dimethoxybutane (**9**).



Reagents and conditions: (a) iPr_2NH, CH_2Cl_2, rt. (82 %); **(b)** $LiAlH_4$, THF, reflux (71 %); **(c)** (i) DMSO, $(COCl)_2$, Et_3N, CH_2Cl_2, -55 °C to r.t.; (ii) CH_3OH, cat. H_2SO_4, r.t. (54 %)

Fig. 5 Synthesis of *N,N*-diisopropylamino-1,1-dimethoxybutane (**10**).

used for oxidation of **15** to aldehyde, which was immediately acetalized to obtain **10** in moderate yield.

Haptens I–IV (**1a–d**) were conjugated with bovine serum albumin (BSA) using the methodology previously employed in our group.[10,14] Conjugates I–IV were submitted to MALDI-TOF analysis and the number of hapten molecules was determined as follows: 13 for conjugate I, 31 for conjugate II, 28 for conjugate III and 37 for conjugate IV.

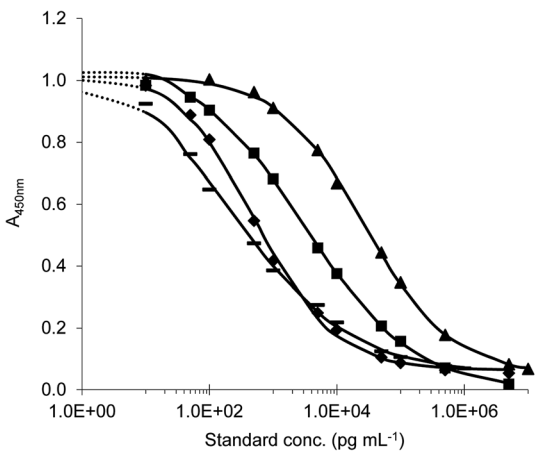


Fig. 6 Typical ELISA standard curves (◆) ELISA I (standard DMT); (▲) ELISA II (standard 5-MeO-DMT); (▬) ELISA III (standard 5-MeO-DMT); (■) ELISA IV (standard DiPT).

Antisera were collected by immunization of rabbits with all of prepared conjugates.[11] The indirect competitive format of ELISA was used for antiserum testing. First, checkerboard titrations were performed and suitable immunoreagent concentrations were determined when the maximum absorbance ranged from 1.2 to 1.9. Then the calibration curves with target analyte were constructed. The antibody with the highest sensitivity to the appropriate analyte was selected (on the basis of the lowest IC_{50} values) for each immunisation conjugate and further tests were made. In this study, four different ELISAs (ELISA I–IV) for the detection of tryptamine-based drugs were successfully developed. Titration standard curves are shown in Fig. 6 and analytical parameters of methods are summarized in the table (Table 1).

The specificity of obtained antibodies was evaluated with 102 new psychoactive substances and non-hallucinogenic compounds: 11 synthetic or natural tryptamines, 18 phenylethylamines, 13 piperazines, 18 synthetic cannabinoids, 27 cathinones, 16 non-hallucinogenic substance. Cross-reactivity values for tryptamines are summarized in Table 2. Positive cross-reactivity values were obtained only for synthetic tryptamines and psilocin. No cross-reactivity was observed with other tested compounds. Our results show that synthesized haptens and immunogens are functional and that they can be used for the production of antibodies. These antibodies can be applied in the development of immunoanalytical methods for the tryptamine-based drug detection.

Table 1 Analytical parameters of assembled ELISAs

	IC_{50}[a] ± SD[b] (ng ml^{-1})	Linear working range (ng ml^{-1})	LOD[c] ± SD[b] (ng ml^{-1})
ELISA I	0.52 ± 0.17	0.11–4.24	0.017 ± 0.005
ELISA II	27.3 ± 5.20	4.08–241.96	0.254 ± 0.059
ELISA III	0.29 ± 0.07	0.03–4.93	0.006 ± 0.002
ELISA IV	3.36 ± 0.28	0.27–45.13	0.034 ± 0.005

[a] IC_{50}, 50% intercept. [b] SD, standard deviation. [c] LOD, limit of detection.

