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Studijní program: Analytická chemie

Mgr. Hana Adamusová

Využití vysokoúčinných separačních metod pro analýzy biologicky aktivních látek

Analysis of biologically active compounds using high performance separation methods

Disertační práce

Vedoucí disertační práce: Doc. RNDr. Zuzana Bosáková, CSc.

Konzultantka: RNDr. Kateřina Hortová, Ph.D.

Praha, 2017

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 17. 7. 2017

Podpis

Tato disertační práce vznikla na základě výsledků získaných v letech 2011 až 2017 během mého doktorského studia na Katedře analytické chemie Přírodovědecké fakulty Univerzity Karlovy v Praze, Hlavova 2030, 128 43, Praha 2 a za mého působení ve společnosti Zentiva, k.s., U kabelovny 130, 102 37, Praha 10 na pozicích Ph.D. Cont a Vývojový analytik junior.

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Tato disertační práce je založena na následujících vědeckých pracích, které byly publikovány v mezinárodních impaktovaných časopisech. Práce jsou seřazeny tak, jak jsou zmiňovány a komentovány v disertační práci.

I. Bosáková, Z.; Tockstein, A.; **Adamusová, H.**; Coufal, P.; Šebková, N.; Dvořáková-Hortová, K.: *Kinetic analysis of decreased sperm fertilizing ability by fluorides and fluoroaluminates: a tool for analyzing the effect of environmental substances on biological events*; Eur Biophys J Biophys Lett. 45 (1) (2016) 71-79.

II. **Adamusová, H.**; Bosáková, Z.; Kozlík. P.; Hortová, K.: *Determination of 17β-estradiol and 17α-ethynylestradiol in mouse fertilizing M2 medium*; In: Proceedings, 9th International Students Conference „Modern Analytical Chemistry“: Praha 2013, p. 26-27, ISBN 978-80-7444-023-6.

III. Bosáková, Z.; **Adamusová, H.**; Bosáková, T.; Tockstein, A.; Šebková, N.; Dvořáková-Hortová, K.: *17β-estradiol signalling is directed by autocatalytic reaction in capacitating sperm*; bude zasláno, červenec 2017 do Scientific Reports.

IV. **Adamusová, H.**; Bosáková, Z.; Coufal, P.; Pacáková, V.: *Analysis of estrogens and estrogen mimics in edible matrices-A review*; J Sep Sci. 37 (8) (2014) 885-905.

V. **Adamusová, H.**; Novotná, N.; Bosáková, Z.; Douša, M.: *Enantiomeric Separation of (R,S)-Aclidinium Bromide with Negatively Charged Gamma-Cyclodextrin by CE*; Chromatographia 80 (4) 2017 559-563.

VI. Douša, M.; Lehnert, P.; **Adamusová, H.**; Bosáková, Z.*: Fundamental study of enantioselective HPLC separation of tapentadol enantiomers using cellulose-based chiral stationary phase in normal phase mode*; J Pharm Biomed Anal. 74 (2013) 111-116.

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1. **Adamusová, H.**; Bosáková, Z.; Kozlík. P.; Hortová, K.: *Determination of 17β-estradiol and 17α-ethynylestradiol in mouse fertilizing M2 medium*; In: Proceedings, 9th International Students Conference „Modern Analytical Chemistry“: Praha 2013, p. 26-27, ISBN 978-80-7444-023-6. (80 %)

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3. **Adamusová, H.**; Bosáková, Z.; Coufal, P.; Pacáková, V.: *Analysis of estrogens and estrogen mimics in edible matrices-A review*; J Sep Sci. 37 (8) (2014) 885-905. (80 %)

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Doc. RNDr. Zuzana Bosáková, CSc.

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Michal Douša, Ph.D.

Předmětová hesla: analytická chemie, separační metody, farmaceutická analýza

Klíčová slova: endokrinní disruptory, estrogenní polutanty, vysokoúčinná kapalinová chromatografie, tandemová hmotnostní spektrometrie, kapacitace spermií, kinetická analýza, kapilární elektroforéza, chirální separace, léčiva

Subject words: analytical chemistry, separation methods, pharmaceutical analysis

Key words: endocrine disrupting chemicals, estrogenic pollutants, high-performance liquid chromatography, tandem mass spectrometry, sperm capacitation, kinetic analysis, capillary electrophoresis, chiral separation, drugs

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Abstrakt

V rámci první části disertační práce byla vypracována nová analytická metoda HPLC-MS/MS pro sledování koncentračních změn 17β-estradiolu (βE2) během kapacitace myších spermií *in vitro*. Kapacitace byla prováděna se třemi počátečními koncentracemi βE2 (200, 20 a 2 μg/L). Pro všechny koncentrace byl pozorován obdobný trend závislosti koncentrace nevázaného βE2 na čase kapacitace. Koncentrace βE2 se snižovala, aby dosáhla svého minima a poté se opět zvyšovala. Pozice minima se lišila pro jednotlivé testované koncentrace βE2. Experimentálně získané výsledky byly podrobeny kinetické analýze. Křivky proložené experimentálně získanými body vykazovaly autokatalytický charakter. Pro shodu mezi křivkami, získanými proložením experimentálními body, a teoreticky vypočítanými křivkami je nutné předpokládat, že prvním krokem je adsorpce βE2 na povrch spermií řízená Langmuirovou izotermou.

Kinetická studie byla použita i pro studium působení fluoridů a fluorohlinitých komplexů na kapacitaci myších spermií. Bylo zjištěno, že teoreticky získané křivky vykazují velmi dobrou shodu s experimentálně získanými body, tento fakt potvrzuje mechanismus kinetiky kapacitace myších spermií.

Ve druhé části této disertační práce byly vyvinuty dvě analytické metody pro chirální separaci aclidinium bromidu (AB) a tapentadolu hydrochloridu (TAP).

Aclidinium bromid působí jako antagonista muskarinových receptorů pro úlevu od symptomů chronické obstrukční plicní nemoci. Separace umožňující stanovení 0,4 % (*S*)-AB v syntetickém vzorku AB pomocí kapilární elektroforézy bylo dosaženo za použití sulfatovaného γ-cyklodextrinu 4,8 % (*w/v*) v kyselém základním elektrolytu dihydrogenfosforečnanu draselného (100 mM, pH = 3,0) na nepokryté křemenné kapiláře s rozšířenou optickou dráhou. Metoda byla validována jako limitní test.

Tapentadol hydrochlorid je centrálně působící analgetikum pro léčbu středně těžké až těžké akutní bolesti nebo chronické bolesti. Molekula TAP má čtyři možné stereoizomery, ale pouze (*R,R)*-izomer je v současné době klinicky používanou formou. Enantioseparace TAP metodou HPLC s rozlišením větším než 2,5 pro všechny z enantiomerů bylo dosaženo při použití kolony Chiralpak AD-H s mobilní fází heptan/propan-2-ol/diethylamin (980:20:1, *v/v/v*). Vyvinutá metoda byla validována dle požadavků směrnice International Conference on Harmonisaton (ICH).

Abstract

In the first part of this doctoral thesis, a new analytical HPLC-MS/MS method for monitoring of concentration changes of 17β-estradiol (βE2) during *in vitro* mouse sperm capacitation was developed. Capacitation was performed for three initial concentrations of βE2 (200, 20 and 2 μg/L). For all the concentrations a similar trend for the total unbound βE2 was observed. In general, the βE2 concentration decreased to reach its minimum and then increased again. The position of the minimum differed for the individual tested βE2 concentrations. Experimentally obtained results were subjected to the kinetic analysis. The curves fitted through the experimentally determined points displayed an autocatalytic character. For the agreement between the curves obtained by fitting through the experimental points and the theoretical calculated curves, it is necessary to assume that the first step is adsorption of βE2 onto the surface of the sperm controlled by Langmuir isotherm.

The kinetic study was also used to study the effects of fluorides and aluminium fluoride complexes on the capacitation of mouse sperm. The experimental points were in very good agreement with the shape of the theoretical curves and this fact verifies the mechanism of the mouse sperm capacitation kinetics.

In the second part of this work, two analytical methods for chiral separation of aclidinium bromide (AB) and tapentadol hydrochloride (TAP) were developed.

Aclidinium bromide acts as a muscarinic receptor antagonist to relieve the symptoms of chronic obstructive pulmonary disease. The separation of 0.4% (*S*)-AB in a synthetic laboratory sample of AB by capillary electrophoresis was achieved using 4.8% (*w/v*) sulphated γ-cyclodextrin in an acidic background electrolyte based on potassium dihydrogen phosphate (100 mM, pH = 3.0) in an uncoated fused silica capillary with extended capillary light path. The method was validated as a limit test.

Tapentadol hydrochloride is a centrally acting analgesic for the treatment of moderate to severe acute pain or chronic pain. The TAP molecule has four possible stereoisomers, but only the (*R,R*)-isomer is currently a clinically used form. Enantioseparation of TAP by HPLC with resolution greater than 2.5 for all of the enantiomers was achieved using a Chiralpak AD-H column with heptane/propane-2-ol/diethylamine mobile phase (980: 20: 1, *v/v/v*). The developed method was validated according to the requirements of the International Conference on Harmonization (ICH).

OBSAH

Seznam zkratek a použitých symbolů

AB	aclidinium bromid
AD	tris(3,5-dimethylfenylkarbamát) amylózy
API	účinná látka
AR	akrozomální reakce
B	relativní koncentrace 17β-estradiolu
BEH	hybridní sorbent (bridged ethylene hybrid)
BPA	bisfenol A
BSA	hovězí sérový albumin (bovine serum albumin)
CD	cyklodextrin
CE	kapilární elektroforéza
cER	cytoplazmatický estrogenní receptor
CSPs	chirální stacionární fáze
DES	diethylstilbestrol
E1	estron
E3	estriol
EDC	endokrinní disruptory
EE2	17α-ethynylestradiol
ESI	ionizace elektrosprejem (electrospray ionization)
EtG	ethylglukuronid
FDA	úřad pro kontrolu potravin a léčiv
HEX	hexestrol
HSS	vysoce odolné silikagelové částice (high strenght silica)
CHOPN	chronická obstrukční plicní nemoc
ICH	International Conference of Harmonisation; mezinárodní směrnice pro regulaci nečistot v léčivech
ICH Q2 (R1)	požadavky a pokyny pro validace analytických metod
ICH Q3A	směrnice pro nečistoty v nových účinných látkách
ICH Q3B	směrnice pro nečistoty v nových léčivých přípravcích
ICH Q6A	testovací postupy a akceptační kritéria pro nové účinné látky a léčivé přípravky; pokyny pro stanovení optické čistoty
LIF	laserem indukovaná fluorescence

LOD	limit detekce
LOQ	limit kvantifikace
LP	léčivý přípravek
mER	membránový estrogenní receptor
MRM	záznam vybraných přechodů mezi prekurzorovými a produktovými ionty (multiple reaction monitoring)
NF	nonylfenol
NP	normální mód HPLC
OD	tris(3,5-dimethylfenylkarbamát) celulózy
OJ	tris(4-methylbenzoát) celulózy
OP	oktylfenol
pTyr	tyrozinová fosforylace
RP	reverzní mód HPLC
S	stupeň aktivity; poměr výchozí a aktuální koncentrace βE2
SBSE	sorpční extrakce na míchadélku (stir bar sorptive extraction)
SPE	extrakce tuhou fází (solid phase extraction)
TAP	tapentadol hydrochlorid
WDT	testování užívání drog a léčiv na pracovišti (workplace drug testing)
WHO	Světová zdravotnická organizace (World Health Organization)
αE2	17α-estradiol
βE2	17β-estradiol
$\beta E2_i$	17β-estradiol v cytoplazmě spermie (inside)

CÍLE PRÁCE

Tato disertační práce je rozdělena na dvě části.

Cílem první části, která se věnuje výzkumu působení vybraných látek s potenciálně negativním dopadem na fertilizační schopnost spermií, bylo zjistit, jak tyto látky mohou ovlivňovat průběh kapacitace myších spermií. Byl sledován vliv fluoridů a fluorohlinitých komplexů na průběh kapacitace myších spermií z hlediska kinetiky a mechanismu působení těchto látek. Pro studium vlivu estrogenního hormonu 17β-estradiolu na průběh kapacitace myších spermií *in vitro* byla vypracována nová analytická metoda vysokoúčinné kapalinové chromatografie s tandemovou hmotnostní detekcí a získané výsledky byly podrobeny kinetické analýze.

Cílem druhé části této práce, která se zabývá analýzou léčiv, byl vývoj analytických metod pro stanovení chirální čistoty nových, farmaceuticky účinných látek (API). Pro tento účel byly vybrány dvě API – tapentadol hydrochlorid a aclidinium bromid.

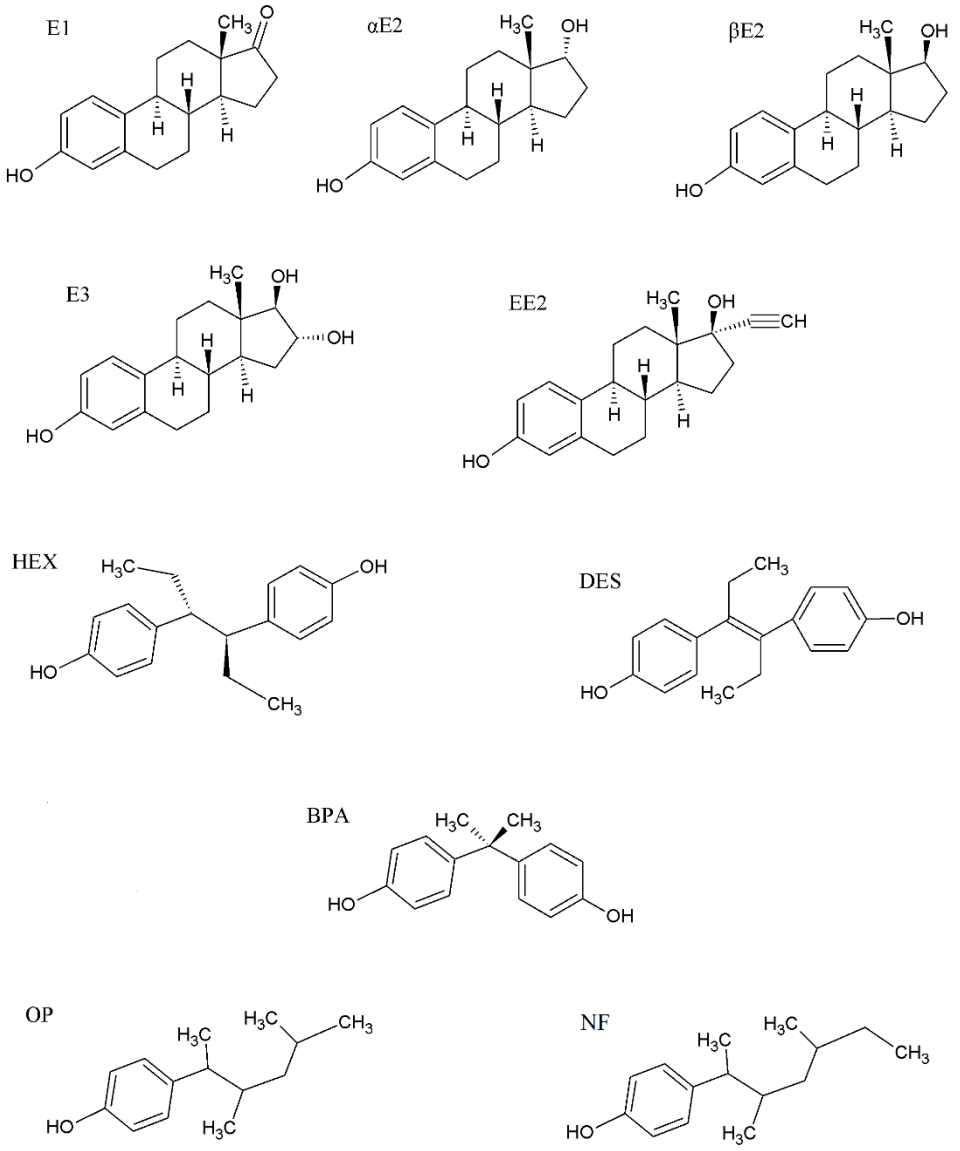
1 ENDOKRINNÍ DISRUPTORY

1.1 Teoretický úvod

První část této disertační práce se zabývá endokrinními disruptory (EDC), zejména estrogeny a látkami, které napodobují jejich chování. V důsledku nejrůznějších lidských aktivit dochází ke kontinuálnímu zvyšování znečištění životního prostředí polutanty a toto znečištění se stává stále komplexnějším. Důležitou skupinou polutantů jsou EDC. Endokrinní disruptory jsou exogenní látky, které mohou narušovat fyziologickou funkci endogenních hormonů a endokrinního systému, což ovlivňuje zdraví a reprodukci u zvířat a lidí. Mohou zasahovat do produkce, uvolňování, metabolismu a eliminace endogenních hormonů nebo mohou napodobovat jejich chování [1]. Endokrinní disruptory se dostávají do životního prostředí jako důsledek zvýšené produkce a konzumace řady léčiv a podpůrných látek v podobě odpadních produktů, které představují toxikologické reprodukční riziko již při velmi nízkých koncentracích [2]. Působení EDC se může týkat různých hormonálních systémů a řada pozorování, týkající se reprodukčního vývoje a pohlavní diferenciace, se zaměřila na zasahování EDC do funkce pohlavních steroidních hormonů [3]. Proto se přítomnost těchto látek studuje jak ve vzorcích ze životního prostředí, tak v potravinách a obalových materiálech.

1.1.1 Estrogeny a látky s estrogenní aktivitou

Mezi EDC se řadí široká škála látek zahrnujících jak endogenní hormony: estron (E1), 17α-estradiol (αE2), 17β-estradiol (βE2) nebo estriol (E3), tak syntetické estrogeny: mestranol, 17α-ethynylestradiol (EE2) a nesteroidní diethylstilbestrol (DES) a hexestrol (HEX) a jejich konjugáty. Mezi důležité EDC patří také aditivita, používaná v průmyslu zpracovávajícím plasty, které mohou působit podobně jako estrogeny, například bisfenol A (BPA), nonylfenol (NF) a oktylfenol (OP). Struktury vybraných EDC jsou ukázány na obrázku 1.1. Z hlediska původu lze endokrinní disruptory rozdělit do čtyř hlavních tříd, které zahrnují (a) farmaceutické výrobky (například steroidy včetně fytoestrogenů, antibiotika, analgetika, protizánětlivé léky a psychofarmaka); (b) výrobky pro osobní hygienu (např. detergenty, syntetické vůně a konzervační látky); (c) pesticidy (například insekticidy, fungicidy); a d) různé průmyslové chemické látky (např. organické sloučeniny kyslíku a polycyklické aromatické sloučeniny) [4].



Obr. 1.1 Struktury vybraných zástupců EDC: estron (E1), 17α-estradiol (αE2), 17β-estradiol (βE2), estriol (E3), 17α-ethynylestradiol (EE2), hexestrol (HEX), diethylstilbestrol (DES), bisfenol A (BPA), oktylfenol (OP) a nonylfenol (NF).

Estrogeny patří do skupiny steroidních látek. Jako prakticky všechny živočišné steroidní látky vznikají i estrogeny biotransformací cholesterolu. Přírodní steroidní estrogeny mají stejný molekulární základ tvořený cyklopentanoperhydrofenanthrenovým (steranovým) skeletem. Jednotlivé látky se liší funkčními skupinami v pozicích C16 a C17. Ze struktur přírodních estrogenů je patrné, že jsou to relativně hydrofobní látky, které nejsou příliš rozpustné ve vodě. Jejich

konjugáty (sulfáty nebo glukuronáty), vzniklé biotransformací, nejsou biologicky aktivní, ale jsou daleko více rozpustné ve vodě [5].

Endogenní estrogenní hormony (E1, αE2, βE2 a E3) jsou u samic syntetizovány převážně z androgenních prekurzorů ve vaječnících, v tukové tkáni a nadledvinách. Jsou to pohlavní hormony, které hrají důležitou roli v menstruačním a estrálním reprodukčním cyklu (označujícím periodické, fyziologické změny v těle samic placentálních savců). Jejich role je komplexní; jsou důležité pro fyziologický vývoj primárních i sekundárních pohlavních znaků, zajišťují reprodukci i řízení menopauzy, metabolismus tuků, syntézu bílkovin, ale ovlivňují i vývoj nemocí jako jsou karcinomy, neurodegenerativní změny nebo kardiovaskulární onemocnění [6, 7].

17β-Estradiol je nejaktivnější endogenní estrogen, regulující rozmnožování jak samic, tak samců, u kterých je syntetizován ve varlatech. Účastní se vývoje a dozrávání vajíček i spermií a jejich vzájemné komunikace před oplozením. Jeho nefyziologicky vysoké dávky však mohou působit zvýšení apoptózy (buněčné smrti) samčích zárodečných buněk a snížení počtu spermií [8, 9]. Estriol je estrogenní metabolit, který se vytváří a vylučuje během těhotenství [10]. 17α-Estradiol je slabým estrogenem, stereoizomerem βE2. Jeho účinnost je 2 krát až 200 krát nižší než účinnost βE2. Díky svým vlastnostem by mohl mít potenciál pro léčbu neurodegenerativních onemocnění (např. Alzheimerovy choroby) [11]. Estrogenem se slabším účinkem je i E1, který je převládajícím estrogenem u žen po menopauze, u kterých klesají hladiny βE2 [12].

17α-Ethynylestradiol byl vyvinut v roce 1938 jako první perorálně účinný estrogen na světě. Je to syntetický estrogenní hormon, odolný vůči jaternímu metabolismu. Jeho hlavní využití je v perorálních kontraceptivech [5, 13].

Diethylstilbestrol je syntetický nesteroidní estrogen, který byl používán k prevenci potratu a dalších komplikací v těhotenství od padesátých do sedmdesátých let 20. století ve Spojených státech a několika evropských zemích. Jeho používání bylo ve většině zemí zakázáno v 70. letech. V roce 1971 vydal Úřadu pro kontrolu potravin a léčiv (FDA) varování o použití DES během těhotenství po tom, co byl zjištěn vztah mezi expozicí tomuto syntetickému estrogenu a vývojem karcinomu vaginy a děložního čípku u mladých žen, jejichž matky užívaly v těhotenství DES. U mužských potomků žen, které v těhotenství užívaly DES, byl zjištěn zvýšený výskyt genitálních abnormalit

a zvýšené riziko karcinomu prostaty a varlat. Bylo prokázáno, že expozice DES *in utero* má karcinogenní, teratogenní a reprodukční účinky [14, 15].

Hexestrol je nesteroidní syntetický estrogen, který byl hojně využíván k urychlování růstu v živočišné výrobě; používá se pro léčbu estrogenní nedostatečnosti ve veterinární medicíně. Nicméně zbytkový HEX může narušovat normální fyziologické procesy a mít teratogenní, mutagenní a karcinogenní účinky na člověka. Proto bylo používání HEX ve většině zemí zakázáno [16, 17]. U myší, intoxikovaných HEX, byly pozorovány menší vaječníky se sníženým počtem žlutých tělísek a vajíček [18].

Bisfenol A je monomer používaný pro výrobu polykarbonátových plastů, využívá se také v epoxidových pryskyřicích, jako potah v kovových plechovkách a jako zubní tmel. Používá se také jako přísada k jiným plastům, jako je polyvinylchlorid; halogenované deriváty BPA jsou široce používány jako zhášeče hoření [19]. Esterová vazba spojující molekuly BPA v polykarbonátu a pryskyřicích prochází hydrolýzou, což vede k uvolnění volného BPA do potravin, nápojů a do životního prostředí. Podle monitorovacích studií je biologicky aktivní BPA téměř všudypřítomný. Bisfenol A byl nalezen v balené vodě a také v obalových materiálech používaných pro skladování potravin [20]. Estery kyseliny ftalové se celosvětově používají jako změkčovadla, které dodávají pružnost a ohebnost pevným polymerům, jako je polyvinylchlorid. Tyto molekuly se vyskytují převážně v průmyslových barvách a rozpouštědlech, ale také v hračkách, přípravcích osobní hygieny a ve zdravotnictví například ve vacích pro krevní transfuze [21].

Alkylfenoly jako NF a OP mají široké průmyslové využití jako detergenty, v latexových barvách a v průmyslu zpracovávajícím plasty. Tyto látky byly detekovány v půdě, povrchové i podzemní vodě, odtocích čistíren odpadních vod, v potravinách i obalových materiálech. Spolu s BPA a dalšími bisfenoly byly tyto látky stanoveny také ve výrobcích osobní hygieny, jako jsou šampony, kondicionéry, mýdla atp. [22-24].

Látky s estrogenní aktivitou mohou ovlivňovat reprodukci u savců. Mnoho desítek let byly estrogeny považovány čistě za ženské hormony, v poslední době se ale ukázalo, že hrají důležitou roli také u mužů [25]. Během posledních několika desetiletí došlo v lidské populaci k nárůstu testikulárních nádorů, defektů ve vývoji reprodukčních orgánů a výrazně se snížila kvalita lidského spermiogramu [26, 27]. Jednou z příčin

může být vedle důsledků negativního životního stylu i přítomnost estrogenních polutantů. V případě některých z nich byl již jejich negativní vliv na reprodukci prokázán (DES) nebo je testován (např. BPA nebo NF) [27-29]. Výskyt estrogenních polutantů také úzce souvisí se zvyšujícím se užíváním antikoncepčních tablet, které dnes obsahují látky s vysokou fyziologickou aktivitou [30]. V literatuře již byla popsána změna pohlaví a reprodukčních funkcí u plazů, ptáků, obojživelníků, korýšů a ryb v důsledku přítomnosti estrogenů v životním prostředí [31]. Za hlavní způsob expozice je považován příjem EDC potravinami [32].

1.1.2 Mechanismus účinku endokrinních disruptorů

Působení hormonů je realizováno jejich vazbou na receptory v buněčné membráně, cytosolu nebo v jádře. Účinky, vyvolané na jaderných hormonálních receptorech, přímo ovlivňují genovou expresi, ale jsou známy i rychlé, negenomické účinky na receptory, asociované s membránou. Membránové receptory jsou spojeny s různými bílkovinami v buňce a vazba na tyto receptory rychlým způsobem mění buněčné odpovědi [33], nicméně důsledkem rychlé signalizace může být aktivace nukleárního transkripčního faktoru, což vede k odezvám, které lze pozorovat až po delší době [34].

Endokrinní systém je nastaven tak, aby reagoval na velmi nízké koncentrace hormonů, což umožňuje ohromnému množství hormonálně aktivních molekul koexistovat v krevním oběhu [35]. Koncentrace βE2 v krevní plazmě samců potkanů je 2 – 25 pg/mL, ale v tekutině *rete testis* (síti kanálků na zadní straně varlete) je 250 pg/mL [36, 37]. Sérová koncentrace βE2 u samic potkanů se pohybuje mezi 30 a 90 pg/mL v závislosti na fázi estrálního cyklu [38]. Podobně u mužů je koncentrace βE2 v krvi z periferních žil kolem 20 pg/mL ale 50krát vyšší (1 ng/mL) ve spermatické véně [39]. Na druhou stranu u netěhotných žen před menopauzou se pohybuje plazmatická koncentrace βE2 od 50 do 400 pg/mL v závislosti na menstruačním cyklu [40].

Je několik důvodů, proč jsou endogenní hormony schopné pracovat při takto nízkých koncentracích: a) receptory, specifické pro daný hormon, mají tak vysokou afinitu, že mohou vázat dostatek molekul hormonu, k vyvolání reakce b) mezi koncentrací hormonu a počtem obsazených receptorů je nelineární vztah a c) nelineární vztah je i mezi počtem obsazených receptorů a největší pozorovatelnou biologickou odezvou [34]. Endokrinní disruptory fungují stejným způsobem; váží se na estrogenní receptory,

čímž vyvolávají kaskádu molekulárních účinků, které nakonec modifikují genovou expresi. Proto jsou účinky estrogenních EDC a molekulární mechanismy jejich působení v některých detailech známy [34].

Již bylo zmíněno, že hormony působí převážně pomocí interakcí s příslušnými receptory. Ty lze rozdělit do dvou velkých skupin a to: a) na membránové receptory, které slouží primárně pro odezvu peptidových hormonů jako je např. inzulin a b) na jaderné receptory, které jsou aktivovány interakcí s malými lipofilními hormony, jako jsou pohlavní steroidní hormony. Endokrinní disruptory mohou působit různými mechanismy, nicméně protože mnoho EDC jsou malé lipofilní látky, upřednostňovaný způsob jejich přímé interakce je skrze jaderné receptory, což pravděpodobně narušuje nebo upravuje následnou genovou expresi. Reprodukční nebo vývojové vady, vzniklé působením EDC, se považují za výsledek narušení funkce estrogenního a/nebo androgenního receptoru, čímž je zároveň narušena normální činnost estrogenních nebo androgenních ligandů [41, 42]. Endokrinní disruptory účinkují i jinými způsoby, které nejsou závislé na vazbě na klasický nebo membránově vázaný receptor pro steroidní hormony [43, 44].

Endokrinní disruptory mohou ovlivňovat metabolismus přirozených hormonů a způsobovat tak rozdíly v množství hormonu, který je dostupný, protože je ho vyprodukováno více (nebo méně) než je obvyklé v normálním stavu nebo protože je hormon rozložen rychleji (nebo pomaleji) než je obvyklé. Ovlivňují transport hormonů, což také může změnit množství hormonu dostupného pro vazbu s receptorem. Endokrinní disruptory mohou mít také účinky nezávislé na známých endokrinních činnostech. Příkladem je vliv endogenních hormonů a EDC na aktivitu iontových kanálů. Bylo prokázáno, že BPA, dichlorodifenyltrichloroethan, DES, NF a OP narušují Ca^{2+} kanálovou aktivitu a Ca^{2+} signalizaci v některých typech buněk [45-48]. Endokrinní disruptory mohou působit aditivně nebo dokonce synergicky s jinými chemickými látkami nebo přirozenými hormony v těle [49].

17β-estradiol, EE2 a DES se chovají jako antagonisté estrogenního receptoru, jako slabí antagonisté tohoto receptoru vystupují BPA a NF [50]. Polyethoxyláty alkyfenolů a deriváty karboxylových kyselin alkylfenolů vykazují také estrogenní aktivitu u ryb, ptáků a savců, spolu s OP a NF jsou o tři až čtyři řády méně účinné než samotný βE2 [51].

U savců mohou látky s estrogenní aktivitou působit mnoho zdravotních problémů, jako je například předčasná puberta u samic, nižší počet spermií u samců, změněná funkce pohlavních orgánů, obezita, změněné sexuálně specifické chování a vyšší výskyt rakoviny prsu, vaječníků, varlat a prostaty. Savci ve stádiu plodu, novorozenci a mladiství jsou velmi citliví na velmi nízké dávky (někdy pikomolární nebo nanomolární) látek majících estrogenní aktivitu. Vzhledem k tomu, že sekrece estrogenních hormonů u dětí před pubertou je velmi malá, je sérová koncentrace βE2 nedetekovatelná [52]. Vystavení dětí působení malým dávkám EDC může způsobit nežádoucí účinky na jejich růst a dospívání. Mnoho z těchto účinků, pozorovaných na zvířatech, se může projevovat také u lidí, protože základní fungování endokrinních systémů je v podstatě stejné napříč obratlovci [53-55].

Biochemické testy zkoumaly kinetiku vazby BPA na estrogenní receptor a zjistily, že BPA se váže jak na α-estrogenní receptor, tak na β-estrogenní receptor s přibližně desetinásobně vyšší afinitou pro β-receptor [56, 57]. Bisfenol A také zahajuje rychlé reakce prostřednictvím estrogenních receptorů, primárně asociovaných s plazmatickou membránou. Podobně jako βE2 způsobuje BPA změny některých buněčných funkcí v koncentracích mezi 1 pM až 1 nM [20]. Účinky expozice BPA mohou být obzvláště škodlivé pro plod, kojence a malé děti, protože nedostatek zpětné vazby reguluje činnost, syntézu a eliminaci hormonů. Kontakt s BPA v této době může vést k nezvratným změnám, které se objevují i s velmi dlouhým zpožděním. Studie na myších ukázala, že už koncentrace BPA 20 ppm v pitné vodě stačí ke genetickým změnám u myších plodů [58].

1.1.3 Kapacitace

Aby savčí spermie získaly schopnost oplodnit vajíčko, musí být nejprve kapacitovány. Kapacitace souhrnně označuje mnoho biochemických a fyziologických změn, kterým spermie savců podléhají během své cesty samičím pohlavním ústrojím [59]. Při kapacitaci nedochází k morfologickým změnám spermie, ale převážně ke změnám chemickým. Kapacitace zahrnuje zejména: a) vzrůst tekutosti membrány hlavičky spermie snížením koncentrace cholesterolu v membráně, b) tok iontů vedoucí ke změně membránového potenciálu, c) fosforylaci mnoha proteinů převážně na

tyrozinových zbytcích (pTyr) spojenou s aktivací signálních kaskád a d) hyperaktivaci a schopnost navození akrozomální reakce (AR) [60].

Kapacitace probíhá *in vivo* v prostředí dělohy nebo vejcovodů vlivem látek vylučovaných těmito orgány. Albumin je hlavním proteinem, který se nachází v samičím pohlavním ústrojí. Pro realizaci kapacitace *in vitro* je zásadní používat médium simulující prostředí samičího pohlavního traktu, a to kapacitační/fertilizační médium, jehož důležitou složkou je i hovězí sérový albumin (BSA), vyvazující cholesterol z plazmatické membrány spermie [59] a vápenaté a hydrogenuhličitanové ionty [61-63]. Během kapacitace může mít BSA i jiné funkce [64], ale jeho schopnost umožňovat tzv. „cholesterolový efflux" je pro kapacitaci nejdůležitější. Pro kapacitaci *in vitro* je nutné spermie nejprve inkubovat v kapacitačním/fertilizačním médiu za teploty 37 °C v atmosféře s 5% CO_2, čímž se simuluje fyziologické prostředí samičího pohlavního ústrojí [59, 60].

Během transportu spermií samičím reprodukčním traktem jsou spermie vystaveny změnám okolního prostředí. Jako následek změn v extracelulární koncentraci iontů dochází ke změnám potenciálu plazmatické membrány spermie [65, 66]. Membrána je propustnější pro K^+ ionty, které proudí z buňky ven. Zároveň dochází k ovlivnění napěťově ovládaných Ca^{2+} iontových kanálů, které se otevírají, a extracelulární vápník vstupuje do buňky. Zvýšení koncentrace Ca^{2+} iontů v buňce je nezbytné pro začátek AR [67]. Akrozomální reakce je vyvrcholením kapacitace a je nezbytným předpokladem pro oplodnění vajíčka spermiemi savců [59]. Spermie se váže k extracelulárnímu obalu vajíčka (*zona pellucida*), přes specifické receptory, které jsou lokalizovány na anteriorní části hlavičky spermie. Během AR dochází k fúzi membrán, která umožní řízenou exocytózu (vylití) lytického váčku tzv. akrozómu, díky které spermie pronikne obaly vajíčka. Tyrozinová fosforylace proteinů bičíku je důležitá pro hyperaktivaci spermie [68, 69] a v oblasti akrozomální části hlavičky je nezbytná pro průběh AR a následnou fúzi membrán spermie a vajíčka. Následkem kapacitace dochází ke změně amplitudy pohybu bičíku spermií – k tzv. hyperaktivaci. Bičík se pohybuje v hlubokých ohybech a jeho pohyb je méně symetrický. Hyperaktivace umožňuje spermii pronikat vysoce viskózním prostředím vejcovodu, kumulárním matrixem a po AR také penetrovat *zona pellucida*. Spermie, u kterých neprobíhá kapacitace, nemají proteiny fosforylované. Tyrozinová fosforylace může být primárním nebo dokonce jediným ukazatelem přenosu

signálu u spermií [60, 70] může být ale i ukazatelem mužské neplodnosti [71]. Výsledkem pTyr je přestavba kostry buňky nazývané cytoskelet, představované polymerizací proteinu aktinu. Je to jeden ze zásadních aspektů, umožňující spemiím získat schopnost oplodnit vajíčko a následně projít AR [72].

Na myších spermiích byl studován vliv estrogenů na průběh kapacitace spermií *in vitro* a AR [73]. V publikaci Šebkové a kol. [73] byla popsána přítomnost β-estrogenního receptoru v distální oblasti nadvarlete a pomocí pozitivní pTyr hlaviček spermie a lysátu celých spermií byl vyhodnocen vliv estrogenních hormonů na kapacitaci myších spermií. Bylo zjištěno snížení počtu spermií schopných projít AR a modifikace fyziologického průběhu kapacitace spermií.

1.1.4 Fluoridy

Lidská populace je vystavována velkému množství faktorů přispívajících k rostoucí neplodnosti. Fluoridy a hlinité ionty představují dva z potenciálních kandidátů ovlivňujících plodnost [74]. Zvýšená expozice fluoridům, která může snadno překročit doporučovanou denní dávku dle Světové zdravotnické organizace (WHO) [75] může mít potenciálně negativní vliv jak na samičí [76-78], tak na samčí plodnost, zahrnující spermatogenezi, genovou expresi, morfologii a motilitu spermií, fertilizační schopnost spermií a chromatinovou strukturu spermie [79-84].

Obecně jak fluoridové, tak hlinité ionty hrají důležitou roli ve vývoji a zdraví jednotlivců. V posledních letech vzrůstá nefyziologický příjem fluoridů způsobený fluorizací pitné vody a soli, použitím fluoridů při prevenci zubního kazu, pediatrickými doplňky a léky obsahujícími fluor (např. antibiotika, cytostatika, protizánětlivé léky a psychofarmaka). Schopnost fluoridových iontů vytvářet komplexy se stopami iontů hliníku vede k působení fluoridů na regulační proteiny, zprostředkovávající přenos buněčných signálů tzv. G-proteiny, stejně tak jako na různé adenosintrifosfatasy a fosfatasy. Tyto fluorokovové komplexy se chovají analogicky jako fosfát a váží se s vysokou afinitou, ale reverzibilně, do míst určených pro vazbu fosfátu [85]. Fluorohlinité komplexy [AlF_i], zejména tetrafluorohlinitan, simulují G-proteiny [86], které jsou součástí různých biologických signalizačních drah včetně těch, zahrnujících kapacitaci spermií. V publikaci [74] byl systematicky studován vliv fluoridů a fluorohlinitanů na spermatogenezi a kapacitaci myších spermií *in vitro* s důrazem na

pTyr v hlavičkách spermií a polymerizaci aktinu. Výsledky této studie ukazují, že schopnost myších spermií úspěšně projít kapacitací *in vitro* byla významně ovlivněna přítomností fluoridů a fluorohlinitanů *in vivo*, i přesto, že nebyly pozorovány fyziologické změny v pTyr nebo polymerizaci aktinu.

1.2 Metody analýzy

Pro analýzy estrogenních polutantů se používají nejčastěji chromatografické metody HPLC a GC, ale své uplatnění zde mají i metody imunochemické. Ty se používají zejména pro rychlý screening a v biologických pokusech.

1.2.1 Kapalinová chromatografie v analýzách EDC

Kapalinová chromatografie je nejběžnější metodou pro analýzy estrogenů a EDC, protože často nevyžaduje před analýzou derivatizaci vzorku, jak je tomu u GC [32, 87]. Podkladem pro tuto disertační práci je mimo jiné i přehledný článek shrnující analýzy EDC v potravinách. Ve vybraných potravinových matricích byly HPLC analýzy nejčastěji prováděny za použití stacionární fáze obsahující jako sorbent navázané C18 řetězce v kombinaci s mobilní fází obsahující acetonitril, vodu a aditiva v různých objemových poměrech. K detekci byla nejčastěji používaná hmotnostní spektrometrie [32].

Kapalinová chromatografie se však používá pro stanovení EDC i v jiných matricích, jako jsou například vzorky ze životního prostředí. Stejně jako u analýz EDC v potravinách jsou nejvíce používanou stacionární fází kolony s C18 stacionární fází a jako mobilní fáze se nejčastěji používají směsi acetonitrilu a vody v různých objemových poměrech. I nejčastěji používaný způsob detekce je pro environmentální matrici a potraviny shodný a je jím MS detekce. Toto téma je přehledně zpracováno například v následujících souborných publikacích [87, 88]. I v nejaktuálnějších publikacích pro separace estrogenů ve vzorcích z životního prostředí bylo použito výše zmiňovaných kombinací stacionární a mobilní fáze [89, 90].

1.2.2 Metody používané v biologických pokusech v přítomnosti EDC

Metody studia vlivu estrogenních hormonů na kapacitaci spermií jsou v literatuře publikovány pouze ojediněle. Pro studium vlivu estrogenů na kapacitaci kančích

spermií *in vitro* bylo použito imunochemických metod (ELISA, průtoková cytometrie). Bylo zjištěno, že stimulační vliv závisí na době kapacitace, konkrétním estrogenu a konkrétním jedinci [91]. Imunochemické metody (imunofluorescenční analýza, imuno-proteinová detekce) byly použity v publikaci [73] pro studium vlivu estrogenů na kapacitaci myších spermií *in vitro* a AR. Bylo zjištěno snížení počtu spermií schopných projít AR a modifikace fyziologického průběhu kapacitace spermií. Při studiu vlivu estrogenů na kapacitaci myších spermií *in vivo* imunochemickými metodami bylo zjištěno, že při podávání βE2 myším samcům během jejich dospívání dochází k předčasné kapacitaci spermií v nadvarleti, což může ohrozit jejich fertilizační schopnost v samičím pohlavním ústrojí [92].

Vliv E3 na kapacitaci myších spermií *in vitro* byl sledován pomocí HPLC metody s UV detekcí, avšak s nízkou citlivostí detekce [42]. Bylo zjištěno, že během kapacitace myších spermií *in vitro* dochází k vyvázání E3 myšími spermiemi. Při použití této metody bylo možné sledovat pouze počáteční koncentraci E3 200 µg/L.

1.2.3 Kinetická analýza

Vývoj a řešení reakčních schémat kinetickou analýzou [93] rychlostních rovnic, nebo jejich integrovaných forem, se často používá pro homogenní chemické reakce, zvláště když se předpokládá, že součástí celkového mechanismu jsou nestabilní látky, které nelze přímo prokázat. Dokonce i komplexní reakční mechanismy mohou být vyřešeny metodami kinetické analýzy [94, 95].

Bylo publikováno pouze několik článků týkajících se aplikace kinetické studie na biologické jevy [96-99]. Kinetická analýza byla v této disertační práci využita ve dvou publikacích, kde bylo studováno, zda chemická kinetika, která je platná pro homogenní chemické reakce, může být aplikována na experimentální data, získaná během kapacitace spermií *in vitro*.

1.3 Výsledky a diskuze – komentáře k publikacím

PUBLIKACE I

Kinetic analysis of decreased sperm fertilizing ability by fluorides and fluoroaluminates: a tool for analyzing the effect of environmental substances on biological events

Bosáková, Z.; Tockstein, A.; Adamusová, H.; Coufal, P.; Šebková, N.; Dvořáková-Hortová, K., Eur Biophys J Biophys Lett. 45 (1) (2016) 71-79.

PUBLIKACE II

Determination of 17β-estradiol and 17α-ethynylestradiol in mouse fertilizing M2 medium

Adamusová, H.; Bosáková, Z.; Kozlík. P.; Hortová, K., In: Proceedings, 9th International Students Conference „Modern Analytical Chemistry“: Praha 2013, p. 26-27, ISBN 978-80-7444-023-6.

PUBLIKACE III

17β-Estradiol signalling is directed by autocatalytic reaction in capacitating sperm

Bosáková, Z.; Adamusová, H.; Bosáková, T.; Tockstein, A.; Šebková, N.; Dvořáková-Hortová, K., bude zasláno, červenec 2017 do Scientific Reports.

PUBLIKACE IV

Analysis of estrogens and estrogen mimics in edible matrices—a review

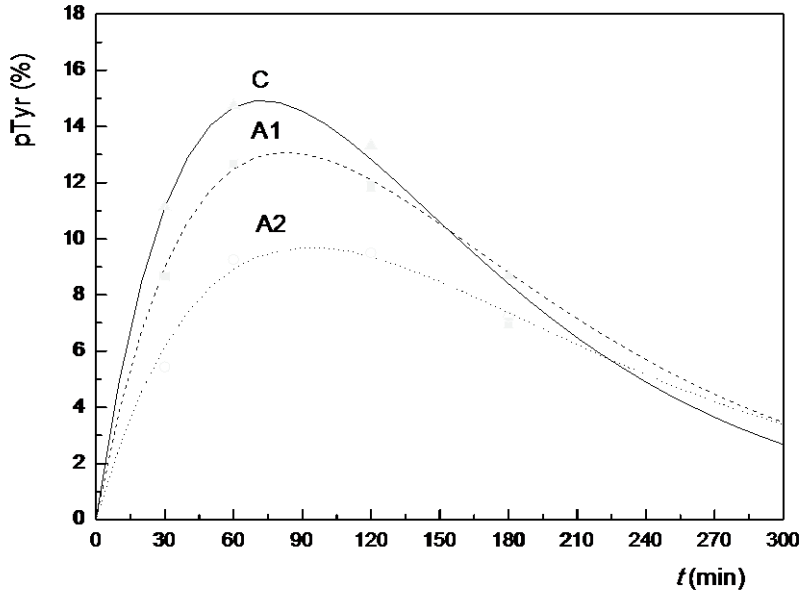
Adamusová, H.; Bosáková, Z.; Coufal, P.; Pacáková, V., J Sep Sci. 37 (8) (2014) 885-905.

1.3.1 Kinetická analýza spermií se sníženou fertilizační schopností způsobenou fluoridy a fluorohlinitany: nástroj pro zjišťování efektu environmentálních látek na biologické jevy – publikace I

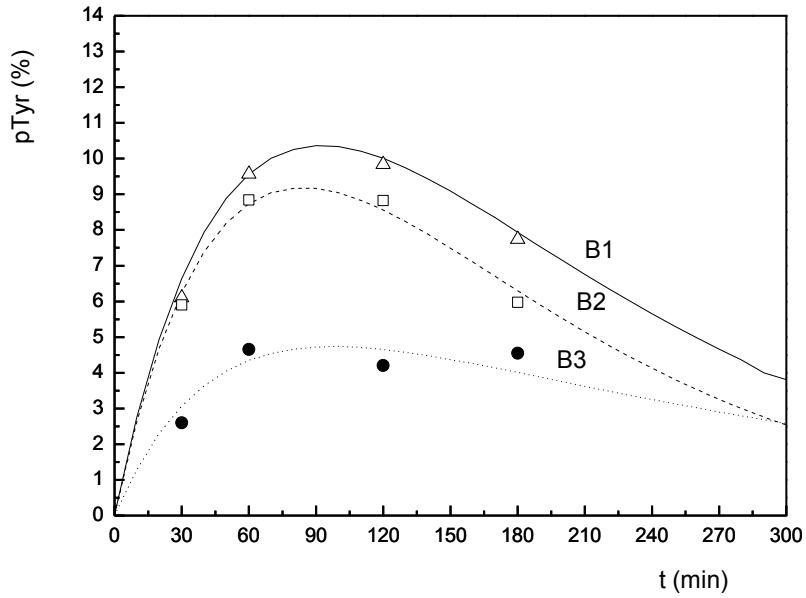
Jak již bylo výše zmíněno, publikace [74] se zabývala vlivem fluoridů a fluoridových komplexů s hlinitými ionty na stupeň pTyr během *in vitro* kapacitace myších spermií. Byla sledována také schopnost spermií projít AR. Tyto biologicky důležité reakce ukazují kvalitu přípravy spermií na oplodnění a zohledňují vnější vlivy faktorů ze životního prostředí.

Cílem publikace I bylo podrobit experimentální data, získaná z předešlé časové závislosti pTyr a AR [74], kinetické studii pro detailnější popis průběhu těchto reakcí. Bylo zjišťováno, zda závislosti a) procenta hlaviček spermií pozitivních na pTyr a b) procenta spermií, které prošly indukovanou AR na čase probíhající kapacitace *in vitro*, vyhovují rovnici nebo systému rovnic chemické kinetiky. Tato publikace byla využita jako pilotní studie pro aplikaci kinetické analýzy na biologické jevy.

Na základě experimentálně získaných dat [74] bylo zřejmé, že počet spermií s pozitivní pTyr je funkcí dvou proměnných, a to času a koncentrace inhibitoru, které nepůsobí simultánně. Experimentálně získané body závislosti procent spermií s pozitivní pTyr na čase kapacitace vykazovaly pro všechny testované inhibitory obdobný průběh, nejprve dosahovaly maxima a poté klesaly k velmi podobným hodnotám, získaným pro všechny typy inhibitorů. Pro popis výše uvedené závislosti byla navržena soustava kinetických rovnic a byly vypočítány příslušné rychlostní konstanty. Výsledky jsou demonstrovány na obrázcích 1.2 a 1.3 pro různé typy a koncentrace inhibitorů. Z obrázků je vidět, že experimentálně získané body vykazují dobrou shodu s teoreticky vypočítanými křivkami, což ukazuje, že navržený model dobře odpovídá realitě.



Obr. 1.2 Závislost procent hlaviček spermií s pozitivní pTyr na čase kapacitace *in vitro* při použití různých koncentrací fluoridů jako inhibitorů; skupina C (bez intoxikace fluoridy; c_F = 0 mol/L), skupina A1 (s nejnižší podanou dávkou fluoridů; $c_F = 5{,}26 \times 10^{-5}$ mol/L) a skupina A2 (s 10krát vyšší podanou dávkou fluoridů; $c_F = 52{,}6 \times 10^{-5}$ mol/L).



Obr. 1.3 Závislost procent hlaviček spermií s pozitivní pTyr na čase kapacitace *in vitro* při použití různých koncentrací fluoridů a hlinitých iontů jako inhibitorů; skupina B1 ($c_F = 5{,}26 \times 10^{-5} + c_{Al} = 3{,}7 \times 10^{-4}$ mol/L), skupina B2 ($c_F = 52{,}6 \times 10^{-5} + c_{Al} = 3{,}7 \times 10^{-4}$ mol/L) a skupina B3 ($c_F = 526 \times 10^{-5} + c_{Al} = 3{,}7 \times 10^{-4}$ mol/L).

Časové závislosti pTyr vyhovují soustavě kinetických rovnic a výsledky ukazují, že kapacitace probíhá v systému dvou následných reakcí 1. řádu. Navržené kinetické rovnice objevují existenci určitých center ve spermiích a jejich nestabilní aktivní formy. Pouze tyto nestabilní, aktivní formy mohou být fosforylovány a následně se rozpadají. Čas odpovídající maximální produkci nestabilního intermediátu je pro spermie pravděpodobně nejvhodnější k získání schopnosti oplodnit vajíčko. Vypočítané inhibiční koeficienty ukazují, že účinnost inhibitoru se zvyšuje se zvyšujícím se obsahem fluoridu v komplexu s hlinitými ionty. Další detaily a výpočty jsou podrobně zmíněny v publikaci I [100].

1.3.2 Stanovení 17β-estradiolu a 17α-ethynylestradiolu v M2 fertilizačním médiu – publikace II

Tato publikace shrnuje pilotní výsledky, které sloužily jako základ pro další měření použité zejména v publikaci III. Výsledky z této publikace byly prezentovány formou přednášky na studentské konferenci 9th International Students Conference 'Modern Analytical Chemistry' konané v roce 2013.

V publikaci Dvořákové-Hortové a kol. [42] byla vypracována metoda HPLC s UV detekcí pro stanovení E3 ve fertilizačním médiu, pomocí níž byla sledována zbytková koncentrace E3 během *in vitro* kapacitace myších spermií. Bylo zjištěno, že během kapacitace dochází k vyvázání E3 myšími spermiemi [42]. Sledování průběhu kapacitace, včetně vlivu estrogenních polutantů na kapacitaci spermií *in vivo*, by nebylo technicky možné, a proto se veškeré experimenty tohoto druhu provádí *in vitro* ve fertilizačním médiu simulujícím *in vivo* prostředí [42]. Detailnější informace o kapacitaci a *in vitro* experimentech je možno nalézt výše v kapitole 1.1.3.

Na základě výsledků získaných pro separaci E3, byl pro oba testované analyty (βE2 a EE2) vybrán reverzní separační mód s použitím SunFire C18 kolony jako stacionární fáze. Tato kolona poskytovala vysokou separační účinnost a symetrický tvar píku. Pro stanovení βE2 a EE2 v M2 fertilizačním médiu byla vyzkoušena UV detekce. Jako mobilní fáze byla testována směs acetonitrilu s vodou v různých objemových poměrech. Optimalizace procesu byla prováděna s cílem dosáhnout dostatečné citlivosti. Nicméně ani βE2 ani EE2 v požadované koncentraci 200 μg/L nebyly detekovány v žádné z testovaných mobilních fází. M2 médium je komplexní směs obsahující anorganické a organické složky, ze kterých zejména BSA (4.0 g/L) může způsobovat obtíže jak při separaci, tak zejména při UV detekci. Při vlnové délce 200 nm byl signál M2 kapacitačního média o dva řády vyšší než signál analytů. Vzhledem k tomu nebylo pro tento účel možné použít UV detekci a pro další měření (publikace III) byla vybrána tandemová hmotnostní detekce, která je schopna mnohem lépe eliminovat vliv matrice a více se přiblížit reálným hodnotám expozice organismů estrogenům [101].

1.3.3 Signalizace 17β-estradiolu je řízena autokatalytickou reakcí v kapacitujících spermiích – publikace III

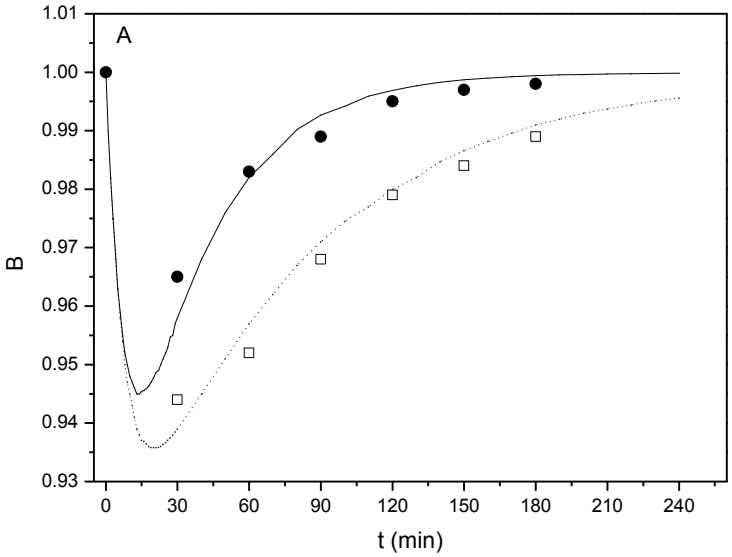
V této publikaci byl sledován průběh kapacitace myších spermií *in vitro* v přítomnosti různých koncentrací βE2. Získaná experimentální data byla následně podrobena kinetické analýze.

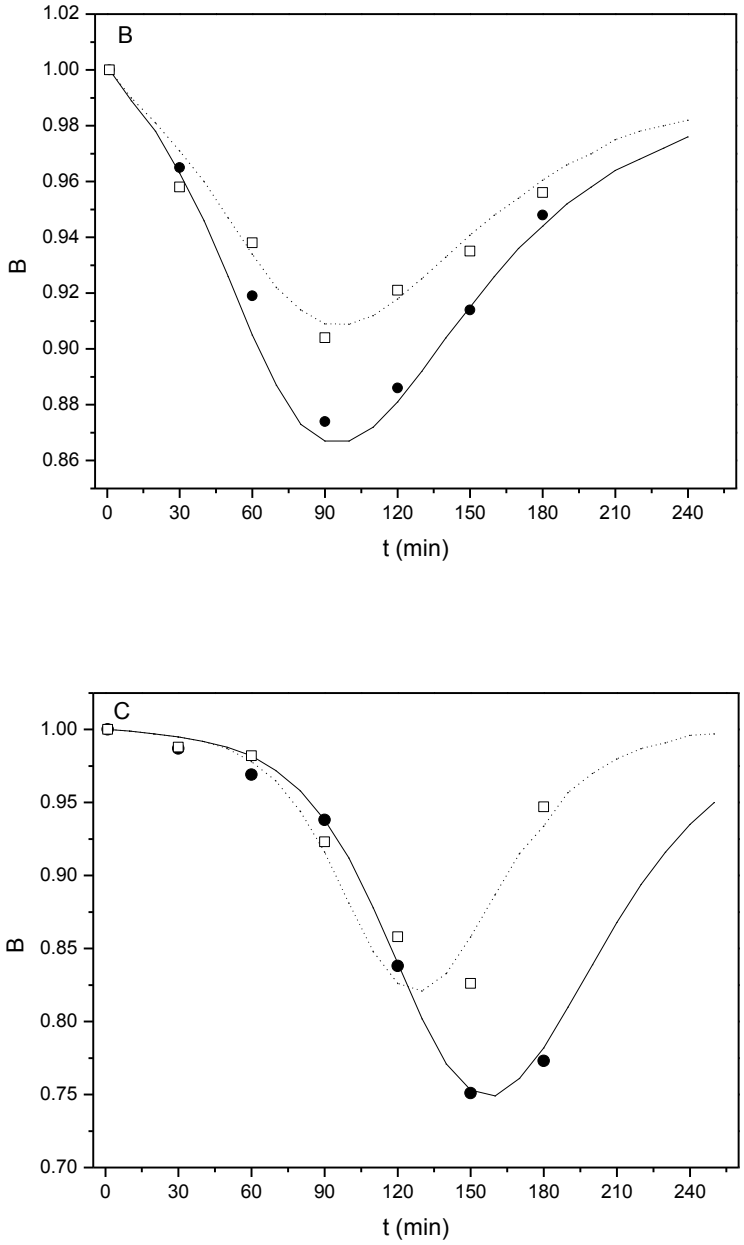
Na základě předchozích výsledků pro analýzu vybraných estrogenů v environmentálních vzorcích vody [102], pro separaci E3 v M2 kapacitačním a fertilizačním médiu [42] a na základě pilotních měření zmiňovaných výše v publikaci II, byla pro sledování koncentračních změn βE2 během kapacitace myších spermií *in vitro* vybrána HPLC-MS/MS metoda s kolonou SunFire C18 jako stacionární fází. Pro izokratickou eluci byla použita mobilní fáze acetonitril/voda 50/50, (*v/v*), obě složky obsahovaly 0,1% roztok mravenčí kyseliny. Jako vnitřní standard byl použit deuterovaný βE2 (βE2-d3). Při použití této metody bylo pro βE2 dosaženo hodnoty LOD 0,3 μg/L.