Table 2 Cross-reactivity data for developed ELISA[a]

	Cross-reactivity (%)			
Compound	ELISA I	ELISA II	ELISA III	ELISA IV
DMT	100.00	53.48	38.89	0.09
5-MeO-DMT	0.57	100.00	100.00	0.04
5-MeO-DALT	1.90	0.95	2.46	0.19
DiPT	2.00	0.09	0.21	100.00
4-HO-MET	2.16	5.87	20.08	0.05
αMT	0.14	0.07	0.04	<0.001
O-Acetylpsilocin	0.06	2.81	23.04	<0.001
Psilocin	3.60	225.71	149.63	0.10
Psilocybin	<0.001	0.07	0.08	<0.001
Serotonin	<0.001	<0.001	<0.001	<0.001
5-MeO-DiPT	0.01	<0.001	0.43	88.43

[a] DMT: *N*,*N*-dimethyltryptamine, 5-MeO-DMT: 5-methoxy-*N*,*N*-dimethyltryptamine, 5-MeO-DALT: *N*,*N*-diallyl-5-methoxytryptamine, DiPT: *N*,*N*-diisopropyltryptamine, 4-HO-MET: 4-hydroxy-*N*-methyl-*N*-ethyltryptamine, αMT: α-methyltryptamine, 5-MeO-DiPT: 5-methoxy-*N*,*N*-diisopropyltryptamine.

4. Experimental

4.1 Synthesis of haptens

4.1.1 Hapten I

*2-(1H-indol-3-yl)-2-oxoacetyl chloride (**3**).* Compound **3** was prepared according to literature[9] from indole (**2**) (2.00 g, 17.1 mmol) as a yellow solid (3.31 g, 93%).

*2-(1H-indol-3-yl)-N-methyl-2-oxoacetamide (**4**).* Glyoxylyl chloride **3** (3.21 g, 15.5 mmol) was added in portions to an ice cold aqueous solution of methylamine (20 ml, 40 wt%) and resulting suspension was stirred at 0 °C for 2 hours. The precipitate was filtered and washed with water and diethyl ether. Recrystallization of the crude product from THF/Et_2O gave the titled compound **4** as a pale yellow solid (2.53 g, 81%). Mp = 214–215 °C (lit.[15] 218–219 °C); ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.75 (d, 3H, J = 4.7 Hz), 7.22–7.30 (m, 2H), 7.50–7.57 (m, 1H), 8.19–8.26 (m, 1H), 8.68 (m br, 1H), 8.77 (s, 1H), 12.21 (s br, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 25.57, 112.19, 112.56, 121.33, 122.53, 123.42, 126.25, 136.27, 138.54, 164.09, 182.08; HRMS (ESI): m/z $[M + Na]^+$ calculated for $C_{11}H_{10}N_2O_2$: 225.06345, found 225.06329.

*N-methyltryptamine (**5**).* The solution of amide **4** (2.00 g, 9.9 mmol) in dry THF (180 ml) was added dropwise to an ice-cold solution of $LiAlH_4$ (3.79 g, 100 mmol) in dry THF (180 ml) under argon atmosphere. After complete addition, the reaction mixture was refluxed for 7 hours. Then the mixture was cooled with an ice bath and decomposed according to Fieser workup. Precipitated salts were filtered of, washed several times with THF and the filtrate was evaporated. The residue was dissolved in dichloromethane (200 ml), washed with water (3 × 200 ml) and brine (150 ml), and the organic layer was dried with $MgSO_4$. Purification of the crude product by column chromatography (CH_2Cl_2 : MeOH, 5 : 1) afforded *N*-methyltryptamine (**5**) as an off-white solid (717 mg, 42%). Mp = 62–64 °C (lit.[16] 81–83 °C); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.44 (s, 3H), 2.88–3.02 (m, 4H), 7.04 (d, 1H, J = 2.3 Hz), 7.09–7.15 (m, 1H), 7.17–7.23 (m, 1H), 7.35–7.39 (m, 1H), 7.62–7.66 (m, 1H), 8.06 (s br, 1H).

*Ethyl 4-[N-[2-(1H-indol-3-yl)ethyl]-N-methyl]aminobutanoate (**6**).* To a mixture of *N*-methyltryptamine (**5**) (523 mg, 3.0 mmol) and NaI (450 mg, 3.0 mmol) in isopropyl alcohol (15 ml) was added DIPEA (620 mg, 835 μL, 4.8 mmol) and ethyl-4-bromobutyrate (878 mg, 645 μL, 4.5 mmol) and resulting solution was heated to 60 °C overnight. Solvent was removed on rotavap. The residue was dissolved in dichloromethane (75 ml) and washed with saturated $NaHCO_3$ solution (75 ml), water (75 ml) and brine (75 ml). Organic phase was dried with $MgSO_4$ and evaporated. Purification by column chromatography (CH_2Cl_2 : MeOH, 10 : 1) afforded the titled compound **5** as a light-brown viscous oil (570 mg, 65%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.25 (t, 3H, J = 7.3 Hz), 1.86 (qui, 2H, J = 7.2 Hz), 2.32–2.41 (m, 5H), 2.52 (t, 2H, J = 7.2 Hz), 2.72–2.81 (m, 2H), 2.92–3.01 (m, 2H), 4.13 (q, 2H, J = 7.3 Hz), 7.03 (d, 1H, J = 2.3 Hz), 7.08–7.14 (m, 1H), 7.15–7.22 (m, 1H), 7.33–7.38 (m, 1H), 7.58–7.63 (m, 1H), 8.09 (s br, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 14.20, 22.29, 22.89, 32.07, 41.92, 56.56, 58.05, 60.32, 111.11, 114.02, 118.69, 119.15, 121.56, 121.87, 127.38, 136.20, 173.55; HRMS (ESI): m/z $[M + H]^+$ calculated for $C_{17}H_{24}N_2O_2$: 289.19105, found 289.19125.

*4-[N-[2-(1H-indol-3-yl)ethyl]-N-methyl]aminobutanoic acid hydrochloride (**1a**, hapten I).* To a mixture of ester **5** (433 mg, 1.5 mmol) in 40% aqueous ethanol (20 ml) was added NaOH (66 mg, 1.65 mmol) and the solution was stirred at r.t. overnight. Then the solution was acidified with 1 M aqueous HCl to pH = 1, evaporated and the residue was purified by reverse-phase flash chromatography (MeOH : H_2O, gradient 5–100% MeOH) to obtain the titled compound **1a** (hapten I) as a white foam (246 mg, 63%). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.83–1,96 (m, 2H), 2.35 (t, 2H, J = 7.3 Hz), 2.80 (s, 3H), 3.06–3.20 (m, 4H), 3.21–3.31 (m, 2H), 6.97–7.04 (m, 1H), 7.06–7.13 (m, 1H), 7.25 (d, 1H, J = 2.3 Hz), 7.37 (d, 1H, J = 8.2 Hz), 7.63 (d, 1H, J = 7.6 Hz), 11.00 (s br, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 19.0, 19.8, 30.8, 39.3, 54.1, 55.2, 109.2, 111.5, 118.3, 118.5, 121.2, 123.2, 126.7, 136.2,

173.6; HRMS (ESI): m/z $[M + H]^+$ calculated for $C_{15}H_{20}N_2O_2$: 261.15975, found 261.15979.

4.1.2 Haptens II–IV

*Ethyl 2-[4-[(tert-butoxycarbonyl)amino]phenoxy]acetate (**16**).* Compound **16** was prepared according to literature[17] in two steps from 4-aminophenol (**17**) (10.09 g, 100.0 mmol) as a white solid (11.29 g, 88% (2 steps)). Mp = 53–55 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.27 (t, 3H, J = 7.2 Hz), 1.48 (s, 9H), 4.26 (q, 2H, J = 7.2 Hz), 4.56 (s, 2H) 6.55 (s br, 1H), 6.78–6.85 (m, 2H), 7.26 (d, 2H, J = 8.8 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 14.1, 28.3, 61.3, 65.9, 80.2, 115.1, 120.3, 132.4, 153.0, 153.6, 169.0; HRMS (ESI): m/z $[M + Na]^+$ calculated for $C_{15}H_{21}NO_5$: 318.13119, found 318.13149.

*2-(4-aminophenoxy)acetic acid (**8b**).* Suspension of **16** (5.91 g, 20.0 mmol) in 1 M hydrochloric acid (50 ml) was stirred at 60 °C overnight. After cooling to r.t., the solution was extracted with dichloromethane (50 ml) and phases separated. Aqueous phase was treated with solid Na_2CO_3 to adjust pH to 4–5 (according to pH paper). The resulting precipitate was filtered off and dried, yielding the titled compound **8b** as an off-white solid (3.03 g, 91%). Mp = 200–215 °C (lit.[18] 200–210 °C); ^{1}H NMR (300 MHz, DMSO-d_6) δ: 4.46 (s, 2H), 6.45–6.52 (m, 2H), 6.58–6.65 (m, 2H).