Aby bylo možné sledovat koncentrační změny βE2 během kapacitace spermií *in vitro*, bylo nezbytné zjistit, kolik βE2, přidaného do M2 média, je k dispozici pro spermie. Jednou z hlavních složek tohoto média je BSA a estrogeny se na něj mohou vázat. Proto bylo důležité určit, kolik βE2 je vázáno na BSA, a kolik je skutečně k dispozici pro využití spermiemi. Toto bylo zjišťováno pro všechny tři testované koncentrace βE2 (200, 20 a 2 μg/L) po *in vitro* inkubaci spermií trvající 1 hodinu v M2 médiu (při 37 °C, 5% CO_2). Tyto vypočtené hodnoty představovaly vstupní koncentrace v čase kapacitace 0 a následně byly změřeny závislosti celkové koncentrace nevázaného βE2 během kapacitace. Průběh kapacitace byl sledován v časovém rozmezí 0 – 180 minut, vzorky byly odebírány ve 30 minutovém intervalu. Sledování kapacitace déle než do 180. minuty nemá žádný fyziologický význam, jelikož kapacitace *in vivo* je dosaženo během 90 minut (nejpozději během 180 minut). Každý experiment byl prováděn pro vzorky s přidanými myšími spermiemi a pro referenční vzorky (blanky) bez přidaných spermií. Analýza pomocí HPLC-MS/MS každého vzorku byla provedena v 5 opakováních.

Pro všechny testované koncentrace βE2 byly hodnoty blanků během kapacitace prakticky konstantní. U vzorků s přidanými spermiemi byly pozorovány obdobné trendy u všech testovaných koncentrací (200, 20 a 2 μg/L), stejně tak u obou kmenů

laboratorních myší BALB/c nebo C57BL/6Nvel jak je vidět z obrázku 1.4. Na obrázku 1.4 je pro lepší porovnání jednotlivých závislostí vynášena relativní koncentrace *B*, což je podíl koncentrace nevázaného, celkového βE2, změřeného v daném čase kapacitace ($t > 0$) a koncentrace nevázaného, celkového βE2, změřeného na začátku kapacitace v čase ($t = 0$). Společným trendem všech závislostí bylo prvotní snižování koncentrace βE2 až k dosažení minima a poté opětovné zvyšování. Pozice minima se však lišila pro jednotlivé testované koncentrace βE2, jak je patrné při porovnání obrázku 1.4 A – C. Pro počáteční koncentraci 200 μg/L docházelo k poklesu na počátku doby kapacitace v rozmezí 0 – 30 minut (obrázek 1.4 A). Pro počáteční koncentraci 20 μg/L byl pokles pozorován později během 60 – 90 minut (obrázek 1.4 B) a závislost získaná pro počáteční koncentraci 2 μg/L vykazovala minimální hodnotu mezi 150 – 180 minutami (obrázek 1.4 C). V rámci jednotlivých testovaných koncentrací βE2 byly pozorovány pouze mírně odlišné rozsahy poklesu mezi spermiemi obou kmenů myší.

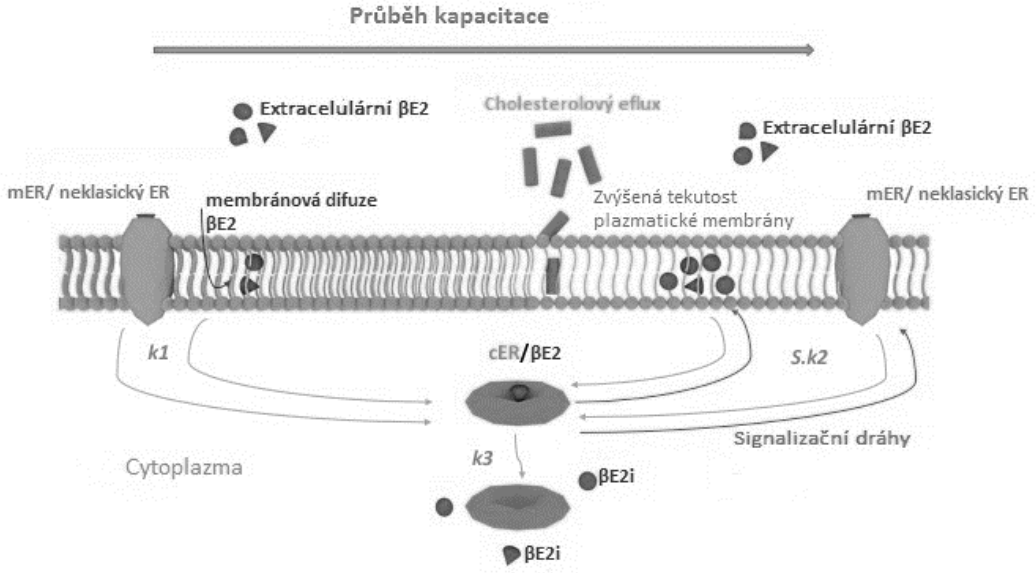




Obr. 1.4 Závislost relativní koncentrace (*B*) βE2 na čase kapacitace. Pro kmen BALB/c je teoreticky získaná křivka zobrazena plnou čarou a experimentálně získané body plnými tečkami. Pro kmen C57BL/6Nvel je teoreticky získaná křivka zobrazena přerušovanou čarou a experimentálně získané body prázdnými čtverečky. Počáteční koncentrace βE2: (A) 200 μg/L, (B) 20 μg/L a (C) 2 μg/L.

Experimentální výsledky získané pro koncentrační změny βE2 v různých časech kapacitace byly podrobeny kinetické analýze. Vzhledem k tomu, že kinetická analýza již byla úspěšně použita pro analýzu dat popisujících sníženou schopnost kapacitace spermií v přítomnosti fluoridů a fluorohlinitých komplexů [100], byla použita také k analýze koncentračních změn βE2 během kapacitace spermií *in vitro*. V předchozí práci [100] byla navržena matematická teorie pro mechanismus, kdy je schopnost dozrávání spermií změněna vnějšími stimuly [74]. Ukázalo se, že chemická kinetika může být aplikována na spermie v roli reaktantu a že kinetická analýza může být užitečným nástrojem pro sledování a predikci specifických molekulárních mechanismů, které se účastní určitých biologických signalizačních cest.

Křivky proložené experimentálně získanými body vykazovaly autokatalytický charakter. Při hledání různých kinetických modelů bylo zjištěno, že pro shodu mezi křivkami, získanými proložením experimentálními body a teoreticky vypočítanými křivkami je nutné předpokládat, že prvním krokem je adsorpce βE2 na povrch spermie řízená Langmuirovou izotermou. Jiné modely (bez adsorpce) vedly ke zcela odlišným výsledkům. Na obrázku 1.4 je vidět, že existuje mírný posun mezi teoretickými křivkami odrážejícími rozdíly v dynamice vazby βE2 mezi dvěma kmeny BALB/c a C57BL/6Nvel. Je však důležité, že teoretické křivky odpovídají experimentálním bodům pro oba testované typy myší, se stejným výsledkem, což dokládá nalezení teoretického mechanismu, který vyhovuje druhově specifickým modifikacím.



Obr. 1.5 Schématická interpretace výsledků kinetické analýzy aplikované na průběh kapacitace spermií *in vitro* v přítomnosti βE2.

Výsledky kinetické analýzy jsou schematicky znázorněny na obrázku 1.5. Jakmile se spermie dostanou do prostředí, kde se nalézá albumin (v případě *in vitro* kapacitace BSA z M2 média), začne proces kapacitace. Extracelulární βE2 se adsorbuje na povrch spermie a interaguje s membránovými (mER) nebo neklasickými estrogenními receptory. Proces adsorpce je řízen Langmuirovou izotermou. Poté projde βE2 do cytoplazmy, kde vytváří s cytoplazmatickým estrogenním receptorem (cER) adukt cER/βE2. Tento děj je pomalý (iniciační), řízený rychlostní konstantou $k1$. Po vzniku prvního aduktu cER/βE2 začne signalizace vedoucí k větší propustnosti plazmatické membrány, ke které přispívá i cholesterolový eflux. Díky tomu může do cytoplazmy pronikat více extracelulárního βE2 membránovou difuzí. Tento děj je rychlý, má autokatalytický charakter a je řízený rychlostní konstantou $k2$ násobenou stupněm aktivity S. Vzniklý adukt cER/βE2 není stabilní a rozpadá se na cER a $\beta E2_i$ (vnitřní βE2, který se již znovu nedostane ven přes plazmatickou membránu) a rozpad aduktu je řízen rychlostní konstantou $k3$. Po rozpadu aduktu jsou cER i $\beta E2_i$ neaktivní. Děje řízené rychlostními konstantami $k1$, $k2 \cdot S$ a $k3$ probíhají simultánně [103]. Pomocí vyvinuté metody HPLC-MS/MS se měří koncentrace celkového nevázaného βE2, což zahrnuje extracelulární βE2 a $\beta E2_i$, který se uvolní z cytosolu během centrifugace po kapacitaci.

Tato publikace ukazuje, že množství βE2 dostupné pro myší spermie během kapacitace *in vitro*, může být kvantifikováno HPLC-MS/MS metodou. Tento nový analyticko-biologický přístup má velký potenciál pro porozumění fyziologickému mechanismu působení steroidních hormonů zahrnujících βE2 nejen pro reprodukční biologii, ale také pro signalizaci v somatických buňkách. Výpočty, vztahující se ke kinetické studii, a další detaily lze nalézt v publikaci III [104].

1.3.4 Analýzy estrogenů a jim podobných látek v potravinách – přehledný článek – publikace IV

V této publikaci je zpracován přehled nejčastěji používaných metod pro stanovení estrogenů a jim podobných látek v potravinách. Stručně je zmíněn mechanismus účinku endokrinních disruptorů. Protože jsou EDC přítomny v matricích ve velmi nízkých koncentracích, je v článku podrobně zpracována příprava vzorku před vlastní analýzou. Ta je důležitým krokem v analýze, jelikož může být zdrojem chyb. Online předúprava vzorku zvyšuje přesnost měření a snižuje dobu analýzy. Aby příprava vzorku nebyla tak drahá a časově náročná, jsou zapotřebí nové selektivní materiály pro extrakci EDC. Pro každou separační metodu jsou analýzy dále rozdělené podle jednotlivých matric vzorků. Detaily z jednotlivých metod jsou pro porovnání shrnuty v tabulkách. Jak již bylo uvedeno výše, je nejčastěji používanou metodou pro stanovení EDC v potravinách HPLC.

Plynová chromatografie se často používá pro analýzy estrogenů a jim podobných látek ve vzorcích z životního prostředí [105, 106]**.** Využití plynové chromatografie v analýzách EDC v potravinách je méně běžné. Některé EDC mohou být analyzovány přímo bez předchozí derivatizace, nicméně konverze na těkavější formy je upřednostňována. Derivatizace často vede k vyšší citlivosti a selektivitě detekce. Bylo pozorováno, že derivatizace zvýšila intenzitu signálu v hmotnostním spektru požadované látky 2 – 12 krát v porovnání s nederivatizovaným vzorkem [107]. Nicméně derivatizace může být pracná, většina derivatizačních činidel je toxická a nestabilní a derivatizace je krok navíc, který může být zdrojem dalších chyb. Nejběžnější derivatizační reakce jsou silylace a esterifikace. Vedou ke zvýšení těkavosti, nižší polaritě a lepší teplotní a katalytické stabilitě, což je potřeba při použití hmotnostní detekce.

Kromě chromatografických metod jsou pro analýzy EDC v potravinách používané i imunochemické metody. Nejběžnějším analytem je BPA, imunochemické metody se používají pro jeho screening v potravinových matricích.

1.4 Použitá literatura I

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PUBLIKACE I

KINETIC ANALYSIS OF DECREASED SPERM FERTILIZING ABILITY BY FLUORIDES AND FLUOROALUMINATES: A TOOL FOR ANALYZING THE EFFECT OF ENVIRONMENTAL SUBSTANCES ON BIOLOGICAL EVENTS.

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ORIGINAL PAPER

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Kinetic analysis of decreased sperm fertilizing ability by fluorides and fluoroaluminates: a tool for analyzing the effect of environmental substances on biological events

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Abstract Fluorides and fluoroaluminates decrease mouse sperm fertilizing potential by modifying the process of sperm preparation for fertilization, so-called capacitation, followed by acrosome reaction (AR). Capacitation was monitored by protein tyrosine phosphorylation (pTyr), and AR was induced consequently. The aim of this study was to apply kinetic analysis to the previously obtained dependences of pTyr and AR at capacitation times, and propose a mathematical theory for a mechanism when sperm maturation ability is amended by external stimuli. The experimental input data, previously obtained, are consistent with the proposed theory and the results of kinetic analysis show that sperm capacitation runs as two subsequent first-order steps. Firstly, an unstable intermediate is formed and then gradually decomposes. The time corresponding to the maximal production of the unstable intermediate is probably most suitable for sperm obtaining the ability to fertilize the egg. The presented calculations indicate that the application of kinetic analysis can serve as a tool to predict or confirm a course of biological events that are modified by external factors, and therefore the proposed theory shall be of interest to a broad scientific audience.

Keywords Kinetics · Fluoride complexes · Capacitation · Tyrosine phosphorylation · Acrosome reaction · Sperm fertilizing ability

Introduction

The human population is being exposed to an enormous variety of factors contributing to ever so growing infertility. Two of the potential candidates to affect fertility potential are fluoride and aluminium ions. Increasing exposure to fluorides, which can easily exceed the recommended daily dose stated by WHO (1987), might have a potential negative impact on both female (Darmani et al. 2001; Chinoy and Patel 2001; Zhu et al. 2014) and male fertility including spermatogenesis, gene expression, sperm morphology and motility, sperm fertilizing ability, and sperm chromatin structure (Ghosh et al. 2002; Pushpalatha et al. 2005; Sun et al. 2010, 2011, 2014; Lu et al. 2014; Kim et al. 2015). Generally, both fluoride and aluminium ions play an important role in the development and health of individual organisms. In recent years, however, there has been a non-physiological increasing fluoride intake in the human population caused by fluoridation of water and salt, dental caries prevention, pediatric supplements, and organic fluorine-containing drugs (e.g., antibiotics, anti-cancer and anti-inflammatory agents, psycho-pharmaceuticals). Dong et al.

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(2004) describe biological fluorination, conversion of inorganic fluoride to organic fluorine, and production of highly valuable organofluorine compounds. The ability of fluoride ions to form complexes with traces of aluminium ions leads to the action of fluorides on guanosine nucleotide binding proteins (G-proteins), as well as on various adenosine 5′-triphosphatases (ATPases) and phosphatases. These fluorometallic complexes act as analogues of phosphate and they bind with high affinity, but reversibly in phosphate sites (Chabre 1990). Aluminium fluoride complexes [AlF_i], especially a tetrafluoroaluminate, therefore simulate various G-proteins (Sternweis and Gilman 1982), which are involved in a variety of biological signaling systems including those of sperm capacitation. In order for mammalian sperm to obtain fertilizing ability, it must undergo the series of maturation events in female reproductive tract so-called capacitation (Yanagimachi 1994). Capacitation of mammalian spermatozoa is a complex process, including sets of molecular changes where protein tyrosine phosphorylation (pTyr, an addition of a phosphate group to the amino acid tyrosine on the specific protein) may be the primary or even the exclusive indicator of a signal transduction pathway in sperm (Naz and Rajesh 2004; Visconti et al. 1995a; Wang et al. 2015) as well as an indicator of male infertility (Kwon et al. 2014). Progress of capacitation is associated with sperm pTyr, resulting in major cytoskeleton rearrangements represented by cytoskeletal protein actin polymerization. This molecular pathway is accomplished in the sperm head through the activation of protein kinase A (Visconti et al. 1995b) and represents crucial aspects enabling sperm to obtain full fertilizing capacity, and subsequently undergo calcium-mediated acrosome reaction (AR) (Baker et al. 2004). AR is the event of lytic enzyme release from the apical acrosomal vesicle and this enables sperm to pass through the layers surrounding the egg and fuse with its plasma membrane (Jin et al. 2011; Yanagimachi 1994).

Our previously published study (Dvořáková-Hortová et al. 2008) systematically focused, for the first time, on the effect of fluorides and fluoride complexes with aluminium ions on the level of pTyr monitored during the capacitation of mouse sperm in vitro. The ability of sperm to undergo AR was also monitored. These are biologically important reactions, which are markers of the quality of preparation of the sperm for fertilization and reflect the effects of external environmental factors.

Therefore, the goal of our work was to subject the experimental data, previously obtained for time dependence of pTyr and AR (Dvořáková-Hortová et al. 2008), to kinetic analysis for more detailed description of the course of these reactions. In other words, the goal was (1) to find out whether the percentage of sperm head positive on protein tyrosine phosphorylation [*pTyr* (%)] and the percentage of sperm that underwent induced acrosome reaction [*AR* (%)] versus the time of ongoing capacitation fit to an equation or a system of equations of chemical kinetics and from these results (2) to induce a consequence for the capacitation. Only a few papers dealing with an application of kinetics on biological events have been published (Mariani et al. 2004; Zhdanov 2006, 2007, 2009). Kinetic analysis has not yet been used for biological reactions in which sperm take part, and we believe that it will be of wider use in the prediction of kinetics of specific molecular mechanisms involved in selected signaling pathways.

It must be emphasized that no new laboratory work was done in this work and all experimental data used in this work as input data for kinetic equations were adopted from the previous published paper (Dvořáková-Hortová et al. 2008). Methods previously used for obtaining dependences of pTyr and AR at capacitation times are briefly described in the section "Materials and methods" for better understanding of biological impact of kinetic analysis.

Materials and methods

Instrumentation and animals

For realization of capacitation in vitro, 35-mm Petri dishes obtained from Corning, NY, USA, were used. A light-inverted microscope from Olympus CX 21 and Olympus epifluorescent microscope were supplied by Olympus, Czech Republic. An NB-203 incubator was purchased from N-BIOTEK, Korea. Laboratory BALB/c mice were purchased from Velaz, s.r.o., Prague, Czech Republic.

Experimental design

Since 1990, drinking water in the Czech Republic has not been fluorinated, therefore fluoride-free tap water was used as drinking water for group C, which served as a negative control. Fluoride concentrations of 1, 10, and 100 ppm (5.26×10^{-5}, 52.6×10^{-5}, and 526×10^{-5} mol l^{-1}) were added to drinking water for groups A_1, A_2, and A_3, prepared according to the standard protocol of the Czech National Hygienic Department, Prague, Czech Republic. Group A_1, receiving a fluoride concentration of 1 ppm (5.26×10^{-5} mol l^{-1}), served as a positive physiological control. Parallel groups (B_1, B_2, and B_3) were exposed to drinking water at the same, above stated, fluoride concentrations, but with the addition of aluminium ions at the physiological concentration of 10 ppm (3.7×10^{-4} mol l^{-1}), according to standards of JECFA (Joint Expert Committee on Food Additives under Food and Agriculture World Organization, 1998), which was the same in all B groups. B_1 group served as a physiological positive double control due to presence of both ions at the physiological concentrations. Experimental

and control groups were represented by male mice. They were exposed to experimental ion concentrations postnataly from day 1 (via mothers' milk), and further through drinking water, continuously for 3 months.

Capacitation

Spermatozoa were recovered from the cauda epididymidis by placing its distal region into a M2 in vitro fertilizing medium for 10 min to incubate at 37 °C in 5 % CO_2 in air. Sperm stock was diluted for the required concentration (5×10^6 ml^{-1}) into a 100-µl drop of M2 medium under paraffin oil in 35-mm Petri dishes. The motility of the sperm population was checked by a light-inverted microscope with a thermostatically controlled stage at 37 °C. Spermatozoa were capacitated for up to 3 h. At half-hour intervals, a drop of spermatozoa suspension was collected, washed in tris-buffered saline (TBS), and smeared onto glass slides.

Tyrosine phosphorylation

Smears of sperm after 30, 60, 120, and 180 min of capacitation in vitro were blocked with 10 % bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1 h, then washed once in PBS and then followed by a 2-h incubation with mouse monoclonal antibody anti-phosphotyrosine (mAb) in 1:200 dilutions. After washing off the primary antibody mAb in PBS, slides were incubated with the secondary antibody Alexa Fluor 488, goat anti-mouse IgG for an hour. Then slides were evaluated under an Olympus epifluorescent microscope for sperm-head phospho-tyrosine staining.

Acrosome reaction

The acrosome reaction was induced at 60, 120, and 180 min of capacitation in vitro by the calcium ionophore A-23187 at a final concentration of 5 µmol l^{-1}, followed by an hour of incubation at 37 °C in 5 % CO_2 in air. The status of the acrosome was evaluated 5 min after adding 2.5 µmol l^{-1} peanut agglutinin (PNA) lectin. Two hundred cells were counted for each group and experimental time point under an Olympus epifluorescent microscope. For further experimental details, see Dvořáková-Hortová et al. (2008).

Application of kinetic analysis

The research and solution of reaction schemes by kinetic analysis (Frost and Pearson 1961) of either the rate equations or their integrated forms are used frequently for homogeneous chemical reactions, especially when they are assumed to be parts of the overall mechanism of an unstable substance, which cannot be directly demonstrated. Even complex reaction mechanisms can also be solved by methods of kinetic analysis (Tockstein 1987, 1992).

In this work, it was investigated whether chemical kinetics, which is valid for homogeneous chemical reactions, can be applied on the experimental data obtained during the capacitation of sperm in vitro. It can be solved by kinetic analysis using either the analysis of rate equations (the dependence of dc_i/dt on various parameters, so-called differential data) or the comparison of experimental $[c_i = f(t)]$ points with theoretical data, obtained either by numerical integration of a set of differential equations or by analytical expression of known function $c_i = f(t)$. In our case, differential data (dc_i/dt) were not available; therefore it was necessary to compare experimental $c_i = f(t)$ data with kinetic theoretical equation created for the given purpose. QBasic software was used for the calculation.

Results and discussion

The published data, taken from the Dvořáková-Hortová et al. (2008), are summarized in Table 1. To enhance the potential for comparison, this publication maintains the same marking of the inhibitors as in Dvořáková-Hortová et al. (2008), however, the concentration composition (originally given in ppm) is expressed in molarities.

On the basis of these data, it is evident that the positive reaction, i.e., the number of protein *pTyr* positive sperm, is a function of two independent variables, which is, the time and the inhibitor concentration, and these variables do not act simultaneously. Therefore, the two phases of the process must be differentiated, namely, the inactivation and the capacitation phases. To maintain a suitable sequence of computations, the capacitation will be addressed first.

Capacitation monitored by pTyr

The shape of the *pTyr* (%) dependence on sperm capacitation time exhibiting an extreme and then, after a certain time, very close values for all the samples (see Table 1) provides a basis for the following scheme: Some sperm contain C_n centers (in the total number C_{no}), which are converted to C_a centers (for list of abbreviations, see Electronic Supplemental Data). Only these C_a centers can be phosphorylated. However, the C_a center is unstable and gradually decomposes to a C_d center. This process can be described by the following system of kinetic equations with the initial conditions for $t = 0$: $C_n(0) = C_{no}$, $C_a(0) = 0$, $C_d(0) = 0$, (Eqs. 1a, 1b):

$$-\frac{dC_n}{dt} = K_1 C_n \tag{1a}$$

Table 1 Fluoride (c_F) and aluminium (c_{Al}) ion concentrations administered to mice, percentage of positive tyrosine-phosphorylated mouse sperm in the acrosome head region during capacitation in vitro, and percentage of acrosome-reacted sperm induced by calcium ionophore (taken Dvořáková-Hortová et al. 2008) ppm converted to mol l^{-1})

Group	$c_F + c_{Al}$ (mol l^{-1})	Tyrosine-phosphorylated sperm (*pTyr*, %), Capacitation time				Acrosome-reacted sperm (*AR*, %), Capacitation time		
		30 min	60 min	120 min	180 min	60 min	120 min	180 min
C	0 + 0	11.12 ± 0.45	14.72 ± 0.47	13.29 ± 0.32	8.66 ± 0.25	71.75 ± 2.20	85.16 ± 1.42	90.16 ± 0.88
A_1	$5.26 \times 10^{-5} + 0$	8.66 ± 0.54	12.66 ± 0.51	11.86 ± 0.37	7.00 ± 0.30	79.23 ± 2.88	74.73 ± 1.52	88.57 ± 0.71
A_2	$52.6 \times 10^{-5} + 0$	**5.44 ± 0.40**	**9.26 ± 0.35**	**9.50 ± 0.32**	7.21 ± 0.33	69.09 ± 1.98	80.32 ± 0.92	85.34 ± 1.12
A_3	$526 \times 10^{-5} + 0$	**5.94 ± 0.24**	**9.68 ± 0.64**	**10.16 ± 0.42**	7.60 ± 0.45	84.99 ± 4.07	91.09 ± 0.85	89.35 ± 1.40
B_1	$5.26 \times 10^{-5} + 3.7 \times 10^{-4}$	**6.10 ± 0.95**	**9.57 ± 1.39**	**9.84 ± 0.43**	7.74 ± 0.40	68.76 ± 1.34	75.46 ± 1.46	89.86 ± 0.99
B_2	$52.6 \times 10^{-5} + 3.7 \times 10^{-4}$	**5.90 ± 0.61**	**8.84 ± 0.39**	**8.82 ± 0.71**	**5 97 ± 0.83**	71.48 ± 1.66	84.41 ± 2.42	83.99 ± 2.14
B_3	$526 \times 10^{-5} + 3.7 \times 10^{-4}$	**2.60 ± 0.73**	**4.65 ± 0.34**	**4.20 ± 0.64**	**4.55 ± 0.89**	73.21 ± 4.52	87.57 ± 2.64	86.69 ± 1.79

Mean ± standard error. Data in bold express significant statistical difference (p level <0.05) between control (C) and other experimental groups for capacitation time 30, 60, 120, and 180 min

$$\frac{dC_a}{dt} = K_1 C_n - K_2 C_a \tag{1b}$$

The solution of the system of linear differential equations for C_a has the following form (Eq. 2a):

$$C_a = C_{no} \frac{K_1}{K_1 - K_2} \left(e^{-K_2 t} - e^{-K_1 t} \right) \tag{2a}$$

If C_a and C_{no} values are related to 100 sperm, then they are expressed as percentages. On setting the first derivative equal to zero, it then holds in the extreme that (Eq. 2b)

$$\frac{dc_a}{dt} = 0 \Leftrightarrow t_{ex}, t_{ex} = \frac{1}{K_1 - K_2} \ln \frac{K_1}{K_2} \tag{2b}$$

In the inflex point, on setting the second derivative equal to zero, it holds that (Eq. 2c)

$$\frac{dc_a}{dt^2} = 0 \Leftrightarrow t_{inf}, \; t_{inf} = 2t_{ex} \tag{2c}$$

Curves were constructed through the experimental points given in Table 1 for groups C, A_1, A_2, and A_3 and constants K_1, K_2, and C_{no} were optimized to attain the best agreement of the experiment with the theory. The results obtained are listed in Table 2 (except for group A_3) and shown in Fig. 1. The agreement of the experimental points (group C, without intoxication by selected fluoride concentrations) with the theoretical $C_a(t)$ curve is very close, as shown by Fig. 1, curve 1. This demonstrates that the above model of capacitation in the absence of fluorides corresponds to reality. The good agreement of experimental points with the $C_a(t)$ curves is observed also for the intoxicated groups A_1 and A_2 (Fig. 1, curves 2 and 3, respectively). It can further be seen (Table 2) that constants K_1 and K_2 are very weakly affected by the presence of fluorides. On the other hand, the C_{no} value is affected substantially. It was found that the initial C_{no} values decreased through the inhibitors effect and the C_{no} value in the absence of fluorides denoted the maximum percentage of sperm capable of activating signaling pathways leading to pTyr.

However, the results obtained for group A_3 (the highest tested fluoride concentration) differ and do not follow the

Table 2 The computed values of rate constants K_1, K_2, and C_{no} values (for the composition of the inhibitors, see Table 1)

Group	K_1 (min^{-1})	K_2 (min^{-1})	C_{no} (%)
C	1.40×10^{-2}	1.36×10^{-2}	40
A_1	1.13×10^{-2}	1.29×10^{-2}	38
A_2	1.05×10^{-2}	1.10×10^{-2}	27

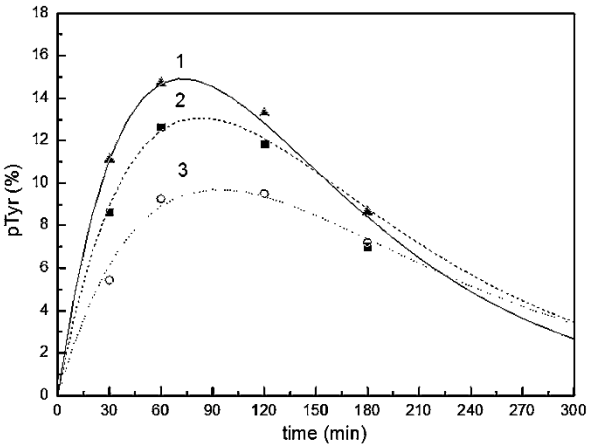

Fig. 1 The dependence of percentage of sperm *pTyr* on capacitation time for groups C (*curve 1*), A_1 (*curve 2*), and A_2 (*curve 3*). Inhibitors: fluoride ions, Curves: *1* $c_F = 0$ mol l^{-1}, *2* $c_F = 5.26 \times 10^{-5}$ mol l^{-1}, *3* $c_F = 52.6 \times 10^{-5}$ mol l^{-1}

trend described for groups C, A_1, and A_2. The C_{no} value for group A_3 is almost the same as the value obtained for group A_2, while the theoretical value (calculated from Eq. 3c) is 6.88. The results obtained for group A_3 may be explained by a well-known biological mechanism, so-called 'paradox effect' or 'dose-dependent effect' of certain substances including fluorides (Burgstahler 2002), where lower concentrations trigger a higher response of the organism compared to exposition to high or extreme concentrations.