General procedure A: preparation of arylhydrazinium chlorides. A procedure from the literature[19] was modified as follows: suspension of aniline acid **8a,b** (1 eq.) in concentrated hydrochloric acid (3 ml mmol^{-1}) was cooled to −5 °C and a solution of sodium nitrite (1.05 eq.) in water (0.5 ml mmol^{-1}) was added dropwise. After complete addition the mixture was stirred for 1 hour at −5 °C and then it was added dropwise to a solution of $SnCl_2 \cdot 2H_2O$ (3 eq.) in concentrated hydrochloric acid (1 ml mmol^{-1}) cooled to −20 °C. After complete addition, the mixture was stirred for additional 2.5 hours at −20 °C and then the suspension was filtered. Solids were washed with cold ethanol and diethyl ether and dried, yielding arylhydrazinium chlorides **7a,b**.

*4-(carboxymethyl)phenylhydrazinium chloride (**7a**).* Was prepared according to general procedure A from aniline **8a** (2.27 g, 15.0 mmol) as a white solid (2.86 g, 94%). Mp = 207–210 °C (lit.[20] 228–229 °C); ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.48 (s, 2H), 6.92 (d, 2H, J = 8.5 Hz), 7.16 (d, 2H, J = 8.5 Hz), 8.20 (s br, 1H), 10.27 (s br, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 39.9, 114.6, 128.1, 129.9, 144.3, 173.0; HRMS (ESI): m/z $[M]^+$ calculated for $C_8H_{11}N_2O_2$: 167.08150, found 167.08124.

*4-(carboxymethoxy)phenylhydrazinium chloride (**7b**).* Was prepared according to general procedure from aniline **8b** (836 mg, 5.0 mmol) as a white solid (937 mg, 86%). Mp = 135 °C (decomp.); ^{1}H NMR (300 MHz, DMSO-d_6) δ: 4.60 (s, 2H), 6.82–6.89 (m, 2H), 6.95–7.02 (m, 2H), 10.63 (s br, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 64.9; 115.0, 116.9, 139.5, 153.1, 170.3; HRMS (ESI): m/z $[M - NH_3]^+$ calculated for $C_8H_8NO_3$: 166.04987, found 166.04977.

*4-Chloro-1,1-dimethoxybutane (**12**).* DIBAL-H (94 ml, 1 M solution in hexane, 94.0 mmol) was added dropwise to a solution of ethyl-4-chlorobutyrate (**11**) (12.30 g, 81.7 mmol) in dry dichloromethane (125 ml) cooled to −78 °C. After addition, the reaction mixture was stirred for half hour at −78 °C and then poured into ice-cold 10% hydrochloric acid (160 ml). Resulting mixture was stirred at 0 °C for 1 hour, then the layers were separated and the aqueous one extracted with dichloromethane (2 × 100 ml). Combined organic layers were washed with brine (300 ml) and dried with $MgSO_4$. After solvent removal, the residue was dissolved in methanol (35 ml) acidified with few drops of concentrated sulfuric acid. The solution was stirred for 18 hours, diluted with dichloromethane (125 ml), washed with 10% aqueous $NaHCO_3$ (100 ml), water (100 ml) and brine (100 ml) and dried with MgSO4. Distillation under reduced pressure gave the titled acetal **12** as a colorless liquid (9.76 g, 78%). Bp = 53–55 °C (5 torr) (lit.[21] 84–86 °C (25 torr)); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.70–1.90 (m, 4H), 3.32 (s, 6H), 3.56 (t, 2H, J = 6.3 Hz), 4.39 (t, 1H, J = 5.4 Hz).

*4-(N,N-dimethylamino)-1,1-dimethoxybutane (**9**).* Compound 9 was prepared according to literature[13] from acetal **11** (4.86 g, 31.8 mmol) as a colorless liquid (4.32 g, 84%) after distillation under reduced pressure. Bp = 59–60 °C (6 torr) (lit.[13] 40 °C (1 torr)).

*4-(N,N-diisopropylamino)-4-oxobutanoic acid (**14**).* To a solution of succinic anhydride (**13**) (10.07 g, 100.0 mmol) in dichloromethane (300 ml) was added diisopropyl amine (21.8 ml, 300.0 mmol) and resulting solution was stirred for 20 hours. Then the mixture was concentrated and 1 M hydrochloric acid (250 ml) was added to the residue. Aqueous layer was extracted with dichloromethane (3 × 150 ml) and combined organic layers were dried with MgSO4. Solvent removal offered the titled compound **14** as a light brown viscous oil (16.50 g, 82%), which solidified upon standing in a fridge. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.16 (d, 6H, J = 6.7 Hz), 1.29 (d, 6H, J = 7.0 Hz), 2.56–2.64 (m, 4H), 3.46 (m br, 1H), 3.95 (sept, 1H, J = 6.7 Hz), 11.02 (s br, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 20.3, 20.6, 29.5, 29.8, 45.9, 48.4, 170.7, 176.7.

*4-(N,N-diisopropylamino)butan-1-ol (**15**).* Suspension of $LiAlH_4$ (6.83 g, 180.0 mmol) in dry THF (300 ml) was cooled with ice bath and then the solution of amide **14** (8.05 g, 40.0 mmol) in dry THF (150 ml) was added dropwise. After complete addition, the reaction mixture was refluxed for 5 hours. Then it was cooled with an ice bath and decomposed according to Fieser workup. Solids were removed by filtration, washed with THF and the filtrate was evaporated. The residue was dissolved in dichloromethane (250 ml), washed with water (3 × 200 ml) and brine (150 ml) and dried with $MgSO_4$. Distillation under reduced pressure gave the titled amino alcohol **15** as a colorless liquid (4.91 g, 71%). Bp= 65–68 °C (0.43 torr); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.04 (d, 12H, J = 6.7 Hz), 1.58–1.71 (m, 4H), 2.43–2.52 (m, 2H), 3.10 (sept, 2H, J = 6.7 Hz), 3.48–3.58 (m, 2H), 6.06 (s br, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 20.0, 27.6, 32.4, 44.9, 47.5, 62.7.

*4-(N,N-diisopropylamino)-1,1-dimethoxybutane (**10**).* Dimethyl sulfoxide (2.56 ml, 36.0 mmol) was added dropwise to a solution of oxalyl chloride (2.57 ml, 30.0 mmol) in dry dichloromethane (60 ml) cooled to −55 °C and the mixture was stirred for additional 10 minutes. Then the solution of alcohol **15** (2.60 g, 15 mmol) in dry dichloromethane (10 ml) was added and the mixture was stirred at −55 °C for additional 15 minutes. Then trimethylamine (8.9 ml, 60.0 mmol) was added and the mixture was allowed to warm to r.t. (1 hour). Reaction mixture was then

poured into water (130 ml), phases were separated and the aqueous one extracted with dichloromethane (2 × 100 ml). Combined organic phases were dried with $MgSO_4$ and evaporated. The residue was dissolved in methanol (50 ml), acidified with concentrated sulfuric acid (2.5 ml) and the mixture was stirred at r.t. for 18 hours. Then the mixture was diluted with dichloromethane (150 ml), cooled with ice bath and 20% NaOH solution was added (100 ml). Phases were separated, the aqueous one was diluted with water (50 ml) and extracted with dichloromethane (2 × 75 ml). Combined organic phases were washed with brine (200 ml) and dried with $MgSO_4$. Distillation under reduced pressure gave the titled amino acetal **10** as colorless liquid (1.74 g, 54%). Bp = 51–55 °C (0.24 torr); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.98 (d, 12H, J = 6.6 Hz), 1.36–1.51 (m, 2H), 1.53–1.63 (m, 2H), 2.39 (t, 2H, J = 7.3 Hz), 2.99 (sept, 2H, J = 6.5 Hz), 3.31 (s, 6H), 4.37 (t, 1H, J = 5.9 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 20.7, 26.1, 30.3, 44.7, 48.2, 52.6, 104.7.

General procedure B: preparation of haptens II–IV. A procedure from the literature[13] was modified as follows: to a 4% sulfuric acid, which was first heated to 50 °C and bubbled with argon, arylhydrazinium chloride **7a,b** (1 eq.) and then amino acetal **9** or **10** (1.2 eq.) were added and the resulting mixture was heated to 80 °C for 3.5 hours. After cooling to r.t., the mixture was neutralized with concentrated ammonia solution. Water was removed under reduced pressure and the residue was treated with ethanol (10 ml mmol^{-1}) and filtered to remove most of the inorganic salts. Filtrate was evaporated, 1 M hydrochloric acid (10 ml mmol^{-1}) was added and resulting solution was evaporated again. Purification of crude product by reverse-phase flash chromatography (water/methanol, gradient 5–100% of methanol) gave haptens **1b–d** (haptens II–IV).