Inactivation

Inactivation of C_{no} centers in sperm is manifested by intense decrease of C_{no} values with increasing concentration of fluorides (see Table 2). Various models of inactivation were investigated and finally the Langmuir isotherm was found to describe the dependence of C_{no} on $[F^-]$ very well (see Eq. 3a).

$$Z_s = \frac{Z}{1 + w[F^-]} \tag{3a}$$

where $C_{no}([F^-]) = Z_s$, $C_{no}([F^-] = 0) = Z$ and w can stand for an inactivation coefficient. This hyperbolic function has the advantageous property that the reciprocal value of $1/Z_s$ versus $[F^-]$ yields a straight line with intercept $1/Z$ and slope w/Z (see Eq. 3b) and satisfaction of this can be readily controlled.

$$\frac{1}{Z_s} = \frac{1}{Z} + \frac{w}{Z}[F^-] \tag{3b}$$

The results of plotting $1/C_{no}$ versus concentration $[F^-]$ exhibit a linear shape. The origin fluoride concentration 1 ppm (Dvořáková-Hortová et al. 2008) corresponds to the concentration $c_F = [F^-] = 5.26 \times 10^{-5}$ mol l^{-1}. The results can be fitted to the straight line P_1, (Eq. 3c) with the intercept 1/40 where the slope 22.88 equals $w/40$. Therefore, for fluorides it holds $w_o = 915$ l mol^{-1}. Relationship (Eq. 3a) is confirmed, and thus the proposed inactivation mechanism by fluorides is confirmed. For the highest fluoride concentration ($c_F = 526 \times 10^{-5}$ mol l^{-1}), C_{no} should equal 6.88. This value is in very good agreement with $C_{no} = 7$ obtained for the same analytical concentration of fluorides (c_F) in group B_3 (see Table 3).

$$\frac{1}{C_{no}} = \frac{1}{40} + 22.88[F^-] \tag{3c}$$

Table 3 The optimized values of constants K_1, K_2, and C_{no} values (for the composition of the inhibitors, see Table 1)

Group	K_1 (min^{-1})	K_2 (min^{-1})	C_{no} (%)
B_1	0.81×10^{-2}	1.4×10^{-2}	38
B_2	1.1×10^{-2}	1.3×10^{-2}	27
B_3	2.0×10^{-2}	0.4×10^{-2}	7

The common effect of fluoride and aluminium ions

The use of Eq. 2a for the data in groups B_1, B_2, B_3 and optimization of constants K_1, K_2 and C_{no} yield the following results given in Table 3 and depicted in Fig. 2. Similar trends (as for groups A but without any exception) were obtained for the groups B_1, B_2, and B_3 intoxicated by fluoroaluminates and it was found again that the initial C_{no} values decreased through the inhibitors effect.

In the above cases, the accurate equilibrium concentration of free fluoride ions, determined by their analytical concentration, $[F^-] = c_F$, was known. This does not hold in the presence of aluminium ions because the distribution between free fluoride and aluminium ions and their complexes is not known. The literature (Harvey 2000) describes AlF_i complexes with overall stability constants $\beta_i = [AlF_i]/[Al] \times [F^-]^i$ for $i = 1$ to 6 with values $\beta_1 = 10^{6.1}$, $\beta_2 = 10^{11.1}$, $\beta_3 = 10^{14.0}$, $\beta_4 = 10^{17.0}$, $\beta_5 = 10^{18.4}$, $\beta_6 = 10^{18.8}$, and $\beta_0 = 1$. The composition is computed as follows: AlF_i are expressed by means of overall stability constants β_i and substituted into the expression for analytical concentrations c_F (Eq. 4a) and c_{Al} (Eq. 4b)

$$c_F = [F^-] + \sum_{i=1}^{6} i[AlF_i] \tag{4a}$$

$$c_{Al} = \sum_{i=0}^{6} [AlF_i] \tag{4b}$$

and from Eq. 4b, $[Al^{3+}]$ is expressed by Eq. 4c, where $\beta_0 = 1$

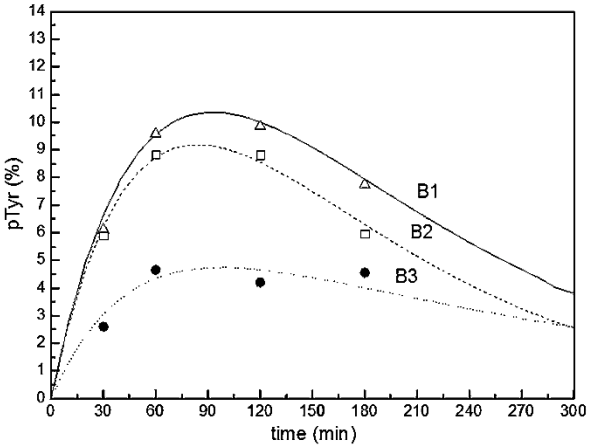

Fig. 2 The dependence of percentage of sperm *pTyr* on the capacitation time for groups B_1, B_2, and B_3. Inhibitors: fluoride and aluminium ions (their analytical concentrations are given in Table 1)

Table 4 The computed compositions of solutions supplied to experimental groups B_1, B_2, and B_3; the concentrations are given in mol l^{-1}; $n = c_{Al}/c_F$

Input parameters	B_1	B_2	B_3
C_{no}	38	27	7
c_F	5.26×10^{-5}	52.6×10^{-5}	526×10^{-5}
c_{Al}	3.7×10^{-4}	3.7×10^{-4}	3.7×10^{-4}
n	7	0.7	0.07
Equilibrium concentrations of inhibitors			
$[Al^{3+}]$	3.18×10^{-4}	1.74×10^{-5}	1.15×10^{-11}
$[F^-]$	1.25×10^{-7}	8.43×10^{-6}	3.87×10^{-3}
$[AlF^{2+}]$	5.13×10^{-5}	1.89×10^{-4}	5.71×10^{-8}
$[AlF_2^+]$	6.57×10^{-7}	1.63×10^{-4}	2.26×10^{-5}
$[AlF_3]$	6.24×10^{-11}	1.04×10^{-6}	6.62×10^{-5}
$[AlF_4^-]$	7.81×10^{-15}	8.78×10^{-9}	2.56×10^{-4}
$[AlF_5^{2-}]$	2.46×10^{-20}	1.86×10^{-12}	2.49×10^{-5}
$[AlF_6^{3-}]$	7.72×10^{-27}	3.94×10^{-17}	2.41×10^{-7}

$$\left[Al^{3+}\right] = \frac{c_{Al}}{\sum_{i=0}^{6} \beta_i \left[F^-\right]^i} \tag{4c}$$

and substituted into Eq. 4a for c_F, where only one unknown $[F^-]$ remains. After rearrangement and introduction of symbols $c_{Al}/c_F = n$, $M_i = \beta_i \times c_F^i$ and relative concentration $\delta = \left[F^-\right]/c_F$ a seventh-grade equation is obtained for δ, i.e., Eq. 4d, where $M_0 = 1$ and $M_7 = 0$

$$\sum_{i=1}^{7} \delta^i \left[M_{i-1} + M_i(i \times n - 1)\right] - 1 = 0 \tag{4d}$$

For the given values of M_i a n, the root of δ is found by the applying of bisection method with a precision of five decimal places in the pre-exponential factor. This yields the concentrations of free fluoride ions $[F^-] = \delta \times c_F$, $[Al^{3+}]$ (Eq. 4c) and $[AlF_i] = \beta_i \cdot [Al^{3+}] \times [F^-]^i$. The results for groups B_1, B_2 and B_3 are listed in Table 4.

Mixed inactivation

During simultaneous action of two or more inhibitors I_0, I_1, I_2 etc., the Langmuir isotherm can be written as Eq. 5a:

$$Z_s = \frac{Z}{1 + \sum_{i=0}^{r} w_i I_i} \tag{5a}$$

where $C_{no}([I_1], [I_2]...) = Z_s$, $C_{no}([I] = 0) = Z$ and w_i is an inactivation coefficient. Because the C_{no} values measured for fluorides alone are identical to those for fluorides in the presence of aluminium ions (see Tables 2, 3), it can be written that (Eq. 5b):

$$\frac{Z}{1 + w_0 c_F} = \frac{Z}{1 + w_0 \left[F^-\right] + \sum_{i=1}^{6} w_i [AlF_i]} \tag{5b}$$

After conversion to the reciprocal values, Eq. 5c can be written:

$$w_0 c_F = w_0 \left[F^-\right] + w_1 \left[AlF^{2+}\right] + w_3 \left[AlF_2^+\right] + \cdots + w_6 \left[AlF_6^{3-}\right] \tag{5c}$$

Therefore, three values are available for c_F, so that Eq. 5c represents a set of three equations for six unknowns, w_1 to w_6. This cannot be solved. Approximate values of some coefficients w can be obtained by the following procedure. It follows from column B_3 of Table 4 that the main components of the solution are $[F^-] = 3.87 \times 10^{-3}$ mol l^{-1} and $[AlF_4^-] = 2.56 \times 10^{-4}$ mol l^{-1} ions. If focused on these two components, then w_4 can be computed for $[AlF_4^-]$ from Eq. 5c, as w_0 is known. The calculation yields $w_4 = 4992$ l mol^{-1}. Hence, $[AlF_4^-]$ is found to be a five-times more effective inhibitor than $[F^-]$. It is found analogously from column B_1 that the only substantial component is $[AlF^{2+}]$ (5.13×10^{-5} mol l^{-1}) and we obtain $w_1 = 938$ l mol^{-1} from Eq. 5c. Finally, it can be seen from column B_2 that the predominant compounds of the solution are $[AlF^{2+}]$ (1.89×10^{-4} mol l^{-1}) and $[AlF_2^+]$ (1.63×10^{-4} mol l^{-1}) and thus $w_2 = 1869$ l mol^{-1} is obtained from Eq. 5c using coefficient w_1. The values of inactivation coefficients (w) calculated for the individual inhibitors show that the inhibitor efficiency increases with increasing fluoride content in the complex with aluminium ions.

Capacitation monitored by the follow-up AR

The published data (Dvořáková-Hortová et al. 2008) of percentage of sperm that underwent induced AR (summarized in Table 1) indicate monotonous growth with increasing capacitation time, as can be seen in Fig. 3. Two very close exponential functions were selected and it can be seen that all the experimental data are contained in this narrow channel, so that hopefully the time dependence of AR (%) vs. t could be expressed for each group A_{1-3} and B_{1-3} by an exponential curve with its own K_1 value. As capacitation is a kinetically first-order reaction (see above) in which the loss of the principal substance W corresponds to the monotonously increasing function, generally expressed by (Eq. 6)

$$W - W(t) = W\left(1 - e^{-Kt}\right) \tag{6}$$

and thus represents the actual value of all the capacitated sperm at time t, it is easy to correlate the time course of the AR with the number of capacitated sperm, e.g., Eq. 7a

$$\%AR = (100 - N)\left(1 - e^{-Kt}\right) \tag{7a}$$

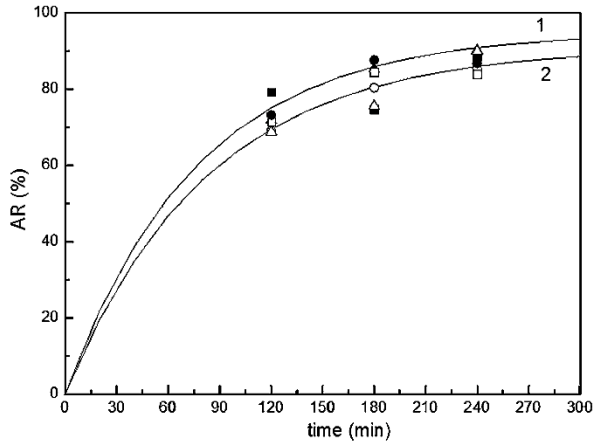

Fig. 3 The dependence of percentage of sperm that completed induced *AR* on monitored time; Point labeling according to groups C (*filled triangles*), A_1 (*filled squares*), A_2 (*unfilled circles*), B_1 (*unfilled triangles*), B_2 (*unfilled squares*), and B_3 (*filled circles*). The curves are plotted according to Eq. (7a), curve (1) for parameters $N = 5$, $K_1 = 1.3 \times 10^{-2}$ min^{-1} and curve (2) for parameters $N = 9$, $K_1 = 1.2 \times 10^{-2}$ min^{-1}

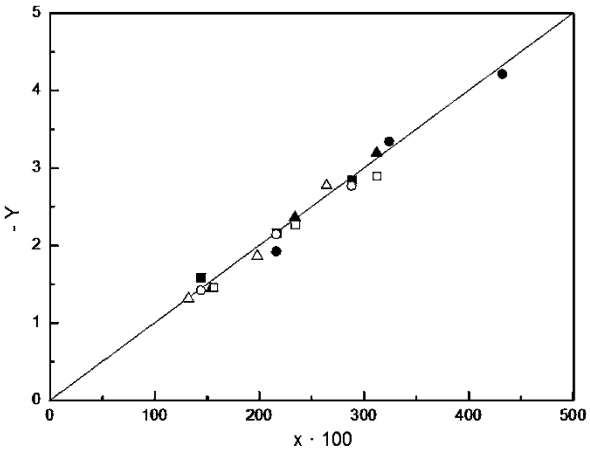

Fig. 4 Linearization of the kinetic equation for the first-order reaction in coordinates Y, X for experimental data, the points are denoted as in Fig. 3. Y-axis was labeled as negative (-Y) in order to present the numbers in standard positive values. The application of higher numerical values X·100 was used in order to present better visualization of the individual points on the X-axis

where N is percentage of sperm that is completely incapable of capacitation.

Because rate constants K_1 for conversion of C_n to C_a centers during capacitation (Eq. 1a; Tables 2, 3) were found for various groups of inhibitors from the data on protein tyrosine phosphorylation, these constants should also describe the course of the AR in the presence of the inhibitors used. For this purpose, the data obtained after a 60-min calcium ionophore inductor action, when the AR was evaluated, were used (Dvořáková-Hortová et al. 2008). Therefore, it was first investigated, whether the experimental data fulfil Eq. 7a, in the best way after conversion to a linear shape in the coordinates of $-\mathrm{Y} = \ln(1-AR/(100\text{-}N))$, $\mathrm{X} = K_1 \times t$ in which a straight line should be obtained, passing through the origin with slope 1. The basic value K_1, required for the formation of products $K_1 \cdot t$ is obtained by comparison of the exponential functions for $t = 240$ and 120 min, Eqs. 7b, 7c.

$$\frac{AR_{(240)}}{100-N} = 1 - e^{-240K_1} \tag{7b}$$

$$\frac{AR_{(120)}}{100-N} = 1 - e^{-120K_1} \tag{7c}$$

Division of both Eqs. 7b,c yields Eq. 7d

$$\frac{AR_{(240)}}{AR_{(120)}} = \frac{1-e^{(-120K_1)^2}}{1-e^{-120K_1}} = 1 + e^{-120K_1} \tag{7d}$$

and thus Eq. 7e

$$-\ln\left(\frac{AR_{(240)}}{AR_{(120)}} - 1\right) = 120K_1$$
$$X = K_1 t = -\ln\left(\frac{AR_{(240)}}{AR_{(120)}} - 1\right) \times \frac{t}{120} \tag{7e}$$

Then it was possible to optimize K_1 so that point $AR_{(180)}$ yields the best possible fit for the experimental value. The result can be seen in Table 5 and Fig. 4 and it is confirmed that Eq. 7a is satisfied in all groups A_{1-3} and B_{1-3}.

Optimized rate constants K_1 for groups A_{1-3} and B_{1-3} were compared with the corresponding values found during protein tyrosine phosphorylation, and the results are given in Table 6. As shown in Table 6, the rate constants obtained for pTyr and AR (for the individual inhibitors) are virtually the same (again with an exception of group A_3) and the capacitation

Table 5 The coordinates for linearization of the first-order reaction

C		A_1		A_2		B_1		B_2		B_3	
−Y	X·100	−Y	X·100	−Y	X·100	−Y	X·100	−Y	X·100	−Y	X·100
1.44	154	1.59	144	1.42	144	1.31	132	1.46	156	1.92	216
2.36	234	2.16	216	2.14	216	1.86	198	2.27	234	3.34	324
3.19	312	2.85	288	2.77	288	2.77	264	2.90	312	4.21	432

Table 6 Comparison of the optimized values of rate constant K_1 for pTyr and AR, where N is percentage of the number of capacitation incapable sperm

	C	A_1	A_2	A_3	B_1	B_2	B_3
$K_1 \times 10^2$ (min^{-1}) for pTyr	1.4	1.1	1.1	1.0	0.8	1.1	2.0
$K_1 \times 10^2$ (min^{-1}) for AR	1.3	1.2	1.2	2.5	1.1	1.3	1.9
N (%)[a]	6	6	~10	8	6	9	~13

[a] Obtained by optimization according to Eq. 7a

is the rate determining step for all the following processes. It can be further seen that the inhibitors increase the percentage of sperm incapable of capacitation N (they do not undergo the AR) and affect the rate of capacitation only slightly.

It must be emphasized that both the types of constants K_1 were each computed from a different curve (curves with an extreme, curves with a monotonous increase) and a different equation and from various experiments. Their good agreement demonstrates the correctness of the kinetic formulation for the shape of the capacitation. The agreement of values for both types of K_1 (see Table 6) verifies that the course of capacitation itself is the same in both cases. The slight differences between K_1 values reflect only the way, how capacitation was terminated.

Conclusions

Even though the topic is very complicated and experimentally demanding, the experimental data (obtained by Dvořáková-Hortová et al. 2008) are extremely well consistent with the proposed theory. The experimental points are in very good agreement with the shape of the theoretical $C_a(t)$ curves and this fact verifies the mechanism of the mouse sperm capacitation kinetics. The time dependences of pTyr satisfy the equation, which is valid for intermediate in two consecutive first-order reactions. The suggested kinetic equations discover the existence of C_n centers in sperm, and their unstable active C_a forms, which can be only phosphorylated and gradually decompose to C_d centers. The time corresponding to the maximal production of the unstable intermediate is probably most suitable for sperm obtaining the ability to fertilize the egg.

A transfer to the fulfilled linear dependence verifies the basic inactivation equation according to Langmuir isotherm. The inhibition coefficients indicate that the inhibitor efficiency increases with increasing fluoride content in the complex with aluminium ions. This supports the theory of the increased biological effect of fluorides in the presence of aluminium ions, where fluorometallic complexes behave analogously to phosphate groups (Chabre 1990) and these complexes imitate G-proteins (Sternweis and Gilman 1982) and inhibit their signaling activity.

Rate constants K_1 and K_2 do not exhibit significant differences among the groups, which demonstrate that the rate of capacitation is only slightly affected by the previous inhibition, which only decreases the initial C_{no} values.

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Compliance with ethical standards

Conflict of interest All authors of this manuscript (Zuzana Bosakova, Antonin Tockstein, Hana Adamusova, Pavel Coufal, Natasa Sebkova, and Katerina Dvorakova-Hortova) declare that no competing financial interests exist.

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PUBLIKACE II

DETERMINATION OF 17β-ESTRADIOL AND 17α-ETHYNYLESTRADIOL IN MOUSE FERTILIZING M2 MEDIUM

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Determination of 17β-Estradiol and 17α-Ethynylestradiol in Mouse Fertilizing M2 Medium

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Keywords
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Environmental estrogens (natural and synthetic) belong to a group of contaminants called endocrine disrupting chemicals [1]. These compounds have adverse effect on the endocrine system of human and animals. They can bind to natural estrogen receptors or block synthesis of endogenous hormones [2–4]. Estrogens are not accumulated in the environment and present at very low concentrations (ng/L), their impact on aqueous organisms and other wildlife and finally on humans could be significant [5]. 17β-Estradiol is a natural endocrine disruptor primarily of female origin. The synthetic 17α-ethynylestradiol is used as a main part of birth control pills. It was shown that estradiol and several estrogenic xenobiotics act towards an increase of germ cell apoptosis and a decrease of sperm count [6].

Capacitation is the key event in the study of sperm behavior prior to fertilization. Only capacitated sperm are sufficiently active and able to fertilize. In vitro experiments, simulating precisely an in vivo environment, are crucial for closer understanding of the process, when sperm gain the ability to fertilize an ovum. It is difficult to study these events in vitro. In order to monitor the effects of exogenous estrogen hormones such as 17β-estradiol and 17α-ethynylestradiol on sperm during in vitro capacitation, it is important to develop analytical method for determination of 17β-estradiol or the 17α-ethynylestradiol in M2 laboratory mouse in vitro fertilizing medium.

Our previous study [7] was focused on determination of free estriol in M2 medium during capacitation of mouse sperm in vitro by HPLC with UV detection. Due to the progressive UV absorption of BSA present in the fertilizing medium [8], the detection of estriol was carried out at 200 nm. Even though this method provided relatively high values of limit of detection and limit of quantification, it enabled only the starting concentration of 200 μg/L to be tested.

Based on the results reported for the separation of estriol by HPLC with UV detection [7], the reversed-phase separation system using SunFire C18 column as the stationary phase was selected for study of both the tested analytes. This column provided a high separation efficiency and symmetrical peak. For determination of 17β-estradiol and 17α-ethynylestradiol in M2 fertilizing medium, the UV detection was applied as the first one. Mixtures of acetonitrile and water in different volume ratios were tested as mobile phases. The optimization procedure was carried out with respect to obtain a sufficient sensitivity. However, neither 17β-estradiol nor 17α-ethynylestradiol was detected in any of tested mobile phases. The M2 medium is a complex mixture containing inorganic and organic components from which especially BSA (4.0 g/L) can cause difficulties during the separation process. At 200 nm, the signal of M2 medium was two orders of magnitude higher than the signal of analyte. Therefore, it was impossible to use the UV detection for this purpose and a tandem mass spectrometric detection, which can eliminate the matrix impact much better, was chosen.

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PUBLIKACE III

17β-ESTRADIOL SIGNALLING IS DIRECTED BY AUTOCATALYTIC REACTION IN CAPACITATING SPERM

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17β-estradiol signalling is directed by autocatalytic reaction in capacitating sperm

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Running title: Kinetic analysis of estradiol signalling in sperm.

ABSTRACT

17β-estradiol (estradiol) is a natural estrogen regulating reproduction including sperm and egg development, sperm maturation called capacitation and sperm-egg communication. However, its high doses can act towards an increase of germ cell apoptosis and decrease of sperm count. Mature spermatozoa are responsive to steroid hormones *via* passive diffusion or through membrane and cytoplasmic receptors enabling them to exert fast non-genomic response and activate crucial signalling pathways. In order to monitor time and concentration dependent binding dynamics of extracellular estradiol during mouse sperm capacitation, a high performance liquid chromatography with tandem mass spectrometry was used to measure sperm response. The course of sperm behaviour was subjected to kinetic analysis, which showed that estradiol is transiently adsorbed on the plasma membrane surface and further displays a signalling autocatalytic pattern. This autocatalytic reaction could explain the crosstalk between both receptor and non-receptor pathways suggesting a novel mechanism utilized by sperm prior to fertilization.

Keywords: 17β-estradiol, sperm, capacitation *in vitro*, HPLC MS/MS, kinetics, autocatalysis

INTRODUCTION

The estrogen cell signalling is a crucial event, and the mechanism is still covered by a veil of mystery. An interaction between the most influential of estrogens called 17β-estradiol (estradiol) and its estrogen receptors (ERs) has been described not only through nuclear receptors (nER) but also membrane (mER) and cytoplasmic (cER) ones. (1). These receptors might be either of the same composition, just translocated form the nucleus to the membrane (2, 3), or they may represent a novel kind of ERs (4, 5, 6). Beside the estrogen-based receptors, the ability of non-ER membrane-associated proteins to bind and transfer estradiol across the plasma membrane (PM) has been described (7). On the top of transportation receptors, the estradiol is also able to penetrate and pass through the PM without any other help (8, 9). Things get even more complicated by introducing two routes of estradiol action, the slow genomic and rapid non-genomic one. The generally excepted model is built on the binding of the intracytoplasmic estradiol to the well-described and understood nER, targeting DNA sequences known as estrogen response elements (EREs) (10) or DNA-binding transcription factors (11, 12, 13) both leading to transcriptional activation of the associated genes (14, 15). This route of action falls into the genomic category and requires cell transcriptional activity and time, which none of these mature spermatozoa possess. In this case, the second non-genomic pathway remains to be the only option for sperm to take, in order to regulate the signalling pathways that are crucial for the maturation processes so called capacitation (16), and which happen in the mammalian female reproductive track. In general, capacitation involves membrane rearrangement, cholesterol efflux, activation of specific signal transduction pathways leading to protein tyrosine phosphorylation and cytoskeleton rearrangements (17, 18, 19). It has been demonstrated that estrogens significantly modulate this process and can also increase its speed (20, 21, 22, 23, 24). For obvious reasons it is difficult to study capacitation events *in vivo*, therefore, understanding the precise *in vitro* conditions, including dynamics, speed and amount of exogenous estrogens that are bound to sperm receptor during capacitation, is needed. Moreover, the mathematical interpretations and general quantified process dynamics would bring another perspective to help explain this biological event. Kinetic analysis data would also serve as a tool for predicting the general hormone-receptor mechanism and provide a new insight into the selected biological reaction. At the moment, the scientific community knowledge stretches as far as to a description of the individual signalling scenarios in both somatic and male germinal cells, but 'what is the mechanism of the action' remains to be answered.

This work aimed: firstly, to develop a sensitive analytical high performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS) method for the determination of total unbounded estradiol concentration in the sperm capacitation *in vitro*; secondly, to deliver, whether the concentration of estradiol versus the time of ongoing capacitation fits to an equation or a system of equations of chemical kinetics. Applying a kinetic analysis to our data obtained by HPLC-MS/MS, measuring the estradiol dynamics during mouse sperm capacitation, leads us to propose a unique autocatalytic estradiol signalling, which could unify all the estradiol actions into one complex event.

RESULTS

HPLC measurement

Based on our previous results reported for the analysis of the selected estrogens in environmental water samples (25) and for the separation of estriol in M2 fertilizing medium (26), HPLC-MS/MS with a SunFire C18 column as the stationary phase was selected for the monitoring of estradiol concentration during mouse sperm capacitation. The optimization procedure was carried out to obtain a high sensitivity. Isocratic elution at a flow rate of 0.8mL/min with the binary solvent system consisted of 0.1% HCOOH in H_2O and 0.1% HCOOH in 100% ACN, 50/50 (*v/v*) was selected. The total analysis run time was 8.0 min. The column temperature was held at 21± 0.5°C. The sample injection was 20µL.

All the instrumental MS-MS parameters were optimized according to previous work (25). The optimimized ESI (+) conditions in MRM mode for estradiol were the following: the capillary voltage 5500V, the nebulizer pressure 60 psi, the gas temperature 350°C, and the nitrogen flow rate 10L/min. The m/z 255.5 > 158.9 transition (fragmentor voltage: 120V, collision energy: 14V) was monitored for estradiol, and the m/z 258.5 > 158.9 transition (fragmentor voltage: 120V, collision energy: 14 V) for deuterated estradiol (estradiol-d_3).

Under optimized separation and detection conditions, the calibration curve for estradiol was measured in the concentration range of 0.5 - 250µg/L and the analyte was tested within a linearity range from LOQ to 250µg/L. Each measurement of the peak area (peak height) being carried out in 5 replicates and the results of the linear regression for peak area versus concentration dependence are listed in Table 1. A satisfactory fit between the experimental points and linear calibration curve was observed. The peak height–concentration dependence was treated by linear regression to determine the limit of detection (LOD) and limit of quantitation (LOQ), as triple and ten-times noise level, respectively. The values obtained for LOD and LOQ are presented in Table 1 as well.

136

137 **Capacitation *in vitro***

138 In order to monitor the effects of estradiol on sperm capacitation *in vitro*, it was essential
139 to find out how much of estradiol, added into M2 *in vitro* capacitating/fertilizing medium, is
140 available for sperm. One of the main parts of this medium is bovine serum albumin (BSA)
141 and estrogens can bind to it, especially during *in vitro* incubation, due to the BSA-estrogens
142 affinity which has been described (27, 28) Therefore, it was important to determine how much
143 of estradiol is bound to BSA, therefore, how much there is actually left for the sperm to
144 utilise. This was determined for all three tested estradiol concentrations (200, 20 and 2μg/L)
145 after 1h *in vitro* sperm incubation in M2 medium (at 37°C, 5% CO_2). These calculated values
146 were taken as the entering concentrations at capacitation time 0 and the dependencies of total
147 unbounded estradiol concentration during capacitation were measured. The process of
148 capacitation was monitored in time range 0 - 180min with a 30min interval between the
149 samples collecting. Extended time over 180min has no physiological relevance, as the
150 capacitation of mouse sperm *in vivo* is achieved within 90min (180min at the latest). Each
151 experiment was carried out for samples with added mouse sperm and for reference samples
152 (blanks) without sperm. HPLC-MS/MS measurements of each sample were done in 5
153 replicates with very good repeatability (the RSD values up to 2.1%). The mean concentration
154 values (*C*, μg/L) of total unbounded estradiol, calculated from five individual samples
155 obtained at each capacitation time, are shown in Figure 1. Solid circles depict the experiments
156 with BALB/c mouse sperm, solid squares with C57BL/6Nvel mouse sperm and open triangles
157 represent the blanks.

158 As it can be seen from Figure 1A – C, all the tested estradiol concentrations in blank
159 samples remain practically constant (open triangles) during capacitation. For samples with the
160 addition of sperm (solid circles and squares), similar trends were obtained for all the tested
161 concentrations (2, 20 and 200μg/L) and both spermatozoa of BALB/c or C57BL/6Nvel mouse
162 laboratory strains. In general, the concentration of total unbounded estradiol decreases to
163 reach its minimum and then increases again. However, the position of the minimum differs
164 for the individual tested estradiol concentrations. For the starting concentration 200μg/L
165 (Figure 1A) the straightforward decrease occurs in the beginning of capacitation time within 0
166 - 30min. The drop of 20μg/L starting concentration can be observed later within 60 – 90min
167 (Figure 1B) and the dependence obtained for starting concentration 2μg/L exhibits its
168 minimum between 150 - 180min (Figure 1C). Only slightly different extents of decline were
169 observed between two different strain origins of spermatozoa within the individual tested

estradiol concentration (solid circles for BALB/c sperm versus solid squares for C57BL/6Nvel sperm).

Kinetic analysis

In the light of the fact that kinetic analysis has already been successfully used for analysis of data describing the reduced capacitation ability of sperm (29), it has also been employed to analyse the reaction of sperm with estradiol. For better comparison of the measured estradiol time-dependent concentrations C_t obtained during capacitation, the relative values $B_t = C_t/C_{t=0}$ were introduced and their values for BALB/c and C57BL/6Nvel mouse strains are given in Table 2.

The curves fitted through the experimentally determined B_t points display an autocatalytic character. In the search for various kinetic models it was found that, for the agreement between the curves obtained by fitting through the experimental points and the theoretical calculated curves, it is necessary to assume that the first step is adsorption of estradiol onto the surface of the sperm controlled by Langmuir isotherm. Other models (without adsorption) lead to completely different results.

The following model in which the symbols of the species also correspond to their molar concentrations was used for the autocatalytic process. The Langmuir adsorption of estradiol onto the surface of sperm PM first occurs with a starting estradiol concentration ($E2_0$), and the surface of PM at these sites (number n_a) becomes more accessible. It holds for n_a that: $n_a = \frac{zw(E2_0)}{1+w(E2_0)}$ where z is the maximum number of adsorption sites ((mER) or membrane non-estrogen receptors or other non-receptor sites) and w is the adsorption coefficient. Well below the saturation point ($1 > w\ (E2_0)$) it holds that $n_a = z\ w\ (E2_0)$. At sites n_a the externally present estradiol (concentration of extracellular estradiol at the time > 0, $(E2)$) reacts with the sperm membrane receptors leading to increased permeability of PM, through which estradiol molecules are transported within the cytoplasm to (cER) and forming the adduct $((E2)/(cER))$. This is connected with a further increase in the PM permeability. The primary penetration of PM corresponds to the following kinetic product, where k_1 is the rate constant corresponding to the formation of transport pathways (narrow channels):

$$k_1\ n_a\ (E2)\ (cER) \qquad (1a)$$

The gradual growth of adducts ($(E2)/(cER)$) in the cytoplasm leads to ever increasing permeability of PM. Activity Γ increases and is proportional to the consumed estradiol $\Gamma = k\,((E2_0) - (E2))$ so that, at the end of the reaction, $\Gamma_\infty = k\,(E2_0)$, the degree of activity S can be defined as: $S = \frac{\Gamma}{\Gamma_\infty} = \frac{(E2_0)-(E2)}{(E2_0)}$

The formation of the adduct ($(E2)/(cER)$) is thus enriched by the autocatalytic reaction, with the corresponding kinetic product:

$$k_2\,(E2)\,(cER)\,S \tag{1b}$$

where k_2 is the rate constant corresponding to the elevated degree of permeability of PM through the formation of adduct ($(E2)/(cER)$) in the cytoplasm. The formed adduct ($(E2)/(cER)$) is not stable and decomposes with the formation of internal estradiol (i.e. inside the cytoplasm, ($E2_i$)) and this kinetic equation corresponds to the kinetic product:

$$k_3\,((E2)/(cER)) \tag{1c}$$

where k_3 is the rate constant corresponding to the decomposition ($(E2)/cER$)) of the adduct. Thus, using (1a-c), we can write for the overall rates of the individual steps:

$$\frac{-d(E2)}{dt} = k_1 z\,w\,(E2_0)\,(E2)\,(cER) + k_2\,S\,(E2)\,(cER) \tag{2a}$$

$$\frac{-d(cER)}{dt} = k_1 z\,w\,(E2_0)\,(E2)\,(cER) + k_2\,S\,(E2)\,(cER) \tag{2b}$$

$$\frac{d((E2)/(cER))}{dt} = k_1 z\,w\,(E2_0)(E2)\,(cER) + k_2\,S\,(E2)\,(cER) - k_3\,((E2)/(cER)) \tag{2c}$$

$$\frac{d(E2_i)}{dt} = k_3\,((E2)/(cER)) \tag{2d}$$

with the initial conditions:

$$(E2)_{(t=0)} = (E2_0),\ ((E2)/(cER))_{(t=0)} = 0,\ (E2_i)_{(t=0)} = 0,\ (cER)_{(t=0)} = (cER_0) \tag{2e}$$

It follows from equations (2a, b) that:

$\frac{d(E2)}{dt} = \frac{d(cER)}{dt}$, and after integration: $(E2) - (E2_0) = (cER) - (cER_0)$ (3a)

adding equations (2a, c, d) yields: $\frac{d(E2)}{dt} + \frac{d((E2)/((cER))}{dt} + \frac{d(E2_i)}{dt} = 0$ and, after integration:

$(E2) - (E2_0) + ((E2)/(cER)) + (E2_i) = 0$ (3b)

In the next step we express from (3a): $(cER) = (E2) - (E2_0) + (cER_0)$, substituting into (2a, c) where simultaneously we write S as $\frac{(E2_0)-(E2)}{(E2_0)}$ and rearrange:

$\frac{-d(E2)}{dt} = (E2)((E2) - (E2_0) + (cER_0))(k_1 z\, w\, (E2_0) + k_2 \left(1 - \frac{(E2)}{(E2_0)}\right))$ (4a)

$\frac{d((E2)/(cER))}{dt} = (E2)((E2) - (E2_0) + (cER_0))(k_1 z\, w\, (E2_0) + k_2 \left(1 - \frac{(E2)}{(E2_0)}\right)) -$

$k_3((E2)/(cER))$ (4b)

$\frac{-d(E2_i)}{dt} = k_3((E2)/(cER))$ (4c)

Internal ($E2_i$) is formed after decomposition ($(E2)/(cER)$) and then it follows from (3b) that:

$(E2_i) = (E2_0) - (E2) - ((E2)/(cER))$ (4d)

In the above-described measuring method, after completion of the reaction by intense centrifugation, the centrifugate contains both internal $(E2_i)$ and extracellular $(E2)$ estradiol and the sperm together with the adduct ($(E2)/(cER)$) remain in the sediment. The measured concentration (C) is proportional to the total unbounded estradiol content:

k ($(E2)$ + ($E2_i$)) and (4d) yields its theoretical value:

$C = k\left((E2_0) - ((E2)/(cER)\right),\ C_0 = k\,(E2_0)$ (5a)

In order to simplify the set of equations (4c) as much as possible, minimise the number of variables and eliminate unknown values of constants z, w and (cER_0), the relative

concentrations will now be introduced: $\varepsilon = \frac{(E2)}{(E2_0)}$, $\alpha = \frac{((E2)/(cER))}{(E2_0)}$, the molar ratio $n = \frac{(E2_0)}{(cER_0)}$, dimensionless time $\tau = t\,(cER_0)\,k_1 z\,w\,(E2_{01}) = t\,(cER_0)\,K_0$, (so that $K_0 = k_1 z\,w\,(E2_{01})$) and also constants $K_2 = \frac{k_2}{K_0}$, $K_3 = \frac{k_3}{(cER_0)K_0}$ and fraction $D = \frac{(E2_0')}{(E2_{01})}$, where it is best to take the highest added estradiol concentration for $(E2_{01})$ and $(E2_0')$ is the currently selected initial estradiol concentration, so that $\frac{(E2_0')}{(E2_{01})}$ can assume values of 1, 0.1 or 0.01. Thus the D values are fixed by dilution.

The $B(t)$ value for the sperm of the BALB/c and C57BL/6Nvel strains of mice can then be expressed as:

$$B(t) = 1 - \frac{((E2)/(cER))}{(E2_0)}, \text{ thus } B(t) = 1 - \alpha \qquad (5b)$$

The set of differential equations (4a - c) then changes to the form:

$$\frac{-d\varepsilon}{d\tau} = \varepsilon\,(n(\varepsilon - 1) + 1)\,(D + K_2(1 - \varepsilon)) \qquad (6a)$$

$$\frac{d\alpha}{d\tau} = \varepsilon\,(n(\varepsilon - 1) + 1)\,\big(D + K_2(1 - \varepsilon)\big) - K_3\alpha \qquad (6b)$$

with initial conditions $\varepsilon_{(t=0)} = 1$ and $\alpha_{(t=0)} = 0$.

The reaction is theoretically terminated for $n < 1$, when $\varepsilon = 0$, $\alpha = 0$ and thus $B_{t\infty} = 1$. For $n > 1$ the reaction is terminated for $(cER) = 0$, or $n(\varepsilon - 1) + 1 = 0$, so that $\varepsilon_{(t=0)} = 1 - \frac{1}{n}$, ($n = \frac{(E2)}{0} = \infty$) and $B_{t\infty} = 1$. According to (5b), it also holds for $\alpha = 0$ that $B_{t\infty} = 1$. Thus all the curves end at limiting value 1. The set of equations (6a, b) are solved by the fourth-order Runge-Kutta method with step of $h = 10^{-4}$. The actual time t is one hundred times greater than the calculated time τ and the results of the calculations are given in Table 3. Values D were determined by diluting the highest employed estradiol concentrations (200µg/L), corresponding to a value of $D = 1$. The molar ratio n was estimated from the shape of curve $B(t)$ for dilution of $D = 1$. The other dilutions are given by a tenth and hundredth of the original value. Constants K_2 and K_3 were obtained by optimisation.

The shapes of the theoretical $B(t)$ curves are shown in Figure 2. It is apparent that the experimental points fit the theoretical $B(t)$ curves well for both tested types of mice. For the

tested samples with the addition of sperm, the formation of the adduct ((*E2*)/(*cER*)) takes place autocatalytically and the curves corresponding to the same dilution (same tested concentrations) of estradiol are of the same type, especially in relation to the t_{min} values. Minima appear on the *B(t)* curves at times t_{min} through the formation and decomposition of ((*E2*)/(*cER*)). Their position in relation to the time at ten-fold and one-hundred-fold dilution of the highest employed concentrations of estradiol (200μg/L), i.e. one tenth or one hundredth of the added amount of estradiol, indicate that the reaction of estradiol with the sperm receptors does not have an integral order because the position of t_{min} would not change for first-order but would change ten-fold for a second-order reaction.

Interestingly, there is a slight shift between the theoretical curves reflecting differences in estradiol binding dynamics between two mouse inbreed strains BALB/c and C57BL/6Nvel supporting the well-known fact of strain/sub-strain specific phenotypic responses for mice of different genetical background. However, and importantly, the theoretical curves $B(t)$ fit the experimental points for both tested types of mice, with the same outcome, which support the evidence of finding theoretical mechanism, which fits to species-specific modifications. The shift in experimental data could be due to the knowledge that C57BL/6Nvel mice have lower fertility efficiency (30) and sperm motility parameters, such as reaching the hyperactivated stage after the sperm capacitation (31).

DISCUSSION

It has been reported that *in vivo*, sperm capacitation happens within 30 – 90min after presenting sperm to female reproductive tract surroundings (18), but beside other factors it also depends on the estrogen concentration. Estradiol concentration in follicular fluid is higher than in the reproductive female tract and comparable with our experimental concentrations (32). Specifically, in females, estradiol concentration in the ovarian fluid is at least two-fold higher compared with that of plasma (33), and it fluctuates during the oestrus, e.g. it is between 145 and 2100pg/ml for mouse and rat (34). Therefore, sperm are expected to be exposed to high concentrations during certain stages of their capacitation in the female reproductive tract (32, 35). Based on these facts, the sperm exposure to estradiol *in vivo* is a common phenomenon and understanding the estradiol action is of great importance.

The schematic and simplified interpretation of the kinetic analysis results applied on data obtained from estradiol action in sperm during capacitation is summarized for better visualization in Figure 3. Capacitating sperm undergo several changes including the plasma membrane reorganization, which changes its fluidity and gets more receiving for the

extracellular estradiol E2, which is passing by diffusion k_1 after its transient initial adsorption onto specific PM adsorbents. These could be represented by mERs and/or non-classical ER. As a complete novelty, we would like to deliver a hypothesis considering the formation of an adduct cER/E2 in the cytoplasm, which, when formed, serves as an autocatalytic agent, signalling towards an increase of PM fluidity $S \cdot k_2$. This signalling event is accompanied by the complex cER/E2 disintegration k_3 and a release of estradiol $E2_i$ remaining in the cytoplasm. The cERs remain internalised within the cytoplasm, however, they lose their receptivity and remain dormant.

One of the criteria of the reliability of kinetic equations is the independence of the rate constants on the concentration conditions. The determined values of the optimised constants K_2 and K_3 fulfil these conditions well over two orders of magnitude. The correctness of the used model is further confirmed by the positions and the shapes of the minima on the $B(t)$ curves, which are in agreement with the experimental values. The areas around the minima are very sensitive to the rate constant values. Hewitt et al. 2005 (7) described two ways of estradiol passing through the plasma membrane, one of which is slow and the other is rapid. In our model, the slow process with the rate constant k_1 occurs simultaneously with the rapid (autocatalytic) process with the rate constant k_2 multiplied by S. The concentration gradient of estradiol is the driving force for its transport.

In conclusion, we delivered the evidence that the level of estradiol available for mouse spermatozoa during capacitation *in vitro*, despite their strain origin, can be quantified by HPLC-MS/MS and the newly developed method represents an important generally applicable tool for studying the amount of biological substances, in our case estradiol. There is a big potential in this new analytical-biological approach for understanding the physiological mechanism of steroid hormones including estradiol, not only in reproductive biology, but also in somatic cell signalling events, both physiological and pathological, targeting mainly cancer cell biology research.

MATERIALS AND METHODS

Chemicals, reagents and animals

Acetonitrile (ACN) for HPLC grade purity, M2 laboratory mouse *in vitro* fertilizing medium with HEPES and deuterated β-estradiol-16,16,17-d_3 (estradiol-d_3) (purity 98%) were purchased from Sigma-Aldrich (Chromasolv, Germany). Ethanol (96%) was obtained from Lach-Ner (Czech Republic). Paraffin oil was delivered by Carl Roth (Germany). Formic acid

(HCOOH) (purity 98 - 100%) and 17β-estradiol (estradiol) (purity 98%) were provided by Merck (Germany). Deionized water (Milli-Q water purification system Millipore, MA, USA) was used in all experiments.

Two laboratory inbreed mouse strains (BALB/c and C57BL/6Nvel) were used for comparative experiments. Mice were purchased from Velaz (Czech Republic) and maintained and housed at the animal facilities of the Faculty of Science, Charles University. All the animal procedures and all the experimental protocols were approved by Local Ethics Committee and carried out in strict accordance with the Animal Scientific Procedure, Art 2010, and subjected to review by this Local Ethics Committee of the Faculty or Science, Charles University, Czech Republic (accreditation no. 247732008-10001).

Instrumentation and chromatographic conditions

The HPLC equipment (Agilent Technologies, Germany) comprised of 1290 Infinity Series LC (a quaternary pump, a degasser, a thermostatic autosampler with a 20μL sample loop and a column oven). Triple Quad LC/MS 6490 tandem mass spectrometer (Agilent Technologies, Germany) with an electrospray ionization interface was used for the detection. Signal was processed and data were handled with the Mass Hunter Workstation Software (Agilent Technologies, Germany). Separation was performed on a SunFire C18 column (150 x 4.6 mm I.D., particle size 5 μm) from Waters (MA, USA)

The MS-MS measurements were performed in the multiple reaction monitoring (MRM) mode using ESI ionization in positive mode (ESI (+)). Nitrogen was used as the collision nebulizing and desolvation gas. Because of complex M2 fertilizing medium containing inorganic and organic components, from which especially BSA (4.0g/L) can cause difficulties during the separation and detection process, the eluate was lead to waste from 0 to 5.5min and to MS detector only from 5.5 to 8.0min.

The stock solution of estradiol at concentration of 200mg/L was prepared by dissolving an appropriate amount of estradiol standard in ethanol. The stock solutions at concentration of 20, 2 and 0.2mg/L were obtained by serial dilutions with ethanol. The stock solutions were stored in the dark at 5°C. Working solutions to obtain the standard points of the calibration curve were prepared by diluting the appropriate ethanolic solutions with M2 fertilizing medium to attain the following concentrations: 0.5, 1, 5, 10, 15, 20 and 25μg/L (from 0.2mg/L ethanolic solution) and 50, 75, 100, 125, 150, 175, 200 and 250μg/L (from 2mg/L ethanolic solution).

Capacitation of mouse sperm *in vitro*

For the realization of capacitation *in vitro* 35mm Petri dishes obtained from Corning (NY, USA) were used. Light inverted microscope Olympus CX 21 and Olympus epifluorescent microscope, were supplied by Olympus (Czech Republic). Incubator NB-203 was purchased from N-BIOTEK (Korea). For *in vitro* cultivation of sperm incubator Telstar Bio-IIA and laminar box-BioTek from N-BIOTEK (Korea) were used.

The working solutions with different dilution (200, 20 and 2μg/L) were prepared by diluting the ethanolic stock solutions with M2 fertilizing medium to attain the required concentration according to following scheme: 1μL of appropriate ethanolic solution was diluted into 1mL of M2 fertilizing medium to minimize the amount of ethanol in biological sample and 100μL of this solution was placed into each of the used Petri dishes and covered with 1mL of paraffin oil. All procedures were realized in a sterile laminar box. Prepared Petri dishes were then placed for 60min to incubate at 37°C in 5% CO_2 in air.

Spermatozoa, which were recovered from the distal region of *cauda epididymidis*, were placed in M2 fertilizing medium into an incubator for 10min at 37°C under 5% CO_2 in air to relax sperm. After that, the concentration of stock sperm in M2 medium was adjusted to $5\times10^6 mL^{-1}$. The motility of the sperm population was checked throughout the whole experiment by a light inverted microscope with a thermostatically controlled stage at 37°C.

Preparation of samples for capacitation was realized in Petri dishes according to the following scheme: after 60min incubation of 100μL of 200, 20 or 2μg/L solution of estradiol in M2 fertilizing medium, 5μL of sperm stock solution was added. For each capacitation time 6 Petri dishes were used, each one contained 105 μL of sample volume covered with 1mL of paraffin oil. Petri dishes with added spermatozoa were again placed into an incubator to capacitate (37°C, 5% CO_2 in air). Spermatozoa were capacitated for up to 3 h. At half hour intervals (0, 30, 60, 90, 120, 150 and 180min after adding sperm) samples were collected. The sample solutions from all Petri dishes (for a given capacitation time) were placed into one micro tube to centrifugate for 10min at 12000rpm to remove the spermatozoa. After centrifugation the supernatant was placed into vial for HPLC-MS/MS analysis. The sample volume was 600μL. To avoid potential systematic errors during sample preparation (partial evaporation of samples during incubation, differences in collection of supernatant after centrifugation etc.), reference samples (blanks) were prepared collaterally with the samples described above, but no spermatozoa were added into the incubated estradiol solution. The matrix effect was measured by comparing the peak response of the extracted blank with the pure sample prepared in M2 medium at a concentration of 2, 20 and 200μg/L and no matrix

effect was observed. To check the correctness of HPLC-MS/MS analysis, 6µL of ethanolic estradiol-d_3 solution at a concentration of 2 mg/L was added into each sample before measurement.

Application of kinetic analysis

The experimental results obtained for the concentration changes of estradiol at capacitation times were subjected to kinetic analysis. In a previous work (29), mathematical theory for the mechanism when sperm maturation ability is amended by external stimuli (36) was proposed. It was shown that the chemical kinetics can be applied to sperm in the role of reactant and that kinetic analysis could be a useful tool for monitoring and predicting the specific molecular mechanisms involved in certain biological signalling pathways. For this calculation, QBasic software was used.

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Author Contributions

ZB designed and supervised HPLC-MS/MS, kinetic analysis part and wrote the relevant part of the manuscript, HA performed the HPLC-MS/MS experiments, AT performed kinetic analysis of obtained data, TB performed the HPLC-MS/MS experiments and prepared Figures 1 and 2, NS performed sperm capacitation experiments, KDH designed and supervised the sperm capacitation and estradiol project, analysed the data, created the schematic data interpretation model Figure 3 and wrote the relevant parts of the manuscript. All the authors' reviewed the manuscript.

Competing financial interests: Authors declare no competing financial interests.

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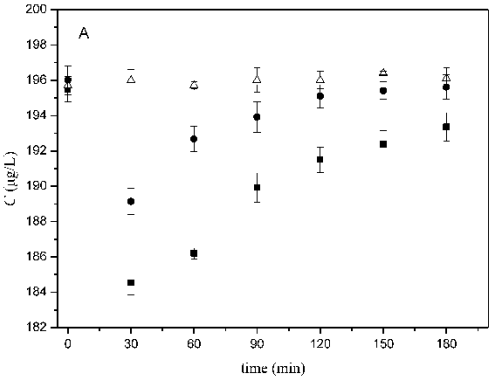
Figures and Tables



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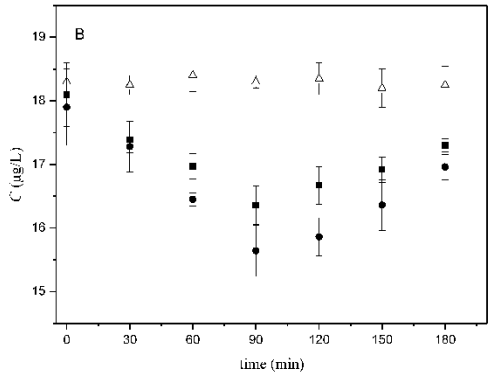

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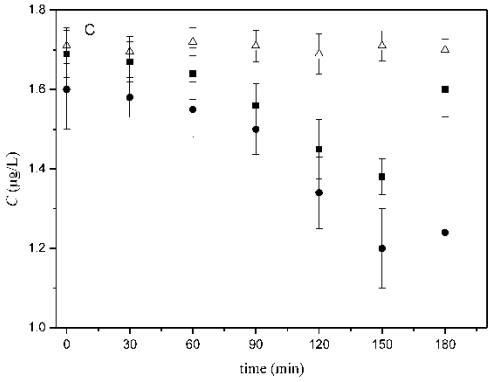

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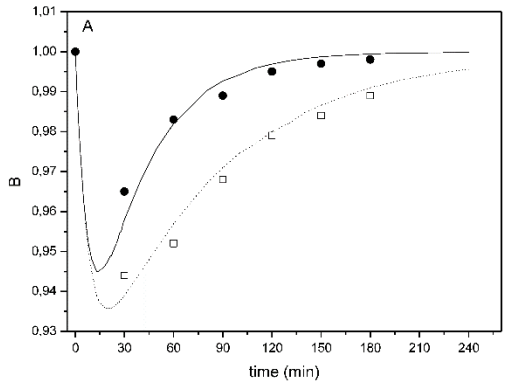
613 Fig. 1. Dependencies of the concentration of total unbounded estradiol on the time of mouse
614 sperm capacitation *in vitro*. The tested concentrations of estradiol in M2 medium were (A)
615 200μg/L, (B) 20μg/L and (C) 2μg/L; samples (solid circles for BALB/c sperm, solid squares
616 for C57BL/6Nvel sperm), blank (open triangles); experimental conditions: 50/50 (*v/v*)
617 ACN/H_2O, both containing 0.1% HCOOH, measured in MRM mode for transition 255.2 >
618 158.9



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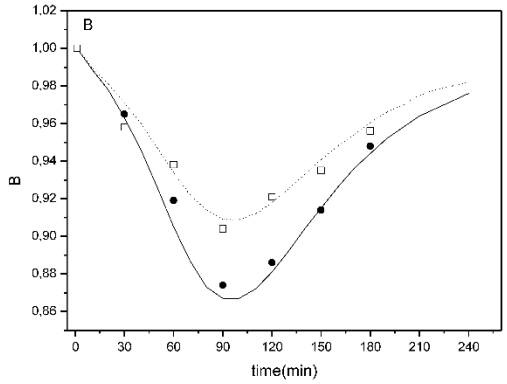

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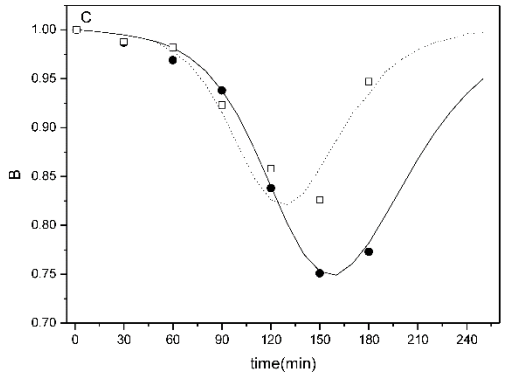

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622 Fig. 2. Theoretical shape of the $B(t)$ curves (solid line for BALB/c, dashed line for
623 C57BL/6Nvel) for the selected dilution D, molar ratio n and optimised values of K_2 and K_3
624 (see Tab. 3) with designation of the points obtained in the experiment (solid circles for the
625 BALB/c experiments, solid squares for the C57BL/6Nvel experiments) for estradiol
626 concentrations (A) 200μg/L, (B) 20μg/L a (C) 2μg/L.

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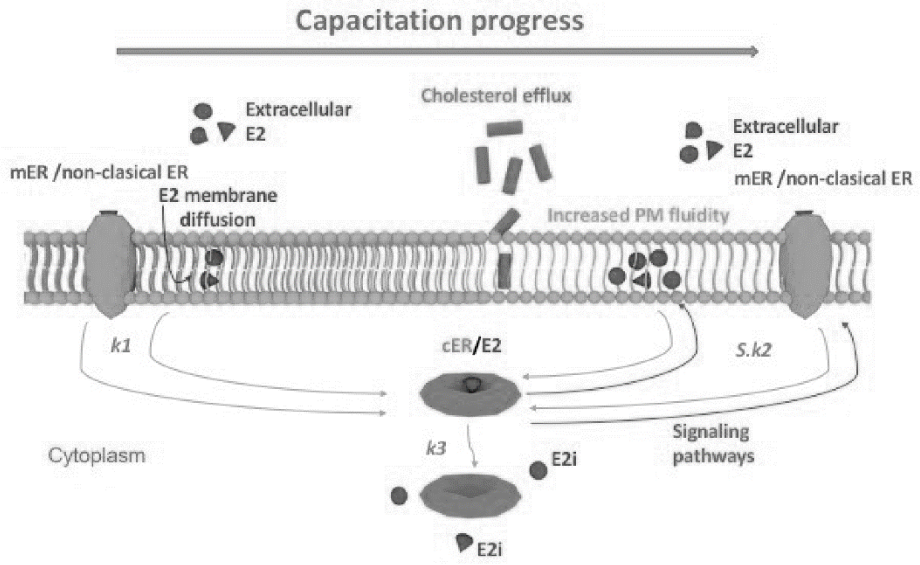

Fig. 3. Schematic interpretation of kinetic analysis results applied on estradiol sperm action using the symbols from kinetic equations.

650 Table 1. Parameters of the calibration curve (standard deviations in parentheses), limit of
651 detection (LOD) and quantitation (LOQ) for estradiol in M2 fertilizing medium, a.u –
652 arbitrary unit.

Compound	Slope (L/μg a.u.·s)	Intercept (a.u.·s)	Correlation coeficient	LOD (μg/L)	LOQ (μg/L)
Estradiol	99.13 (1.07)	-18.66 (1.46)	0.9996	0.3	1.1

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Table 2. Relative concentration values *B* calculated from the measured estradiol time-dependent concentrations *C* (see Fig. 1A - C) obtained during capacitation for three tested estradiol concentration and two laboratory inbreed mouse strains.

Capacitation time (min)	*B*					
	2µg/L		20µg/L		200µg/L	
	BALB/c	C57BL/6N	BALB/c	C57BL/6N	BALB/c	C57BL/6N
0	1.000	1.000	1.000	1.000	1.000	1.000
30	0.987	0.988	0.965	0.958	0.965	0.944
60	0.969	0.982	0.919	0.938	0.983	0.952
90	0.938	0.923	0.874	0.904	0.989	0.968
120	0.838	0.858	0.886	0.921	0.995	0.979
150	0.751	0.826	0.914	0.929	0.997	0.984
180	0.773	0.947	0.958	0.956	0.998	0.989

704 Table 3

705 Calculated constants for three tested estradiol concentrations and two laboratory inbreed
706 mouse strains.

Constants	2 μg/L		20 μg/L		200 μg/L	
	BALB/c	C57BL/6N	BALB/c	C57BL/6N	BALB/c	C57BL/6N
D	0.01	0.01	0.1	0.1	1	1
n	0.12	0.12	1.2	1.2	12	12
K_2	4.5	5.5	4.5	4.0	4.0	2.0
K_3	3.5	6.5	4.3	6.0	3.0	1.3

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PUBLIKACE IV

ANALYSIS OF ESTROGENS AND ESTROGEN MIMICS IN EDIBLE MATRICES—A REVIEW

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Review

Analysis of estrogens and estrogen mimics in edible matrices—A review

This review provides a brief survey of the biological effects of selected endocrine-disrupting compounds that are formed after internal exposure of organisms. Further, the present analytical methods available for the determination of these compounds in foodstuffs are critically evaluated. The attention is primarily devoted to the methods for sample pretreatment, which are the main source of errors and are usually the most time-consuming step of the whole analysis. This review is focused on selected natural and synthetic estrogens, estrogen conjugates, and chemical additives used in the plastic industry that can act as estrogen mimics.