*2-[3-[2-(N,N-dimethylamino)ethyl]-1H-indol-5-yl]acetic acid hydrochloride (**1b**, hapten II).* Was prepared according to general procedure B from arylhydrazinium chloride **7a** (608 mg, 3.0 mmol) and acetal **9** (580 mg, 3.6 mmol) as a colorless glassy solid (475 mg, 56%). ^{1}H NMR (300 MHz, CD_3OD) δ: 2.87 (s, 6H), 3.11–3.21 (m, 2H), 3.30–3.39 (m, 2H), 3.66 (s, 2H), 7.07 (dd, 1H, J_1 = 8.5 Hz, J_2 = 1.5 Hz), 7.15 (s, 1H), 7.30 (d, 1H, J = 8.5 Hz), 7.51 (s, 1H); ^{13}C NMR (75 MHz, D_2O) δ: 20.0, 41.4, 42.6, 57.5, 108.3, 112.2, 118.7, 123.6, 124.6, 125.6, 126.7, 135.5, 178.3; HRMS (ESI): m/z $[M]^+$ calculated for $C_{14}H_{18}N_2O_2$: 247.14410, found 247.14413.

*2-[3-[2-(N,N-dimethylamino)ethyl]-1H-indol-5-yloxy]acetic acid hydrochloride (**1c**, hapten III).* Was prepared according to general procedure B from arylhydrazinium chloride **7b** (328 mg, 1.5 mmol) and acetal **9** (290 mg, 1.8 mmol) as a colorless glassy solid (238 mg, 53%). ^{1}H NMR (300 MHz, D_2O) δ: 2.72 (s, 6H), 2.90–3.00 (m, 2H), 3.08–3.18 (m, 2H), 4.26 (s, 2H), 6.73 (dd, 1H, J_1 = 8.8 Hz, J_2 = 2.1 Hz), 6.99 (d, 1H, J = 2.1 Hz), 7.10 (s, 1H), 7.21 (d, 1H, J = 8.8 Hz); ^{13}C NMR (75 MHz, D_2O) δ: 20.1, 42.7, 57.4, 67.7, 100.9, 108.2, 112.3, 112.9, 124.8, 126.6, 131.7, 152.0, 177.2; HRMS (ESI): m/z $[M]^+$ calculated for $C_{14}H_{18}N_2O_3$: 263.13902, found 263.13912.

*2-[3-[2-(N,N-diisopropylamino)ethyl]-1H-indol-5-yl]acetic acid hydrochloride (**1d**, hapten IV).* Was prepared according to general procedure B from arylhydrazinium chloride **7a** (203 mg, 1.0 mmol) and acetal **10** (262 mg, 1.2 mmol) as a white solid (207 mg, 61%). ^{1}H NMR (300 MHz, CD_3OD) δ: 1.26 (d, 12H, J = 6.5 Hz), 2.83–3.04 (m, 4H), 3.48 (sept, 2H, J = 6.5 Hz), 3.57 (s, 2H), 6.93 (s, 1H), 7.11 (dd, 1H, J_1 = 8.5 Hz, J_2 = 1.5 Hz), 7.24 (d, 1H, J = 8.5 Hz), 7.38 (s, 1H); ^{13}C NMR (75 MHz, CD_3OD) δ: 18.1, 24.9, 46.6, 49.2, 55.9, 110.2, 112.3, 119.1, 124.4, 124.5, 128.1, 129.8, 136.7, 180.9.

5. Conclusion

We successfully used haptens with novel structures to produce polyclonal antibodies against various tryptamines. The constructed ELISAs have low detection limits. Some of the antibodies show good reactivity not only with the target analytes, but also with psilocin and 5-MeO-DiPT. Although the antibodies have not yet been characterized in complex matrices, they appear to be suitable for the development of immunochemical assay kits. In our next work, we will focus on the establishment of an ELISA for the detection of tryptamines in human body fluids. We believe that the outcome of our work could lead to LFIA kits designed for the on-site testing of NPS users.

Conflicts of interest

None.

Acknowledgements

This work was supported by the Ministry of Interior of the Czech Republic (projects VG20122015075 and VI20172020056) and co-funded with financial support from specific university research (MSMT No. 20/2015, MSMT No. 20-SVV/2016 and MSMT No. 20-SVV/2017).

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