Keywords: Endocrine-disrupting compounds / Food analysis / Instrumental methods
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1 Introduction

The human organism is affected by many factors, acting both from outside, e.g. by the environment, and internally, e.g. by foodstuffs. A group of substances that involve both these kinds of exposure is called endocrine-disrupting compounds (EDCs). EDCs are substances that "interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the development, behavior, fertility, and maintenance of homeostasis" (normal cell metabolism) [1]. The endocrine system thus can be affected in various ways, from bonding of a xenobiotic to receptor sites to hindering of the synthesis by the organism itself. Ingestion of EDCs through food is considered the major exposure route.

EDCs are present as contaminants of the environment and represent preferably a reproduction risk. They are known to elevate plasma vitellogenin. Vitellogenin is normally synthesized in mature females, and male and juvenile fish only produce background levels of this protein. However, upon exposure to exogenous estrogen or an estrogen mimic, male and juvenile fish will be induced to synthesize it as well. EDCs are also suspected to lead to decreased gonadosomatic index and sperm motility [2–6].

EDCs involve a wide range of substances containing natural and synthetic estrogens, estrogen conjugates, and chemical additives used in plastics industry that can act as estrogen mimics. EDCs can be divided into four main classes comprising (i) pharmaceuticals (e.g. steroids including phytoestrogens, antibiotics, analgesics, anti-inflammatory and psychotropic agents); (ii) personal-care products (e.g. surfactants and synthetic fragrances and preservatives); (iii) pesticides (e.g. insecticides, fungicides); and (iv) miscellaneous industrial chemicals and byproducts (e.g. organic oxygen compounds and polycyclic aromatic compounds) [7]. This review is focused on the selected groups of EDCs (Table 1) tightly connected with edible matrix analysis.

Significant parts of the EDC group are exogenous estrogens [8]. They are discharged into the environment as a result of an increasing application of drugs and the subsequent excretion of their metabolites. As the human population grows and the livestock industry increases, there is an increase in the discharge of synthetic and natural estrogens. These substances have recently been found in foodstuffs.

Phytoestrogens are present in food that we consume. Phytoestrogens, especially from soya, are the major dietary

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Abbreviations: ACN, acetonitrile; **AP**, alkylphenol; **ASE**, accelerated solvent extraction; **BP**, biphenol; **BPA**, bisphenol A; **BPF**, bisphenol F; **BPZ**, bisphenol Z; **BSTFA**, *N,O*-bis(trimethylsilyl)trifluoroacetamide; **DES**, diethylstilbestrol; **DIE**, dienestrol; **DLLME**, dispersive liquid–liquid microextraction; **E1**, estrone; **E3**, estriol; **EDC**, endocrine-disrupting compound; **EE2**, 17α-ethynylestradiol; **ER**, estrogen receptor; **GCB**, graphitized carbon black; **HEX**, hexestrol; **ILs**, ionic liquids; **LLE**, liquid–liquid extraction; **LVI-PTV**, large-volume injection programmable temperature vaporization; **MCNT**, magnetic carbon nanotubes; **MIPF**, molecularly imprinted polymer filament; **MIP**, molecularly imprinted polymer; **MISPE**, molecularly imprinted solid-phase extraction; **MRM**, multiple reaction monitoring; **MSPD**, matrix solid-phase dispersion; **MSPE**, magnetic SPE; **MSTFA**, *N*-methyl-*N*-trimethylsilyl trifluoroacetamide; **NP**, nonylphenol; **OP**, octylphenol; **P4**, progesterone; **PDMS**, polydimethylsiloxane; **PIL**, polymeric ionic liquid; **RIA**, radioimmunoassay; **SBSE**, stir bar sorptive extraction; **SPME**, solid-phase microextraction; **TMCS**, trimethylchlorosilane; **αE2**, 17α-estradiol; **β-CD**, β-cyclodextrin; **βE2**, 17β-estradiol; **UHPLC**, ultra high performance liquid chromatography

This article is dedicated to the memory of Professor Karel Štulík.

Table 1. Selected groups of EDCs frequently analyzed in edible matrices

Classes	Selected compounds
Pharmaceuticals (steroids)	Synthetic estrogens (e.g. EE2, DES, DIE, HEX)
	Natural estrogens (e.g. E1, βE2, αE2, E3, P4, 4-OHE2, 2-MeOE2)
	Androgens (e.g. T, 17ENT, 17αT, TB, 17αMT)
Personal-care products (surfactants)	APs (e.g. NP, OP)
Industrial chemicals	BPA, BPF

T, 17β-testosterone; TB, 17β-tenbolone; 17αMT, 17α-methyltestosterone; 17αT, 17α-testosterone; 17ENT, 17α-nortestosterone, 2-MeOE2, 2-methoxyestradiol; 4-OHE2, 4-hydroxyestradiol.

source of estrogenicity. Genistein, the main component of soya foodstuff, or resveratrol, occurring in various plants and also in wine, belong here. While industrial endocrine disruptors have been studied for several years, natural compounds like phytoestrogens remain less investigated [9–11].

Alkylphenols (APs) such as nonylphenol (NP) and octylphenol (OP) are widely used in emulsifiers, antistatic agents, demulsifiers, and solubilizers, and are found commonly in wastewater [12]. Bisphenol A (BPA) is a high-volume production monomer ($>2.5 \times 10^6$ kg/year) used in polycarbonated plastic, in polystyrene resins, and as dental sealants. It is also used as an additive to other plastics such as polyvinylchloride, and halogenated derivatives of BPA are widely used as flame retardants [13]. BPA has recently been found in packed water and also in the packaging materials used for foodstuff storage.

Phthalate esters have been used worldwide as softeners to impart flexibility, pliability, and elasticity to otherwise rigid polymers such as polyvinylchloride. These molecules are found mostly in industrial paints and solvents but also in toys, personal-care products, and medical devices such as intravenous tubing and blood transfusion bags [14].

For the analysis of EDCs in edible matrices, LC and GC are most frequently used especially with MS or MS/MS detection. These separation techniques provide high separation efficiency in combination with required sensitivity and selectivity. An important advantage of LC over GC presents no need for derivatization steps. Immunochemical methods such as ELISA or radioimmunoassay (RIA) using highly specific antibodies are applied much less frequently. The assays are highly selective and easy to perform, but the instability of natural antibodies limits their applications to some extent. With a relatively low cost, they are useful for developing high-throughput screening methods. They are suitable for analysis of human body fluids in epidemiological studies and screening of large sample populations [15].

The aim of this work is to detail recent methods used for the determination of EDCs in edible matrices. EDCs are represented mainly by steroids, APs, and BPA (for their structures see Fig. 1). First, the mechanism of endocrine disruption action is discussed. Special attention is placed on samples' pretreatment. The application of LC, GC, and immunochemical methods for analysis of EDCs in edible matrices is summarized in Tables 2–4.

2 The mechanism of endocrine-disruption action

The endocrine system is particularly tuned to respond to very low concentrations of hormone, which allows an enormous number of hormonally active molecules to coexist in the circulation. The typical physiological levels of the endogenous hormones are extremely low, e.g. in the range 10–900 ng/L for 17β-estradiol (βE2) and 300–10.000 ng/L for testosterone. Steroid hormones in the blood are distributed into three phases: (i) free, i.e. bioavailable, representing the unconjugated form; (ii) bound to low-affinity carrier proteins such as albumin; and (iii) inactive, representing the form that is bound to high-affinity binding proteins such as sex hormone binding globulin or α-fetoprotein [16].

There are several reasons why endogenous hormones are able to act at such low circulating concentrations: (i) receptors specific for the hormone have such high affinity that they can bind sufficient molecules of the hormone to trigger a response; (ii) there is a nonlinear relationship between hormone concentration and the number of bound receptors, and (iii) there is also a nonlinear relationship between the number of bound receptors and the strongest observable biological effect. Thus, even a moderate difference in hormone concentration in the low-dose range can produce substantial changes in receptor occupancy and therefore generate significant variations in biological effects [17].

Hormones act mainly through interactions with their cognate receptors, which can be classified into two large groups: (i) membrane-bound receptors, which respond primarily to peptide hormones such as insulin, and (ii) nuclear receptors, which are activated by interaction with small lipophilic hormones such as sex steroid hormones. EDCs may possess multiple mechanisms of action; however, because many EDCs are small lipophilic compounds, one privileged route is through their direct interaction with a given nuclear receptor, which presumably perturbs or modulates downstream gene expression. For example, most EDCs associated with reproductive and developmental defects are thought to result from EDCs interfering with the function of the estrogen receptor (ER) and/or androgen receptor, thereby disrupting the normal activity of estrogens and androgens ligands [11, 18].

EDCs also have other effects that are not dependent on binding to either classical or membrane-bound steroid hormone receptors [19, 20]. EDCs can influence the metabolism of natural hormones, thus producing differences in the amount of hormone that is available for binding either because more (or less) hormone is produced than in a

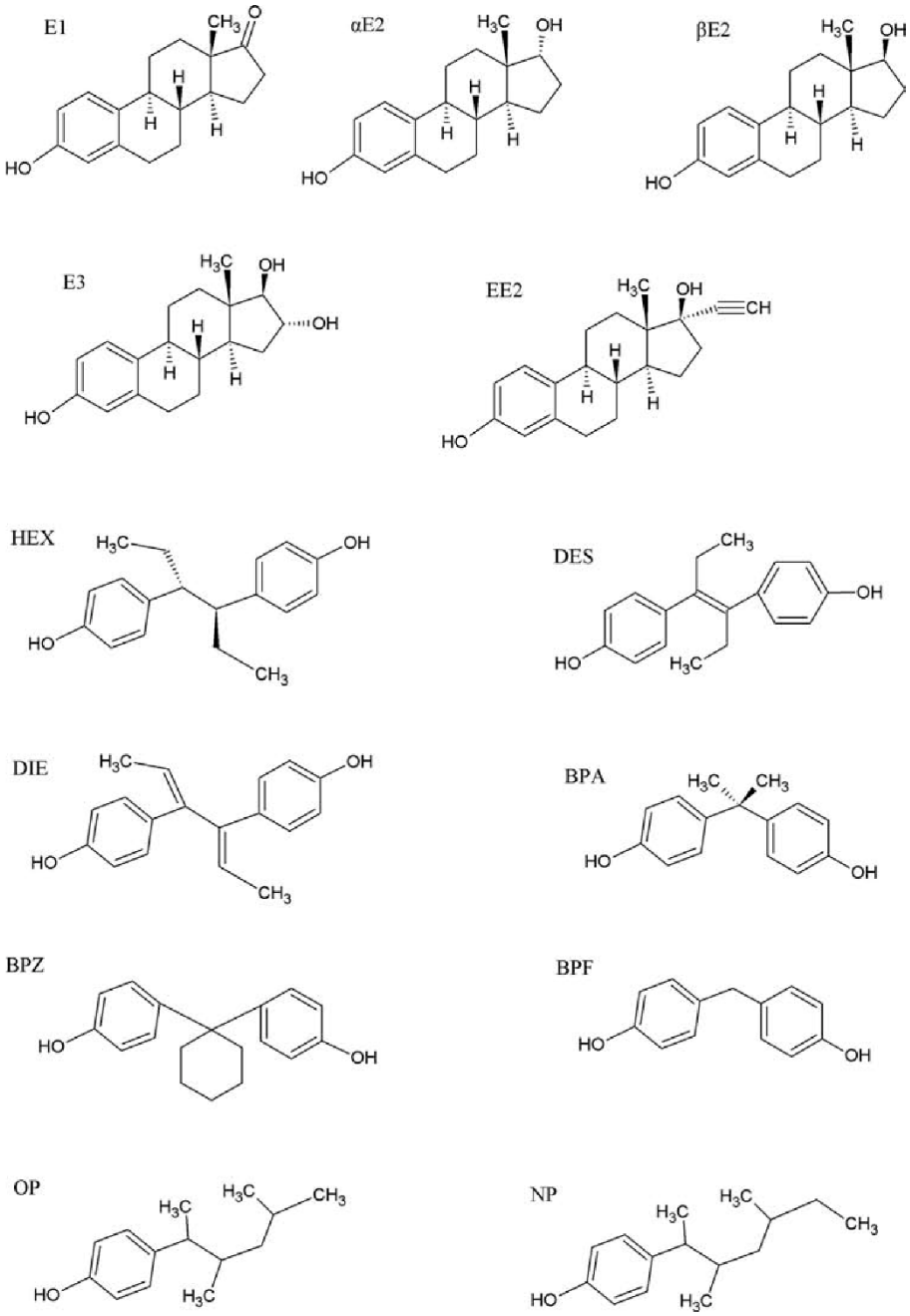

Figure 1. Structures of EDCs discussed in this article.

typical system or because the hormone is degraded faster (or slower) than is normal. Other EDCs influence the transport of hormone, which can also change the amount of hormone that is available for receptor binding. EDCs can also have effects that are independent of known endocrine actions. One example is the effect of endogenous hormones and EDCs on ion channel activity. BPA, dichlorodiphenyltrichloroethane, diethylstilbestrol (DES), NP, and OP have been shown to disrupt Ca^{2+} channel activity and/or Ca^{2+} signaling in some cell types [21–24]. There is also evidence that EDCs act additively or even synergistically with other chemicals and natural hormones in the body [25–27].

In mammals, chemicals having estrogenic activity can produce many health-related problems, such as early puberty in females; reduced sperm counts; altered functions of reproductive organs; obesity; altered sex-specific behaviors; and increased rates of some breast, ovarian, testicular, and prostate cancers. Fetal, newborn, and juvenile mammals are especially sensitive to very low (sometimes picomolar to nanomolar) doses of chemicals having estrogenic activity [28]. In

Table 2. Examples of LC analysis of EDCs

LC

Matrix	Compound	Stationary phase	Detection	Sample pretreatment	LOD	Refs.
Milk and milk products						
Milk; Iran	E1, αE2, βE2, E3	Luna C-18 (150 × 2.0 mm id, particle size 5 μm)	MS/MS	Extraction, derivatization with dansyl chloride, SPE	E1 5 ng/L, αE2 5 ng/L, βE2 5 ng/L, E3 10 ng/L	[56]
Milk; Spain	Pregnenolone, P4, 17-OHP4, (17-OHP5)	Synergi Fusion RP 100A (50 × 2.0 mm id, particle size 2.5 μm)	MS/MS	Hydrolysis, precipitation, derivatization	P4 64.2 ng/L, 17-OHP4 48.9 ng/L, pregnenolone 51.5 ng/L, 17-OHP5 53.4 ng/L	[108]
Milk, USA	E1, βE2, E3, EE2	Beta basic C18 (100 × 2.1 mm id, particle size 3 μm)	MS/MS	SPE	E1 6 ng/L, βE2 10 ng/L, E3 10 ng/L, EE2 37 ng/L	[70]
Milk; China	E1, βE2, E3, DIE, HEX, DES, EE2	Shim-pack VP-ODS (150 × 2.0 mm id, particle size 5 μm)	MS/MS	MSPE	E1 5.1 ng/L, βE2 14.9. ng/L, E3 31.3. ng/L, DIE 28.5 ng/L, HEX 15.2 ng/L, DES 66.7 ng/L, EE2 43.9 ng/L	[81]
Milk; China	E2, EE2, HEX	Home-packed C18 column (250 × 4.6 mm id, particle size 5 μm)	Fluorescence 280/310 nm	MSPE	E2 1.21. μg/L, EE2 2.35 μg/L, HEX 1.97 μg/L	[83]
Human breast milk; Japan	BPA	Vydac protein and peptide C18 (150 × 4.6 mm id)	Fluorescence 350/475 nm	SPE, LLE, derivatization with labeling reagent	0.11 ng/mL	[109]
Milk powder; China	E3, βE2, αE2, equilin, EE2, E1, DES, DIE, HEX	Acquity UPLC HSS T3 (100 × 2.1 mm id, particle size 1.8 μm)	UPLC-Q-TOF	Extraction, SPE	E3 0.26 μg/kg, βE2 0.19 μg/kg, αE2 0.20 μg/kg, equilin 0.11 μg/kg, EE2 0.21 μg/kg, E1 0.22 μg/kg, DES 0.14 μg/kg, DIE 0.30 μg/kg, HEX 0.19 μg/kg	[111]
Pasteurized, homogenized whole milk; China	BPA, E1, E3, βE2, DES, EE2	Symmetry C18, (150 × 2.0 mm id, particle size 5 μm)	MS (Q)	Online SPE tailored made C30	BPA 0.2 ng/mL, E1 0.08 ng/mL, E3 0.3 ng/mL, βE2 0.1 ng/mL, DES 0.05 ng/mL, EE2 0.2 ng/mL	[131]
Egg products						
Egg products; China	Estrogens, androgens	Acquity BEH C18 (100 × 2.1 mm id, particle size 1.8 μm)	UPLC ESI MS	SPE	0.007–0.76 μg/kg	[48]
Meat						
Meat: pork, mutton, beef, duck, chicken, fish; China	BPA, NP, OP	Symmetry C18 (150 × 2.1 mm id, particle size 3.5 μm)	MS/MS	ASE followed by SPE	BPA 0.3 μg/kg, NP 0.05 μg/kg, OP 0.1 μg/kg	[61]
Meat; Belgium	34 anabolic steroids, E1, E2, E3, DIE, HEX	Hypersil Gold C18, (100 × 2.1 mm id, particle size 1.9 μm)	UPLC MS/MS	Extraction, SPE	E1 0.29 μg/kg, E2 0.14 μg/kg, E3 0.29 μg/kg, DIE 0.13 μg/kg, HEX 0.32 μg/kg	[55]

Table 2. Continued

LC

Matrix	Compound	Stationary phase	Detection	Sample pretreatment	LOD	Refs.
Meat: bovine; Germany	17ENT, 17α-testosterone (17αT), 17β-boldenone, 17β-nortestosterone, T, TB, EE2, methylboldenone, 17α—methyltestosteron (17αMT), stanozolol	Luna C-18 (150 × 2.0 mm id, particle size 5 μm)	MS/MS	Extraction, SPE	17ENT 0.48 μg/kg, 17αT 0.43 μg/kg, 17beta boldenone 0.42 μg/kg, 17beta-nortestosterone 0.40 μg/kg, T 0.58 μg/kg, TB 0.47 μg/kg, EE2 0.79 μg/kg, methylboldenone 0.15 μg/kg, 17αMT 0.55 μg/kg, stanozolol 0.30 μg/kg	[117]
Fish, shrimp						
Fish, shrimp (seafood); China	βE2, E3, 4-OHE2, (2-MeOE2	VP-ODS-C18 (150 × 4.6 mm id)	Fluorescence	Dispersive SPE	βE2 0.02 μg/L, E3 0.02 μg/L, 4-OHE2 0.44 μg/L, 2-MeOE2 0.02 μg/L	[122]
Miscellaneous matrices						
Porcine: meat, kidney, liver, chicken, milk; China	βE2, E3, E1, DES, HEX, DIE	CAPCELL PAK Phenyl (250 × 2.0 mm id, particle size 5 μm)	MS/MS	SPE	1–100 ng/kg	[82]
Dairy products: whole milk, yogurt, meat: beef, chicken, pork; China	βE2, E3, P4, boldenone, T, penconazole	Symmetry C18 (150 × 4.6 mm id, particle size 5 μm)	UV	Microextraction, SPE	βE2 0.015 μmol/kg, E3 0.025 μmol/kg, P4 0.04 μmol/kg, T 0.03 μmol/kg, boldenone 0.03 μmol/kg, penconazole 0.025 μmol/kg	[123]
Packaging materials						
Canned food: vegetables, meat, fish, pulses, microwave containers, soft drink cans; Spain	BPA	C 18 (150 × 4.6 mm id)	Fluorescence 275/300 nm, GC/MS confirmation	Samples filled with MiLLiQ water—autoclaved/ microwaved	LOQ 22.8 ng/mL	[129]
Baby bottles water carboys; USA	BPA	Shandon hypercarb S (150 × 4.6 mm id, particle size 7 μm)	Fluorescence 235/317 nm, GC/MS confirmation	Samples in contact with water and food simulants under time and temperature conditions, in some cases SPE	2 ng/mL in EtOH simulants and water, 100 ng/mL in fruit juices, infant formula, Miglyol	[130]

T, 17β-testosterone; TB, 17β-tenbolone; 17ENT, 17α-nortestosterone; 17-OHP4, 17-hydroxyprogesterone, 17-OHP5, 17-OHP5, 17-hydroxypregnenolone; 2-MeOE2, 2-methoxyestradiol; 4-OHE2, 4-hydroxyestradiol.

Table 3. Examples of GC analysis of EDCs

GC

Matrix	Compound	Stationary phase	Detection	Sample pretreatment	LOD	Refs.
Milk and milk products						
Cow´s and goat´s milk, human milk from volunteers; Spain, Morocco	EE2, E1, βE2	DB-5 (30 m × 0.25 mm id, thickness 0.25 μm)	MS	SPE, derivatization with BSTFA, TMCS	E1 1.0 ng/kg, βE2 1.2 ng/kg, EE2 1.1 ng/kg	[72]
Butter; New Zealand, France, China, Argentina, Denmark	EE2, αE2, βE2, E1, E3	DB-5MS (30 m × 0.25 mm id, thickness 0.25 μm)	MS	Solid-phase dispersion extraction, derivatization HFBA with ACN mixture	EE2 + E3 0.5μg/kg, E2 0.3 μg/kg, E1 0.2 μg/kg	[80]
Powdered milk and infant formulas; Taiwan	BPA and phytoestrogens	DB-5MS (30 m × 0.25 mm id, thickness 0.25 μm)	MS	SPE, derivatization with BSTFA, TMCS	LOQ 1.0 ng/g	[143]
Milk, egg, and meat						
Milk, eggs, meat, kidney; France	αE2, βE2, E1, androgens	ZB-5MS (30 m × 0.25 mm id, thickness 0.25 μm)	MS/MS	Extraction, SPE, derivatization with MSTFA, BSTFA, PFBBr, TMIS, DTE	0.01 μg/kg	[139]
Milk, eggs; France	E1, αE2, βE2, T, 17αT, dihydroepiandros-tendione, 4-androstendione	ZB-5MS (30 m × 0.25 mm id, thickness 0.25 μm)	MS/MS	SPE, derivatization with MSTFA, BSTFA, PFBBr, TMIS, DTE, HPLC fractionation	Estrogens 5 ng/kg in milk, 30 ng/kg in eggs, androgens 10 ng/kg in milk, 50 ng/kg in eggs	[144]
Beef meat; Germany	βE2, αE2, E1, E3, T, P4	BPX-5 (30 m × 0.22 mm id, thickness 0.25 um)	MS	Extraction, derivatization with DTE, MSTFA, TMIS; SPE	E1 0.02 μg/kg, βE2, αE2 0.04 μg/kg, E3 0.06 μg/kg	[145]
Meat: beef and veal, pork, chicken, turkey, hen, fish, milk, eggs, plants, beef, pork liver; Germany	αE2, βE2, E1, E3, androgens, P4	DB-5MS (30 m × 0.25 mm id, thickness 0.25 μm)	MS	Extraction, derivatization with MSTFA, TMIS, DTE	0.01 – 0.3 μg/kg	[146]
Meat: beef, pork, chicken, lamb, internal organs: chicken, pig liver; Taiwan	HEX, DES, DIE, βE2, androsterone (anabolic steroids)	DB-5 (30 m × 0.25 mm id, thickness 0.25 μm)	Ion-trap MS, MS/MS	SPE, derivatization with DTE, MSTFA, TMIS	HEX + androsterone 0.2 μg/kg, DES, DIE, bE2 0.1 μg/kg	[147]
Kidney fat, meat; Belgium	DES, HEX, DIE, EE2 (estrogens, gestagens, androgens)	SGE BPX-5 (25 m × 0.22 mm id, thickness 0.25 um), nonpolar 5% phenyl-polysilphenylene-siloxane	MS, MS/MS	Extraction, SPE, derivatization with MSTFA	0.5 μg/kg	[119]
Beef meat, liver, kidney, perinatal fat; France	αE2, βE2, E1	Sac.5 (30 m × 0.25 mm id, thickness 0.25 μm)	MS	Extraction, SPE, derivatization with MSTFA and TMIS	E2 in matrix 1 ng/kg	[52]
Canned products and packaging materials						
Cans of different types of food, corn, tomato, paste, stew and tuna fish near their expiration dates; Iran	BPA, BPF	HP-5 (30 m × 0.25 mm id, thickness 0.25 μm)	MS	SPME	0.1 μg/kg	[148]
PVC films, PVC dishes for food packaging; Czech Republic	NP	DB-5MS (30 m × 0.25 mm id, thickness 0.25 μm)	MS	Extraction	5 μg/g	[149]

Table 3. Continued

GC							
Matrix	Compound	Stationary phase	Detection	Sample pretreatment	LOD		Refs.
Plastic films for deep freeze and also other packaging; Slovenia	NP	ZB-5MS (60 m × 0.25 mm id, thickness 0.25 μm)	Ion-trap MS, MS/MS for quantitation)	Extraction, derivatization with MSTFA	LOQ 0.1 ng/μL		[150]
Canned fruits and vegetables, Spain	BPA, BPF, BPZ, BP	HP-5MS (30 m × 0.25 mm id, thickness 0.25 μm)	MS	SBSE	BP and BPF 0.9 ng/L, BPZ 1.7 ng/L, BPA 2.5 ng/L		[107]
Packages – food cans; Japan	BPA	DB- 5, capillary column of cross linked 5% phenyl methyl siloxane	MS	SPE, derivatization with BSTFA	0.05 μg/L		[151]

DTE, dithioerythritol; HFBA, heptafluorobutyric acid anhydride; PFBBr, pentafluorobenzylbromide; PVC, polyvinylchloride; T, 17β-testosterone; TMIS, trimethyliodosilane.

prepubertal children there is a little secretion of estrogens, and serum βE2 concentration is undetectable (<2 pg/mL) in a conventional enzyme immunoassay [29]. Therefore, the exposure to small doses of estrogens may have adverse effects on growth and maturation in prepubertal children. Exposure to exogenous estrogens through intake of commercial milk produced from pregnant cows has spread around the world since the 1970s. The intake of pregnant cow's milk is one of the causes of early sexual maturation in prepubertal children [30].

APs present in fish, avian, and mammalian cells can mimic the effects of βE2 by binding to the ER. OP was 10- to 20-fold more potent than NP and probably more potent than the other APs studied by Soto et al. [31]. The AP polyethoxylates and AP carboxylic acid derivatives are also estrogenic active, but these, together with OP and NP, are from three to four orders of magnitude less potent than βE2 itself [32].

Biochemical assays have examined the kinetics of BPA binding to ERs and have determined that BPA binds both ERα and ERβ, with approximately tenfold higher affinity to ERβ [33–35]. The effects of exposure to BPA can be particularly harmful to the fetus, infants, and young children, because of lack of feedback regulating the activity, synthesis, and elimination of hormones. Contact with BPA at that time may lead to irreversible changes appearing even after much delay. A study on mice revealed that a concentration of BPA as low as 20 ppm in drinking water is sufficient to bring about genetic changes in mice fetuses. Although BPA concentrations in the food products are low, they are used daily or a few times a day, so the dose accumulates [36]. DES was banned as a performance enhancer in most countries in the 1970s because the clinical use of this compound in women (to prevent miscarriage) led to particular kinds of vaginal cancer in some of their daughters [37].

3 Sample pretreatment

The first step of any analysis is the isolation of the component of interest from the sample matrix into an injectable solution at concentrations detectable by the selected analytical separation system. An edible matrix is complex and can interfere with detection of analyzed compounds present in low concentration. Attention has to be paid to minimizing background contamination through laboratory materials like solvents, SPE columns, glassware, plastic ware and other reagents, and laboratory tools [10].

The simplest way is the isolation of analytes by various extraction techniques. The proper sample treatment can considerably improve the LOQ. The sample pretreatment techniques should use a minimal sample amount; be reproducible, robust, simple, cost effective, time efficient and safe; and have a limited number of steps [38]. Treatment of the sample prior to analysis itself is an important step that is often the main source of errors in quantitative analysis. The analytical methods available are often insufficiently sensitive and thus a preconcentration step is necessary, which is often

Table 4. Examples of immunochemical methods for analysis of EDCs

Immunomethods						
Matrix	Compound	Stationary phase	Detection	Sample pretreatment	LOD	Refs.
Milk products						
Packages, milk; Belgium	BPA	Indirect competitive ELISA	UV/VIS	Spiked with methanolic BPA solution		[155]
Milk, cream, butter; USA	βE2, E1	RIA, ^{125}I labeled E2	Gamma counter	Extraction, SPE (Sephadex LH-20)	LOQ 0.13 pg/mL	[156]
Human colostrum; Japan	BPA	ELISA—EcoAssay BPA kit	UV/VIS 450 nm	SPE (Oasis HLB)	0.3 ng/mL	[154]
Packaging material						
Bottles; USA	BPA	Competitive ELISA	UV/VIS 450 nm	Standardized washing/rinsing procedure	0.06 ng/mL	[153]
Reusable bottles from diffenrent materials; USA	BPA	BPA ELISA assay	UV/VIS 450 nm	Standardized washing/rinsing procedure	0.05 ng/mL	[157]
Meat						
Chicken meat, liver; China	DES	ELISA vs. LC/MS/MS	UV/VIS 492 nm, MS/MS	Homogenization, SPE, extraction	0.07 ng/mL ELISA, 4 ng/kg chicken meat, 30 ng/kg liver tissues LC/MS/MS	[160]
Various matrices						
Food: cereals, vegetables, fruits, dairy products, meat and protein foods, oils, fats; Spain	E1	RIA		Extraction	0.2 μmol/kg of fresh material	[161]
Human plasma—general population and dialysis patients, ovarian follicular and seminal fluids from infertile couples; France	BPA	RIA vs. LC, ^{125}I labeled BPA, C18 Nucleosil	Gamma counter, fluorescence 275/310 nm	Extraction	0.08 μg/L	[15]

followed by purification. Sample pretreatment and preconcentration depend on analyte properties, their concentration; on the matrix complexity; and on the final analytical method. A number of papers deal with the pretreatment of samples from aquatic system (see e.g. refs.[38–41]).

Less attention is paid to pretreatment of edible matrices containing estrogenic compounds. Sample pretreatment for edible matrices usually involves homogenization, hydrolysis (if required), extraction/clean-up, enrichment, and derivatization when needed. Fats, oils, and butter are considered to be homogeneous. Meat and fish products are initially blended and homogenized, then ground with anhydrous sodium sulfate or lyophilized (e.g. refs.[42–46]). The fruit and vegetable samples are cut into small pieces and homogenized with 1 M HCl [47]. It was observed that acid pretreatment increased the recoveries; it is assumed that the enzymes are inhibited by acid and thus the enzymatic degradation of estrogenic compounds is prevented. Homogenized solid samples are extracted using an organic solvent. The selection of the solvent is critical, as it affects the yield of the analytical procedure. For the extraction of sex hormones from eggs, various solvents have been tested [48]. Methanol provided the highest recoveries. It has been found that there was emulsion formation when diethyl ether was used as the extraction solvent. Acetonitrile (ACN) yielded cleaner extracts but the extraction efficiencies were lower compared to methanol. On the contrary, acetone provided the highest recovery for the extraction of estrogens from vegetables and fruits [47].

Food samples contain various amounts of proteins and lipids. Proteins can simply be removed by precipitation with ACN or TFA. The most complicated matrices are those with high lipid content (e.g. meat and seafood, milk and milk products, eggs and fat from food of animal origin). Egg fat is composed mainly of phospholipids, triacylglycerolipids, phosphocholine lipids, and cholesterol. Their presence may unfavorably affect the clean-up and preconcentration procedures. It is quite complicated to extract the EDCs without coextraction of lipids, which are usually difficult to remove from the extract and may harm the detection system. To separate lipids from the extracts, liquid–liquid partitioning [49, 50], saponification [51] or acid treatment, freezing-lipid filtration, matrix solid-phase dispersive (MSPD) extraction, and multiple clean-up methods [52–54] are applied. Homogenized samples can be defatted with hexane followed by liquid–liquid extraction (LLE) with diethyl ether [55].

Wang et al. have published a new method for lipid removal from egg products [48]. The method is based on depositing lipids by divalent metal ions, such as Zn^{2+}. Based on this principle, a method of eliminating lipids using $ZnCl_2$ depositing filtration to quantify sex hormones in high lipid-containing samples by UHPLC–MS/MS (UHPLC is ultra high performance liquid chromatography) has been developed, and the method has successfully been applied to real samples.

Estrogens and estrogen mimics are most often determined in milk at low concentrations, in the presence of many accompanying compounds. Estrogens are present in milk mostly as conjugated metabolites (glucuronated and sulfonated estrogens), which represent about 85% of the overall amount of estrogens [56]. Conjugated estrogens may deconjugate to free estrogens and thus become active. Therefore, it is important to determine free and also conjugated estrogens. When GC is used, the conjugated estrogens must be deconjugated to free estrogens, whereas HPLC can analyze both the free and conjugated estrogens simultaneously. Hydrolysis is carried out either enzymatically by glucuronidase [57] or by an acid [58].

Solvent extraction is a time-consuming method that often requires a large amount of organic solvent, or needs a specific apparatus for processing and more energy. In recent years, ultrasound-assisted extraction has attracted growing interest, as it is an effective method for the rapid extraction of a number of compounds from food, with extraction efficiency comparable to that of classical techniques [59]. It offers an increased yield of extracted components and increased rate of extraction, reduction in extraction time and higher processing throughput. Ultrasound can enhance existing extraction processes. Similar advantages, e.g. reduction in solvent consumption and automation of sample handling, offers accelerated solvent extraction (ASE) [60]. ASE has been applied for analyzing BPA, OP, and NP in meat by LC–MS/MS [61]. Other authors [62] have extracted APs from fish liver using ASE and Florisil clean-up, comparing the efficiency of ASE with conventional Soxhlet extraction. Carabias et al. [63] determined the levels of BPA and NP in cereals using ASE. ASE and LC–MS/MS have been used for simultaneous determination of BPA, OP, and NP in powdered milk and infant formulas [64]. The milk samples were blended with RP silica C_{18} and anhydrous sodium sulfate. The analytes were extracted using the ASE with ethyl acetate as a solvent at 70°C. Vegetable samples were divided by filtration into liquid and solid parts. Liquid matter was cleaned up by SPE followed by treatment with neutral alumina. Solid matter was extracted by a mixture of acetone and methanol, using ASE, followed by cleanup with neutral alumina and C_{18} cartridges [65]. Diatomaceous earth was added to the ASE cells as a desiccant.

3.1 SPE

SPE is the most common preconcentration method, because it is simple, many solvents are available, and the consumption of solvents is small. SPE is thus used in most cases as the primary preconcentration technique. The most common sorption mechanism is based on hydrophobic interactions and thus alkyl-bonded silicas are encountered most often. With strongly polar and low molecular mass substances, the OasisTM HLB sorbent (a copolymer of *N*-vinylpyrrolidone and divinylbenzene) gave good results [66]. For the simultaneous extraction of conjugated and free estrogens with a wide range of hydrophobicity, two cartridges with different sorption mechanisms had to be used [67–69]. An example is the preconcentration and treatment of a milk sample prior to HPLC–MS/MS, involving precipitation of proteins by ACN with acetic acid, SPE on two precolumns containing sorbents based on the hydrophilic–lipophilic balance (OasisTM HLB) and amine-functionalized packing materials (StrataTM Amino SPE), with elution by ethyl acetate and methanol (9:1, v/v) and by methanol with 5% ammonium hydroxide, by evaporation of the eluate and dissolution of the residue in H_2O/methanol (95:5, v/v), or 100% methanol [70]. Sample losses during pretreatment have been observed. It has been proven that evaporation of the SPE eluate to complete dryness should be avoided to prevent irreversible adsorption of the parent estrogens on the test tube surfaces. The importance of using stable isotope-labeled surrogates to compensate for analyte losses during the sample preparation has been emphasized. The recoveries ranged from 72 to 117% for the free estrogens (estrone (E1), βE2, estriol (E3), and 17α-ethynylestradiol (EE2)), and 62 to 112% for seven conjugated metabolites. The three doubly conjugated, highly polar metabolites have given lower recoveries (≤43%), due to poor retention in SPE.

Two SPE cartridges, the first with C_{18} RP and the second with silica gel, have been applied for sample pretreatment prior to analysis of phytoestrogens in milk, cereals, and baby food. The second cartridge efficiently eliminated the naturally occurring estrogens [14]. Anabolic hormones were extracted from muscle (pork, beef, and shrimp), milk, and pig liver with methanol after enzymatic hydrolysis and purified using two SPE cartridges with a graphitized carbon black (GCB) and an NH_2 sorbent, followed by LC–ESI-MS/MS [71]. The combination of GCB–NH_2 cartridges exhibited high recovery and excellent matrix clean-up for most analytes; high sensitivities for DES, hexestrol (HEX), and dienestrol (DIE) during LC–MS/MS analysis; and convenience of operation of the GCB cartridge over C_{18} and HLB cartridges. LOQs were between 0.04 and 2.0 μg/kg and average recoveries were in the range from 76.9 to 121.3%.

Many advantages over off-line combination of preconcentration and separation are provided by direct coupling of SPE with a separation method. Dynamic flow-through systems simplify the preseparation step and permit automation of the whole separation system. The advantages of dynamic SPE over static SPE include the reduced sample, sorbent, and eluent consumptions; a higher sample throughput; better sensitivity; and improved accuracy. A dynamic SPE–GC–MS method, including precipitation of proteins and a clean-up in the SPE module and derivatization

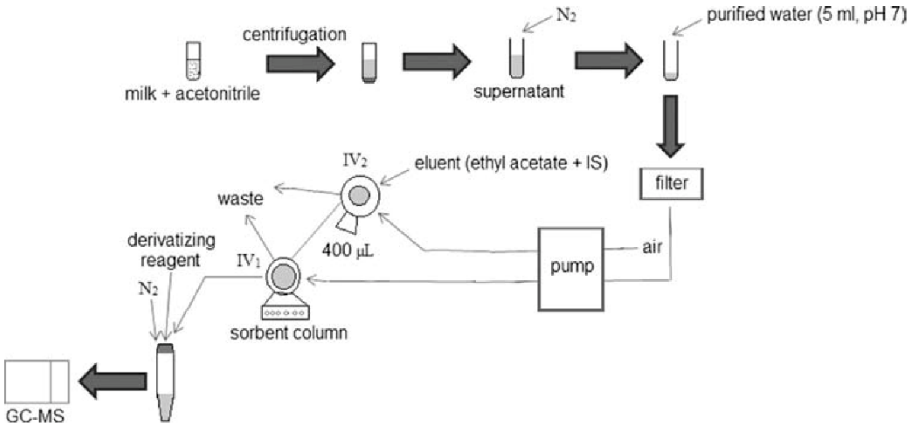

Figure 2. Dynamic flow system for determination of various types of compounds in milk including steroid hormones. IV, injection valve; IS, internal standard. Figure is drawn according to Ref. [72].

with a *N,O*-bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (BSTFA + 1% TMCS) mixture (Fig. 2), has been applied to simultaneous determination of various types of pharmaceutically active substances, including steroid hormones in milk samples from different sources (cow, goat, lactating women). The method features good linearity, accuracy, and precision. High recoveries between 91 and 104% have been obtained [72].

Triacontyl-bonded silica (C_{30}) has been recommended as automated SPE material coupled to HPLC–MS for online extraction of estrogens and BPA in bovine milk [73]. This material allows large sample volume injection (ca. 1 mL) and a high speed of analysis. The milk sample was pretreated with ACN for protein precipitation and then treated with a weak anion exchanger sorbent for the removal of polar impurities. Under the optimized conditions, the recoveries for all analytes ranged from 71.4 to 97.1%.

The development of automated and miniaturized sample-preparation methods with reduced or eliminated solvent consumption has become a dominant trend in analytical chemistry in the past decades. Solid-phase microextraction (SPME) is one of these miniaturized methods. The main advantage of SPME in comparison to SPE is lower consumption of solvents. Online connection of SPME is possible both in GC (the analytes sorbed on the SPME fiber are thermally desorbed in the injection block) and LC. In LC, the analytes can be desorbed by solvents from the fiber either statically or dynamically, by passing the mobile phase through the desorption chamber. The static desorption mode has yielded better results than the dynamic mode for estrogenic compounds in aqueous sample using polyacrylate fibers [74]. The LODs have been between 0.3 and 1.1 µg/L using UV detection and between 0.06 and 0.08 µg/L with electrochemical detection. A poly(acrylamide vinylpyridine-*N,N*-methylene bisacrylamide) monolith, synthesized inside a capillary, has been selected as the extraction medium in an SPME–HPLC method for the analysis of four estrogens [75]. SPME has been applied for online HPLC–MS/MS of five estrogens: E1, βE2, E3, EE2, and DES. A 34- to 90-fold higher sensitivity than that obtained with direct sample injection into the separation system has been attained [76].

Dispersion of adsorbent directly into a biological matrix is a part of the matrix solid-phase dispersion (MSPD) method, which is a combination of sample homogenization, cellular disruption, extraction, purification, and preconcentration in one step. MSPD as a sample-preparation method was first introduced in 1989 [77]. MSPD is usually applied to solid and semisolid samples, including animal tissues and foods with a high lipidic content. For biological tissues, 0.1–5 g samples are blended with an adsorbent that destructs tissues and releases the analytes. As performed in SPE, the mixture is then packed into a cartridge and eluted with a few milliliters of solvent. Chen et al. applied MSDP for the analysis of tissue samples of fish and clams [78]. MSPD is characterized by a reduced consumption of organic solvents, provides high extraction yields, and offers a considerable degree of selectivity [79]. Eight free progestogens were extracted from eggs by MSPD [54]. MSPD with a matrix based on multiwalled carbon nanotubes was applied for the extraction of hormones, including EE2, 17α-estradiol (αE2), E3, βE2, E1, medroxyprogesterone, progesterone (P4), and norethisterone acetate in butter samples [80]. Multiwalled carbon nanotubes have an extremely large surface area and strong adsorption properties due to a hydrophobic surface and a structure with an inner cavity.

3.2 New materials for SPE

Great attention is paid to research into new materials for SPE. This group includes, e.g. nanocomposites of polypyrrole-coated magnetite nanoparticles with a highly π-conjugated structure and hydrophobicity. These materials consist of monodisperse and sphere-like nanoparticles with a mean size of about 65 nm with polypyrrole encapsulated on the surface. They were used as a magnetic SPE (MSPE) sorbent for the extraction of estrogens from milk samples [81]. Estrogens present in milk are captured in the polypyrrole-coated magnetite nanoparticles sorbent without having to precipitate proteins prior to extraction. The extraction is very fast (ca. 3 min). The recoveries of estrogens from milk samples were in the range of 83.4 to 108.5%. Similar sensitivities for

determination of estrogens in milk using preconcentration on the SPE Oasis column in combination with LC–MS/MS have been reached [70, 82]. However, the precipitation of proteins prior to extraction has been required.

Magnetic carbon nanotubes (MCNT) were applied as sorbents for the MSPE of estrogens in milk samples [83]. When carbon nanotubes and magnetic nanoparticles are mixed in a solvent, the magnetic nanoparticles were wrapped in the carbon nanotube bundles and MCNT are formed that can be separated from the solvent by a magnet. MCNT have large surface area and high affinity toward various organic compounds. The reproducibility of the MSPE with different batches of MCNT was acceptable with RSD values <3.6%.

The disadvantage of conventional SPE materials is low selectivity, which results in the coextraction of interferents from the matrix. The highly specific materials include immunosorbents and imprinted polymers. Immunosorbents are prepared by binding the antibody specific for the analyte on a suitable sorbent. They can preferably be used for the extraction of polar substances, because the interaction with the antigen excludes hydrophobic interactions and thus nonpolar substances are not coextracted. Disadvantages of immunosorbents are their low stability and high cost. Immunosorbent binding capacity can be increased by using antibody fragments instead of whole antibodies. This will increase the number of binding sites [84]. Immunosorbents were used, e.g. to extract mycoestrogens from meat-based baby products and milk [85] and to selectively capture the βE2 and E1 from wastewater [86]. High selectivity of immunosorbents is preferable when using ESI-MS detection. By removing the interference that reduces the ionization and isobaric noise in single/selected ion monitoring mode, a reduction of detection limits has been achieved.

Sol–gel immunosorbents have been proposed for sample clean-up in the determination of BPA in food and urine [87]. In sol–gel immunoaffinity columns, the antibodies are physically entrapped in the pores of a silica network instead of being covalently bound to a solid support material, as it is the case in most of the commonly used immunoaffinity columns. The simple way of production, possibility to control the pore size of the silica network by the aging time and the high storage stability and reusability of the columns are the main advantages of sol–gel immunoaffinity columns over columns prepared by covalent immobilization [88]. The preparation of sol–gel immunoaffinity columns is described in detail in Ref. [87].

Other highly selective sorbents are molecularly imprinted polymers (MIPs), also called artificial antibodies. They are highly cross-linked synthetic polymers that are characterized by high selectivity. The other advantages of MIPs besides selectivity include resistance to elevated temperature and pressure, chemical inertness, high reproducibility under harsh operating conditions, low cost, and relative ease of preparation. They have, however, several drawbacks, including instability caused by template bleeding and the necessity to carefully optimize synthesis and operation of MIPs to lower the extent of nonspecific binding [89]. Template bleeding, which is caused by incomplete removal of the template from the MIP, complicates analysis at trace concentration level. This problem can be solved by using a molecule of similar structure to analyte as a template [90]. In the case of its bleeding, it may be separated in the subsequent chromatographic analysis. It was also found that the modification of the MIP by polar monomers suppresses matrix interferences.

A highly selective macroporous MIP/cryogel composite sorbent permitting high flow rates has been prepared for the removal of endocrine-disrupting trace contaminants [91]. An MIP with βE2 as the template has been incorporated into a macroporous polyvinyl alcohol gel at subzero temperatures. Flow rates ten times higher than those used with a normal MIP can be applied. An MIP, synthesized by ultrasonic irradiation, with methylacryloxypropyl modified attapulgite (a magnesium aluminium clay) as a matrix, β-naphthol as the template molecule, acryloyl-β-CD (where β-CD is β-cyclodextrin) as the functional monomer, and *N,N′*-methylenebisacrylamide as the cross-linking agent, has been applied as the packing material for online SPE of E1, βE2, E3, and DES present in milk samples. Compared to polymers prepared by the traditional heat method, the MIP synthesized by ultrasonic irradiation had better recognition selectivity, faster adsorption kinetics, and shorter preparation time. The LODs for these estrogens were in the range 1–8 ng/g and reproducibility expressed as RSDs ($n = 6$) was better than 5.1% with milk samples spiked at 100 and 1000 ng/g of each analyte [92]. The MIP layer at the silica nanoparticles surface with E3 as a template has been prepared and applied as absorbent in dispersive SPE coupled with LC to determinate trace E3 and βE2 in milk tablets [93]. The methacrylic groups of functional monomer at the silica nanoparticles surface act as reactive sites to induce imprinting polymerization. The MIP had an excellent affinity, recognition selectivity, and fast kinetics. The high recoveries of 89.1–93.5% were achieved with RSDs <9.4%.

An MIP-coated fiber was prepared by using silica fiber as substrate, EE2 as pseudo template, 2-(trifluoromethyl) acrylic acid and acrylic acid as composite functional monomers, and ethylene glycol dimethacrylate as the cross-linking agent and applied for determination of estrogens in milk powder [94]. The MIP-coated fiber possessed excellent stability, rebinding capacity, selectivity, and kinetics. The LODs of the HPLC–UV method were in the range 0.83–2.5 ng/mL; the recoveries were 86–92, 88–97, 90–93, and 95–99% with RSD <6.7% for E1, βE2, E3 and EE2, respectively.

MIPs as SPE sorbents have been applied for sample clean-up in the analysis of BPA. An MIP with BPA as the template and 4-vinylpyridine as the functional monomer has been developed as a selective sorbent for the determination of several phenolic compounds (BPA, bisphenol F (BPF), and 4-nitrophenol) and phenoxy acid herbicides in honey [95]. The recoveries achieved were in the 81–96% range. By applying the MIP pretreatment prior to LC with ion-trap MS, the LODs achieved in commercial honey samples were in the 0.1–3.8 ng/g range, with RSDs of 12–24%.

Another application of MIPs as SPE sorbents for the determination of BPA in milk and bottled water is described in Ref. [96]. MIPs were prepared by two synthetic routes: semicovalent and noncovalent methodology. Polymers prepared with noncovalent mode were proven more effective. The developed sample preparation was simple and efficient comprising only milk dilution and SPE with MIP prior to LC–MS analysis. The method provided quantitative BPA recoveries, very good reproducibility (percentage RSDs lower than 7%), and low LOD (0.2 ng/g). Low flow rates in the elution step enhanced extraction recovery. The MIPs exhibited high breakthrough volumes (>500 mL of water), high mass capacity (>10 ng/mg of MIP sorbent), good linearity, and good stability.

SPME preconcentration with a fiber coated with a controlled thickness of MIP has been described [97]. SPME fiber was inserted into a larger bore capillary and a polymerization mixture with BPA as a template was introduced into the resulting interspace. After a running polymerization, the upper tube was removed. SPME fiber modified with BPA-imprinted polymer was tested for the determination of BPA in tap water, urine, and liquid milk. Recovery of BPA from milk was 87.5%, with an LOD of 38.9 ng/mL.

A novel sample-preparation technique, dynamic liquid–liquid–solid microextraction, was developed and online coupled to HPLC for direct extraction, desorption, and analysis of trace estrogens in complex samples [98]. The experimental setup consists of the aqueous donor phase, the organic medium phase, and the molecularly imprinted polymer filaments (MIPFs) as solid acceptor phase. The organic solvent with lower density was added on the top of the aqueous sample. The extraction of analytes on MIPF is carried out by circulating the organic solvent through the MIPFs inside a tube. The sorbed analytes on fiber are desorbed online in the HPLC system. The method was applied for the analysis of five estrogens in aqueous samples and in real samples including urine, milk, and skin toner, using βE2 as MIPF template. The enrichment factors of 51–70 were reached, with LODs of 0.08–0.25 μg/L and precision 4.5–6.9% for aqueous samples. Recovery and reproducibility for real samples were 81.9–99.8% and 4.1–7.9%, respectively.

Polymeric ionic liquids (PILs) have been recommended as coating materials in SPME. Ionic liquids (ILs) possess high thermal stabilities, negligible vapor pressures, and unique solvation properties. PILs exhibit unique material properties while retaining the solvation properties inherent to ILs. The stability of sorbent coatings based on these materials enabled the reusability of SPME. The tuneability of the PIL monomer provides for the selectivity of the sorbent. A highly hydrophobic PIL, poly(1-vinyl-3-hexadecylimidazolium) bis[(trifluoromethyl)sulfonyl]imide, has been applied as the SPME sorbent coating for the extraction phenolic contaminants in aqueous samples [99].

Dispersive liquid–liquid microextraction (DLLME) is a preconcentration technique based on the utilization of a mixture of a nonaqueous miscible extraction solvent (organic solvent) and an aqueous miscible polar solvent (methanol, ACN, or acetone). Analytes are enriched in the low volume of extraction solvent (μL), which is dispersed into the bulk aqueous solution, and separated by centrifugation [100]. DLLME has proven to be an efficient extraction technique due to the high contact surface of fine droplets of extraction solvent and analytes, which leads to increased mass transfer processes. ILs have been applied in DLLME for determination of endocrine-disrupting phenols in environmental samples [101]. The method is characterized by average relative recoveries of 90.2% and enrichment factors ranging from 140 to 989.

In the framework of green analytical chemistry, a clean sample-preparation technique, stir bar sorptive extraction (SBSE), has been developed. SBSE is a solventless sample-preparation method for the extraction and enrichment of organic compounds from aqueous matrices. The mechanism is the same as in SPME [102, 103]. The method is based on sorptive extraction of analytes with a magnetic stirring rod coated with an adsorbent phase, usually polydimethylsiloxane (PDMS). The stir bar is subsequently removed and the sorbed compounds are then either thermally desorbed, and analyzed by GC–MS, or desorbed by means of a liquid, for improved selectivity or for interfacing to an LC system. The extraction efficiency depends on the partitioning coefficient of the analyte between the polymer coating and the sample matrix and by the phase ratio of the polymer coating and the sample volume. Compared to SPME, higher sensitivities are obtained due to the larger amount of polymeric phase. Other advantages of SBSE include a large linear range, the possibility to extract several samples at the same time, and omission of time-consuming preparation steps. The overview of the SBSE method and its applications in trace analysis in environmental, food, and biomedical samples can be found in Refs. [102, 104, 105].

A sol–gel technique was used for the preparation of a stir bar coated with a composite composed of polydimethysiloxane and β-CD (PDMS/β-CD) [106]. Good thermal stability and solvent resistance of the stir bar were found thanks to chemical binding formed between the stationary phase and the glass substrate. The PDMS/β-CD-coated stir bar had better selectivity to polar compounds and higher extraction capacity compared to the PDMS-coated stir bar. The PDMS/β-CD-coated stir bar coupled to HPLC has been applied to the determination of estrogens in environmental water, BPA in drinking water and in leachate of one-off dishware. SBSE in combination with thermal desorption GC–MS has been applied for simultaneous determination of BPA, BPF, bisphenol Z (BPZ), and biphenol (BP) in canned beverages and vegetables [107]. New commercially available thermal desorption units facilitate the hyphenation of GC and SBSE, providing higher repeatability and sensitivity.

4 LC

LC is the most common method for residual analysis of estrogens and feminizing chemicals (altogether EDCs). In this

section, we will briefly introduce HPLC methods to separate and detect estrogens and other EDCs from edible matrices and food contact materials. Because of the low levels of analytes of interest content (μg/kg to ng/kg) and the complexity of bio-sample matrices, the analysis of hormones and hormone-like substances is a challenging task [82]. The HPLC analyses were conducted using mostly octadecyl-bonded stationary phases, in combination with mobile phases containing ACN, water, and additives in different volume ratios. The combination of HPLC with mass spectrometric detection is mostly used.

4.1 Milk and milk products

Milk occupies an important position in human diet. It is reported that 75% of milk is produced from pregnant cows in the modern dairy industry [56, 70]. Milk matrix is complex, composed of proteins and lipids. Therefore, sample clean-up pretreatment before LC–MS analysis is essential (see Section 3) [73]. An HPLC method using HLB and amino SPE preconcentration has been described for analysis of free estrogens (EE2, βE2, E1, and E3) and conjugated estrogen metabolites in milk [70]. Analyses were performed on triple quadrupole MS and negative mode ESI has been the preferred mode of ionization in LC–MS/MS. All analytes were monitored using multiple reaction monitoring (MRM) mode providing the LODs for free estrogens ranged from 6 to 37 ng/L. Estrogen metabolites were detected along with the free estrogens and the highest concentration was found for E1 from 23 to 67 ng/L.

The determination of selected progestogens (pregnelonone, P4, 17α-hydroxyprogesterone, 17α-hydroxypregnenolone) in bovine milk from Spain was carried out [108]. Enzymatic hydrolysis and derivatization proceeded before analysis. C_{18} stationary phase and water/methanol mixture with 0.1% formic acid as a mobile phase were applied. Compounds were analyzed by selected reaction monitoring mode of triple quadrupole with positive ESI ionization. Natural hormones are nonpolar compounds and their ionization is usually difficult when using ESI sources. Derivatization can change the chemical structure of analytes leading to higher ionizable molecules. One of these derivatization procedures is oxime formation, which enables the improvement of ketosteroid analysis with ESI-MS/MS. Results obtained in real skimmed milk samples were above decision limit in almost all cases.

E1, βE2, αE2, and E3 were determined in processed and in raw milk from the Netherlands [56]. The samples were hydrolyzed, extracted with hexane, passed through a C_{18} SPE column, derivatized with dansyl chloride in acetone and finally purified using C_{18} SPE. The analysis was conducted using C_{18} stationary phase and mobile phase consisting of ACN/water mixture with formic acid in both components. MS/MS detection was performed in positive MRM mode—the sensitivity was improved by derivatization. Concentrations in processed milk were below the LOD for αE2 and E3 and ranged from 8.2 to 20.6 ng/L for E1 and βE2 depending on the fat content in samples. The method was also applied to the determination of selected estrogens in milk from gestating cows in the first, second, and the third trimester. Values for E3 were again below LOD, other compounds were found in the range from 9.2 to 10 ng/L in the first trimester and from 21 to 118 ng/L in the third trimester. The level of estrogens is rising during ongoing pregnancy, thus the results correspond to this fact.

Online SPE coupled with HPLC–MS has been applied to determine E3, βE2, E1, DES, EE2, and BPA in bovine milk. Different brands of pasteurized, homogenized whole milk samples (3.5% of fat) were purchased from retail markets in Beijing, China. The samples were preconcentrated on a tailor-made C_{30} extraction SPE column, separated on C_{18} stationary phase and detected by negative ESI ionization and single quadrupole MS [73]. MSPE has been used for the extraction of E1, βE2, E3, DIE, HEX, DES, EE2 in three kinds of milk purchased in Wuhan, China. The HPLC–MS/MS separation using negative ESI ionization was performed on Shim-Pack VP-ODS column with a mixture of ACN/water as the mobile phase. The amount of estrogens ranged from 0.05 to 0.25 μg/L, detailed results are in Ref. [81].

MCNT as a part of MSPE represent a new approach in sample preparation and it has been used for estrogen extraction in milk samples bought in local supermarkets in Wuhan, China [83]. The separation of βE2, EE2, and HEX was achieved on a C_{18} column in combination with fluorescence detector. The linearity range of the proposed method was 5–2000 mg/L with correlation coefficients of 0.9983–0.9994. The LOD for three estrogens ranged from 1.21 to 2.35 mg/L. The intra- and interday RSDs were <9.3%. No positive samples have been found in the analysis.

BPA is a lipophilic compound with the potency to partition into fat and breast milk. The monitoring of human breast milk contamination with BPA is very important, because breast milk is the first food for newborns and thus they could be affected in the critical growth processes. Therefore, breast milk samples from twenty-three lactating women in Nagasaki, Japan, have been investigated for trace analysis of BPA [109]. The samples were fractionated by SPE, then cleaned up using LLE and derivatized with 4-(4,5-diphenyl-1H-imidazol-2-yl)benzoyl chloride. The separation was conducted on a C_{18} column and eluted with ACN/acetate buffer/methanol mobile phase coupled with fluorescence detection. BPA in milk samples was in the range 0.28–0.97 μg/L.

UHPLC is preferable to conventional LC. It generates narrower peaks, which facilitates better resolution of the analytes from the interferences present in the matrix and shortens the chromatographic run [110]. Chromatographic separation of E3, βE2, αE2, equilin, EE2, E1, DES, DIE, and HEX was carried out on an UPLC HSS T3 column. Analytes were eluted by ACN and water mixture and analyzed by negative-ion mode of ionization followed by Q-TOF mass spectrometric detection. Using gradient-elution program, the estrogens can be baseline-resolved in 8 min, with total chromatographic analysis time of 13 min. This method was applied to the analysis of milk powder samples of four different brands purchased

from local supermarkets in Shanghai, China. No estrogen of interest was detected in these samples [111].

4.2 Egg products

Egg products are foods containing high lipids. Most of the methods for the isolation of sex hormones from lipids in extracts are time consuming, costly and use large amounts of organic solvents. In Ref. [112], it was reported that lecithin can be deposited by divalent metal ions such as Zn^{2+}. Based on the same principle, an UHPLC–MS/MS method was developed to quantify sex hormones after $ZnCl_2$ eliminating of lipids [48]. Delipidated samples were cleaned up using C_{18} and NH_2 SPE. LC separation was achieved on an UPLC BEH C_{18} column coupled with tandem mass spectrometer. Negative ESI ionization mode has been found useful for quantitation of EE2, E3, E1, αE2, βE2, DES, DIE, and HEX. Twelve real samples of eggs commercially available from local market in Zhejiang, China, were tested and E1, αE2, and βE2 were found. The levels varied from 0.05 to 1.72 μg/kg.

4.3 Meat

The use of anabolic steroids for growth promotion purposes in meat-producing animals results in the improvement of muscle growth, leaner meat, and a higher feed conversion efficiency [113, 114]. The use of anabolic steroids for fattening purposes has been banned in the European Union since 1986 [115] and therefore, their content in meat has to be monitored. The difficulties in monitoring these compounds are caused by the complexity of the matrix [55]. In order to enhance the extraction efficiency and selectivity, MIPs were introduced into the SPE procedure (see Section 3) [116]. Most interfering compounds can be removed in C_{18} SPE but molecularly imprinted SPE (MISPE) can selectively enrich βE2 in the samples. A sensitive method for quantification of NP, OP, and BPA in pork, fish, mutton, chicken, beef, and duck meat from a Beijing supermarket is described in Ref. [61]. An ASE followed by SPE clean-up and LC–ESI-MS/MS analysis was used. Among the samples, BPA was detectable in 13 of 27 samples with concentrations ranging from 0.33 to 7.08 μg/kg. OP was found only in three samples and NP in 21 samples.

For the simultaneous determination of 34 anabolic steroids (10 estrogens, 14 androgens, and 10 gestagens), a UHPLC method has been used. The separation was carried out on a C_{18} column and the mobile phase was composed of water and methanol. All analytes can be separated in a total run time of 8 min. Analysis was performed on a triple quadrupole mass analyzer fitted with atmospheric pressure chemical ionization source operating simultaneously in positive and negative ion mode. Bovine muscle tissues of different meat brands were obtained from three local supermarkets (Merelbeke, Belgium) [55]. A validated method has been described for analysis of androgenic and estrogenic steroids (e.g. 17α-testosterone, 17α-nortestosterone, trenbolone, EE2) in bovine muscle. The sample preparation consisted of several steps starting with enzymatic degradation followed by an LLE, a defattening step followed by two SPE with HLB and amino-propyl material. The analysis was performed on a C_{18} column, and the mobile phase contained a mixture of water and ACN. The detection was carried out in selected reaction monitoring mode of a triple quadrupole mass spectrometer with atmospheric pressure chemical ionization in positive mode [117].

A simple, sensitive, and selective HPLC–UV method has been developed and applied to the analysis of four estrogens, namely βE2, DIE, DES, and HEX, in pork and chicken samples. After PDMS/β-CD SBSE, the separation was performed on a C_{18} column (Lichrospher ODS) with methanol and 10 mmol/L NaH_2PO_4 (60:40, v/v, pH = 3) as the mobile phase. Under the optimal experimental conditions, the LODs ranged from 0.21 (DIE) to 1.6 μg/L (βE2). The enrichment factors ranged from 18.9 to 50.1. The four target estrogens were not detected in the pork and chicken samples [118].

4.4 Fish and shrimp

Estrogens have been used also in aquatic animals to gain weight; nowadays, these compounds are banned in foods of animal origin [82, 119]. In study concerning fish and shrimp tissues, samples were extracted using dispersive SPE and cleaned up prior to the HPLC. The dispersive SPE was designed for samples containing at least 75% moisture because water could make pores more accessible and consequently provide effective extraction [120, 121]. The evaluation of the suitability of the whole procedure was tested with four estrogens, namely βE2, E3, 4-hydroxyestradiol, and 2-methoxyestradiol. Estrogens were separated through the VP-ODS-C_{18} column by methanol/water mobile phase coupled with fluorescence detection for confirmation and quantitation. The application of this method on real samples has shown that recoveries and procedural precisions appear higher in shrimp than in fish samples due to the less complex matrix [122].

4.5 Miscellaneous matrices

A validated method using off-line MISPE coupled with HLPC–UV method enabled the analysis of trace βE2 in dairy and meat products. Whole milk, yogurt, beef, chicken, and pork samples bought from a local supermarket (Wuhan, China) were analyzed. The method consisted of extraction by MISPE βE2 followed by separation on a C_{18} column with a binary mobile phase ACN/water. The most of the selected products contained trace βE2 (0.116–0.461 nmol/kg) [123]. In another study, a comprehensive LC–ESI-MS/MS method has been developed to detect βE2, E1, E3, DES, DIE, EE2, HEX in porcine meat, liver and kidney, chicken and milk. The samples were preconcentrated using HLB SPE cartridge and cleaned up by amino-propyl SPE. The separation was conducted using a phenyl column coupled to a mass detector operating in negative ESI MRM mode. Androgens were

also determined by this method employing a positive ESI mode [82]. As pointed out above, BPA and also NP and OP are lipophilic compounds. Therefore, they can easily contaminate foods of animal origin, which are considered to represent the most important source of human exposure to many organic pollutants.

4.6 Packaging material

As was mentioned previously, BPA is a starting material for polycarbonate and epoxy resin synthesis. Epoxy resins are used as a plastic coating in the food packaging industry. BPA has been detected in canned food, microwave containers, autoclavable flasks, and baby bottles [124–128]. BPA migration levels from food cans and microwave containers were measured using a C_{18} column and an ACN/water mixture as a mobile phase coupled to a fluorescence detector. A total of 45 brands of canned food, 20 plastic microwave containers, and 20 soft drink cans from supermarkets were analyzed. The study estimated the dietary intake of BPA by pregnant women from canned foods in Southern Spain. Migration limits were below the EU limit, but the effect of low-dose exposure remains of concern [129].

An HPLC method for the determination of BPA in reusable baby bottles, water carboys, and other homeware often made of plastics is described in Ref. [130]. The separation system consisted of Hypercarb S graphitized carbon column and methanol/water/ACN mixture as the mobile phase with fluorescence detector. BPA was determined in the polycarbonate baby bottles and cups at levels ranging from 7 to 58 μg/g. BPA concentrations in the bottled waters ranged from 0.1 to 4.7 ng/L. The concentration of BPA in the water increased with the contact time. Confirmation was performed using a GC–MS method. Methods of applying the PDMS/β-CD-coated stir bar extraction for the determination of estrogens in environmental water, BPA in drinking water and in leachate of one-off dishware coupled with HPLC were presented in Ref. [106]. Diamonsil C_{18} and Phenomenex C_{18} columns were used for separation of estrogens (E1, βE2, E3, and EE2) and BPA, respectively. The mobile phase consisted of ACN/acetate buffer (45:55, v/v, pH = 3.5) for estrogens and of methanol/water (70:30, v/v) for BPA. The LODs were within the range 0.04–0.11 μg/L for estrogens with UV detection and 8 ng/L for BPA using fluorescence detection. Samples of river and lake water were analyzed by the proposed method. E3 was detected in lake water, but not quantified. Samples of drinking water show higher concentration for purified water from plastic container than for tap water. BPA was detected in leachate of a paper cup and a food box [106]. Examples of the LC analysis of EDC are presented in Table 2.

5 GC

GC is often used for analysis of estrogens and estrogen mimics in environmental samples (for reviews see e.g. Refs. [40,132–134]). Papers dealing with the determination of EDC in edible matrices are less common. Several EDCs can be analyzed by GC directly, without derivatization; however, their conversion into a more volatile state is usually preferred. Derivatization usually leads to higher sensitivity and selectivity of the detection. It has been observed that derivatization increased the mass spectral signal intensity of the component of interest 2–12 times compared to an untreated sample [135]. Derivatization is focused on specific groups of analytes; related compounds may be excluded from analysis [136].

Nevertheless, derivatization may be laborious, most of the derivatization reagents are toxic and instable and derivatization is an extra step, which may be a source of additional errors. Derivatization is most frequently performed in compounds containing active hydrogen, e.g. the phenolic–OH group of EDCs. The most common derivatization reactions are silylation and esterification. They lead to increased volatility, reduced polarity, and improved thermal and catalytic stability, which is important from the viewpoint of mass detection. Additionally, silylation leads to the formation of more favorable diagnostic fragmentation patterns. Derivatization applied prior to GC analysis is discussed in detail in, e.g., Ref. [39].

A new silylation reagent, dimethyl-(3,3,3-trifluoropropyl)silyldiethylamine, has been proposed for the determination of natural (E1, βE2 and E3) and synthetic (EE2 and DES) estrogens prior to GC/MS analysis. The dimethyl(3,3,3-trifluoropropyl)silyldiethylamine derivatives exhibited good separation properties (low retention times) and ionization properties. The reagent possesses strong nucleophilic properties and the reaction is completed at 30°C in 30 min [137].

5.1 Milk and milk products

Hormones (E1, βE2, EE2) and other pharmacologically active compounds were determined in food samples including whole, raw, half-skim, skim, and powdered milk from different sources (cow, goat, and human breast) [72]. The procedure involves deproteination of the milk, followed by sample enrichment; clean-up by continuous SPE; and conversion into their silyl derivatives with a mixture of BSTFA and 1% TMCS to improve volatility and thus sensitivity in the subsequent GC–MS analysis. The method provides a linear response over the range 0.6–5000 ng/kg with LODs from 0.2 to 1.2 ng/kg depending on the particular analyte.

Nonsteroidal estrogens in breast milk, plasma, urine, and hair were analyzed by the GC–MS method in the selective ion monitoring mode after SPE using an Oasis HBL column and derivatization with MSTFA/ammonium iodide/dithioerythritol [138]. The LOD ranged from 0.2 to 3 μg/L (or μg/kg). The method is characterized by high sensitivity, good precision, and specificity.

A method for the analysis of E1, βE2, αE2, EE2, E3, medroxyprogesterone, P4, and norethisterone acetate in butter samples has been published [80]. The method includes

MSPD extraction of the target analytes from butter samples, derivatization of hormones with heptafluorobutyric acid anhydride/ACN mixture, and determination by GC–MS. Ethyl acetate was used as an elution solvent. The recoveries of hormones obtained by analyzing the five spiked butter samples were from 84.5 to 111.2% and RSDs from 1.9 to 8.9%. LODs and LOQs were in the range 0.2–1.3 and 0.8–4.5 μg/kg, respectively.

5.2 Milk, egg, and meat

Different derivatization agents have been applied for the determination of the main sex steroid hormones in milk, egg, and meat [139]. Milk and egg samples were separated into androgenic and estrogenic fractions. Androgens were derivatized with a mixture of an MSTFA/dithiothreitol and trimethyliodosilane. The halogenated *N,O*-bis(trimethylsilyl) trifluoroacetamide/pentafluorobenzylbromide reagent was preferred for derivatization of the estrogen fraction of milk and egg samples, because of increased specificity and sensitivity when negative chemical ionization is used [140]. For meat samples, derivatization was performed with MSTFA/trimethyliodosilane/dithiothreitol for both estrogen and androgen fractions. Sensitive fluorescence detection can be applied after derivatization of estrogen molecules with *p*-nitrobenzoyl chloride [58].

5.3 Fish

BSTFA with 1% TMCS in pyridine was used for derivatization of E1, βE2, EE2, E3, and mestranol for their determination in environmental samples (estuarine water, wastewater, fish bile, and fish homogenate) [141]. Using GC–MS with a large-volume injection programmable temperature vaporization (LVI-PTV), approximately ten times lower LODs (0.04–0.15 ng/L for water samples, 0.04–0.67 ng/g for fish bile, and 0.1–7.5 ng/g for fish homogenate) were obtained than those obtained by means of a common split/splitless inlet. Problems arising due to the use of plastic material during the derivatization step and pyridine during LVI-PTV injection were solved by using glass vials during derivatization and by exchange of pyridine for hexane before LVI-PTV–GC–MS analysis.

5.4 Vegetable and fruits

The analysis of many types of estrogenic contaminants (including BPA, APs, and steroid estrogens) in vegetables and fruit was achieved using an isotope dilution technique and GC coupled with MS [47]. The isotopically labeled standards of related environmental estrogens were used to form the following analyte/surrogate pairings: OP/$^{13}C_6$-4-*n*-NP, 4-*n*-NP/$^{13}C_6$-4-*n*-NP, 4-NP/$^{13}C_6$-4-*n*-NP, BPA/$^{13}C_{12}$-BPA, E1/$^{13}C_6$-E1, αE2/$^{13}C_6$-β-estradiol, βE2/$^{13}C_6$-β-estradiol, 17EE2/$^{13}C_2$-EE2, and E3/D_4-E3. Plant samples were homogenized, extracted ultrasonically with acetone, treated with acid, evaporated to dryness, and derivatized with BSTFA and TMCS. Acid hydrolysis releases free compounds from their conjugates and greatly increases peak intensities for the analytes. Isotope dilution technique accounts for extraction inefficiency and loss of analytes through sample preparation. Recoveries of the spiked analytes were greater than 90%. LOQs ranged from 0.04 to 0.60 μg/kg.

5.5 Canned products

BPA, BPF, BPZ, and BP were determined using SBSE in combination with thermal desorption-GC–MS [107]. Two derivatization procedures were tested, *in situ* acetylation and in-tube silylation, and compared to analysis without derivatization. The conversion to acyl derivatives gave the best analytical parameters and was applied to commercially canned beverages, as well as the filling liquids of canned vegetables, providing detection limits between 4.7 and 12.5 ng/L. The intraday and interday precisions expressed as RSD were lower than 6%. Recovery studies at two concentration levels, 0.1 and 1 μg/L, were performed providing recoveries in the range 86–122%.

5.6 Soya products

A simple and robust GC–MS method employing isotope dilution is described for determination of phytoestrogens in biological samples and food matrices [142]. Samples are hydrolyzed with β-glucuronidase, the aglycones are extracted, the phytoestrogen fraction isolated by chromatography on Sephadex LH20, derivatized with BSTFA and analyzed by GC–MS. Examples of the GC analysis of EDCs are presented in Table 3.

6 Immunochemical methods

BPA presents the most common analyte for immunochemical determinations. DES and estrogens are analyzed by immunochemical methods less frequently. Due to the extensive use of polycarbonate plastic and epoxy resins, humans are widely exposed to BPA [152]. Its extensive use in plastics leads to widespread environmental contamination [153]. Therefore, it is important to investigate the human exposure to BPA [154]. It may occur through direct contact with BPA, or by consumption of food or drinks that have been exposed to material containing BPA [15].

6.1 Milk products

The specific BPA immunoglobulins were isolated from the egg yolk and were used as antibodies in indirect ELISA for determination of BPA in milk [155]. The assay was evaluated

for pasteurized milk with various fat contents obtained from retail shops in Ghent, Belgium. Similar competition curves were obtained for skimmed, semiskimmed, or whole milk, due to the presence of milk proteins. The results confirm that the fat content is of no importance with regard to the assay performance. It could mean that BPA is partially absorbed by the fat globule. Used antibodies were able to penetrate within the interface of the fat globules and aqueous phase thus inducing the immunochemical reactions.

The presence of E1 and βE2 in raw whole cow's milk has been demonstrated by RIA and the effects of pasteurization–homogenization on estrogen contents were studied [156]. E1 and βE2 were quantified in fresh milk after pasteurization and homogenization from organic or conventional skim, in 1%, 2%, and whole milk, as well as in half-and-half, cream, and butter samples. Prior to RIA analysis using ^{125}I-labeled βE2, samples were subjected to organic solvent extraction and chromatography and LODs ranged between 20 and 30 pg/g depending on product. Commercial milk (7 unique brands of organic labeled milk and 11 unique brands of conventional milk) with various amounts of fat were purchased from grocery stores and E1 and βE2 were quantified from 71 unique milk samples. Average concentrations found for E1 were 2.9, 4.2, 5.7, 7.9, 20.4, 54.1 pg/mL and 118.9 pg/g in skim; 1%, 2%, and whole milk; half-and-half, cream, and butter samples, respectively. Concentrations obtained for βE2 were lower: 0.4, 0.6, 0.9, 1.1, 1.9, 6.0 pg/mL and 15.8 pg/g in skim; 1%, 2%, whole milk, half-and-half, cream, and butter samples, respectively. This work confirmed that the amount of fat significantly affects E1 and βE2 concentrations in milk. The concentration of E1 and βE2 increases with an increased concentration of fat. Pasteurization–homogenization did not significantly affect βE2 concentrations in milk.

In Ref. [154], the concentrations of BPA in human colostrum were measured by competitive ELISA. The aim of the work was to understand the present status of BPA burden in human breast milk. Samples were collected within three days after delivery from 101 healthy mothers and were pretreated by ACN and SPE extraction. The mean recovery of spiked samples was 102 ± 19.0% and the detection limit of the method was 0.3 ng/mL. The ELISA was performed using an EcoAssayBPA kit, which was able to detect glucuronide-conjugated BPA as well as free BPA. BPA was detected in all of the 101 samples and its concentrations were in the range 1–7 μg/L.

6.2 Packaging material

BPA contamination of water from different types of reusable drinking bottles was determined using ELISA, previously validated to specifically detect BPA in water samples [157]. Reusable bottles were obtained from retail sources: polycarbonate, copolyester and stainless-steel bottles, aluminum epoxy resin lined and EcoCare™-lined bottles. In the water samples, ELISA shows a high correlation with the GC–MS/MS method. Increased levels of BPA were detected in samples from all four different polycarbonate bottles. These results confirmed that BPA can leach into liquids from new polycarbonate bottles at room temperature. Conversely, no BPA was observed to migrate from EcoCare™-lined aluminum, stainless-steel, or Tritan™ plastic bottles after a 120 h incubation period. In epoxy resin lined bottles, initially exposed to boiling water, the migration of BPA increased significantly.

A similar study has been carried out [153] in which the ELISA method has been applied to the determination of BPA released from polycarbonate bottles used for consumption of water and other beverages. New bottles, obtained from a national retail supplier (NJ, USA) and used ones were compared. Used bottles were collected from anonymous donors (OH, USA) and described as having been normally used for between one and nine years. Bottles filled with water were left up to incubate for (i) seven days at room temperature and (ii) 24 h to study the effect of boiling water. Relative cross-reactivity of the ELISA, for endocrine disruptors and chemicals structurally related to BPA (100% reactivity), has been determined; the most significant cross-reactivity was observed for bisphenol B (15.6%) and bisphenol E (6%). Cross-reactivity for NP was 0.19% and <0.05% for diethylhexylphthalate, βE2, and E1. The LOD for BPA was determined as 0.05 ng/mL. The mean content of BPA estimated for the water samples collected following seven days of incubation from the high-density polyethylene bottles was 0.14 ng/mL. However, detectable levels of BPA were identified in all the water samples collected from either the new or used polycarbonate bottles. Since the values calculated for rates of migration from new or used bottles were not significantly different from each other, the average rate of migration from all bottles was calculated as 0.49 ng/h (SD ±0.17). The exposure to boiling water (100°C) increased the rate of BPA migration by up to 55-fold and the results were highly reproducible.

6.3 Meat

The traditional methods for analysis of DES, which is illegally used as a growth promoter in cattle to increase weight [158, 159], are GC and LC. For monitoring of DES residues in edible matrix, an immunosorbent method appeared suitable. Direct competitive ELISA, using polyclonal rabbit antisera, was developed for a simple, fast, and sensitive assessment of DES in chicken meat and liver tissues, at 0.07 ng/mL detection limit. The method was verified by LC–MS/MS. For detailed sample preparation, see Ref. [160].

6.4 Various matrices

E1 is known to induce an increase in body weight in rats. E1 esters can be found in food and they may alter the mechanism of body weight control. In Ref. [161], the total content of E1 in food was measured by RIA (with LOD of 0.2 μmol/kg for fresh material) and estimated the influence of this hormone on development of obesity. Eight categories of food were

analyzed: chocolates, fish, vegetables, fruits and nuts, pastry, eggs, meat and meat products, dairy products, oils and fats. The lowest content of E1 was found in vegetables—almost at the detection limit (<0.25 μmol/kg in potato, tomato, and lettuce); the highest contents 7.81 ± 1.93, 9.15 ± 3.25, and 11.44 ± 3.04 μmol/kg were determined in Parmesan cheese, butter, and pork lard, respectively. For detailed results, see Ref. [161]. Examples of the immunochemical analysis of EDCs are presented in Table 4.

7 Concluding remarks

An increased attention is paid to the determination of endocrine-disrupting chemicals due to their harmful effects on living organisms. Their analysis is usually carried out in environmental samples but their amount in food, especially in baby food, is of great concern. EDCs are present in samples at very low concentrations and therefore their analyses require time-consuming pretreatment and sophisticated instrumentation. To make the sample pretreatment less costly and time-consuming, new selective materials for EDCs extraction are required. Online sample pretreatment improves the analysis precision and decreases the analysis time. Continuous progress in column technology in LC and in MS instrumentation leads to improved sensitivity of measurements. GC or HPLC combined with MS/MS is mostly employed for the analysis of EDCs, but immunochemical methods also are used especially for the analysis and screening of BPA in edible matrices.

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2 ANALÝZY LÉČIV

Druhá část této disertační práce se týká analýzy léčiv. Budou zde popsány metody pro chirální separace léčiv za použití vysokoúčinné kapalinové chromatografie a zónové kapilární elektroforézy (CE). Stručně bude zmíněna UHPLC separace léčiv (zejména psychofarmak), drog a ethylglukuronidu (EtG) ve slinách, využitá pro epidemiologickou studii na Norském institutu veřejného zdraví.

Regulace nečistot léčiv se řídí mezinárodními směrnicemi ICH, lékopisy, předpisy Evropské lékové agentury a FDA. V České republice je celostátně závazným normativním předpisem Český lékopis v aktuálním vydání, který přispívá k zajištění bezpečných, účinných a jakostních léčiv [1]. International Conference of Harmonisation definuje nečistotu v API jako jakoukoliv její složku, která není chemickou entitou definovanou jako API [2]. Podobně nečistotou v léčivém přípravku (LP) je jakákoli jeho složka, která není chemickou entitou definovanou jako API nebo excipient v LP [3]. Bezpečnost LP závisí nejen na toxikologických vlastnostech samotné API, ale také na nečistotách, které obsahuje. Identifikace, kvantifikace a kontrola nečistot v API a LP jsou tedy důležitou součástí vývoje léků a regulačního hodnocení. Směrnice ICH Q3A a Q3B řeší otázky týkající se regulace nečistot v API a LP [4]. Na základě ICH Q3A mohou být nečistoty API zařazeny do následujících kategorií: organické nečistoty (zejména vstupní reaktanty, meziprodukty, vedlejší produkty a degradační produkty), anorganické nečistoty (např. soli, katalyzátory, těžké kovy) a zbytková rozpouštědla [2]. V základní směrnici ICH Q3A nejsou popsány zkoušky pro ověření chirální čistoty. Optické nečistoty je potřeba stanovit stejně jako nečistoty ostatní. Pokyny pro jejich stanovení uvádí směrnice ICH Q6A [5]. Požadavky a pokyny pro validace analytických metod uvádí směrnice ICH Q2 (R1) [6].

Stereochemie má u léčiv svůj zásadní význam. Téměř polovina všech léčiv v současné době je chirální, nicméně pouze 25 % je podáváno jako čistý enantiomer. Je známo, že ve většině případů jsou farmakologické vlastnosti omezené pouze na jeden z enantiomerů, přičemž druhý enantiomer nemá buď žádný účinek, může mít vedlejší účinky, nebo může být dokonce toxický [7]. Enantiomery se mohou lišit v absorpci, distribuci, vazbě na proteiny, receptorové afinitě a mohou se lišit také svým metabolismem [8]. Optická čistota se rutinně stanovuje u léčiv, kde každý z enantiomerů vykazuje jinou farmakologickou aktivitu nebo toxicitu. Chirální separace

je proto nezbytná ve farmaceutických analýzách pro získání bezpečného a požadovaného enantiomeru [9]. Nejčastěji používanými separačními technikami pro chirální analýzy jsou HPLC a CE [10].

Seznam publikací, které budou komentovány ve druhé části této disertační práce:

PUBLIKACE V

Enantiomeric separation of (*R,S*)-aclidinium bromide with negatively charged γ-cyclodextrin by CE

Adamusová, H.; Novotná, N.; Bosáková, Z.; Douša, M., Chromatographia 80 (4) 2017 559-563.

PUBLIKACE VI

Fundamental study of enantioselective HPLC separation of tapentadol enantiomers using cellulose-based chiral stationary phase in normal phase mode

Douša, M.; Lehnert, P.; Adamusová, H.; Bosáková, Z., J Pharm Biomed Anal. 74 (2013) 111-116.

PUBLIKACE VII

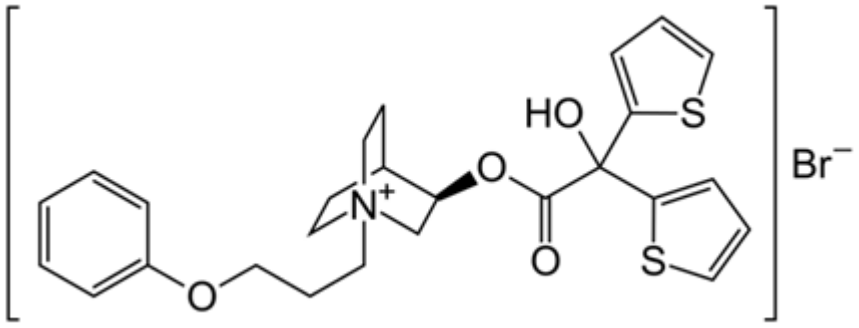
Use of alcohol and drugs by employees in selected business areas in Norway: a study using oral fluid testing and questionnaires

Edvardsen, H. M. E.; Moan, I. S.; Christophersen, A. S.; Gjerde, H., J Occup Med Toxicol. 10 (2015) 10.

Poděkování H. Adamusové za HPLC analýzy, vykonané v rámci ERASMUS stáže v Norsku (Oslo, Norský institut veřejného zdraví).

2.1 Aclidinium bromid

Chirální aclidinium bromid se jako API používá v (3*R*) konfiguraci, jehož struktura je uvedena na obrázku 2.1. Je relativně nově (2012) schváleným antagonistou muskarinových receptorů pro úlevu od symptomů chronické obstrukční plicní nemoci (CHOPN) [11, 12].



Obr. 2.1 Struktura 3(*R*)-aclidinium bromidu (3-{[hydroxy(di-2-thienyl)acetyl]oxy}-1-(3-phenoxypropyl)-1-azoniabicyklo[2.2.2]oktan bromid).

Chronická obstrukční plicní nemoc je charakterizována omezením průtoku vzduchu v dýchacích cestách, které není plně reverzibilní. Je spojena se zvýšenou chronickou zánětlivou reakcí na cizí částice a plyny. Chronickou obstrukční plicní nemoc způsobuje především kouření, z dalších faktorů může k vývoji tohoto onemocnění přispívat zejména pracovní expozice [13]. Mezi symptomy CHOPN léčené AB patří zúžení průdušek, chronická bronchitida a rozedma plic [12]. Pro léčbu CHOPN je možné použít také ostatní dostupné antagonisty muskarinových receptorů jako je tiotropium bromid, glycopyrronium bromid, umeclidinium bromid, nebo β-2-adrenergní agonisty [14, 15]. Jak aclidinium, tak glycopyrronium představují výkonné antagonisty muskarinových receptorů s podobnou kinetickou selektivitou pro M_3 versus M_2 receptor, aclidinium má delší disociační poločas na M_3 receptorech a déle trvající bronchodilataci než glycopyrronium. Systémové anticholinergní vedlejší účinky aclidinia mohou být oproti glycopyrroniu nižší díky jeho kinetické selektivitě a rychlé hydrolýze [16]. Více o souvislostech AB a léčby CHOPN lze nalézt např. v publikacích [13, 14].

Doposud nebyla publikovaná žádná práce týkající se enantioseparace AB. Bylo publikováno několik klinických studií porovnávajících inhalační léčiva jako je

glycopyrronium bromid, tiotropium bromid a AB [14, 16, 17]. V literatuře je možné najít metody kapalinové chromatografie a to jak HPLC tak UHPLC pro stanovení AB a jeho metabolitů v plazmě. Stanovení aclidinia, tiotropia a ipratropia v inkubačních vzorcích ze stabilitních plazmatických studií byly provedeny pomocí UHPLC s MS detekcí. Pro detekci byl využit trojitý kvadrupól s elektrosprejem jako iontovým zdrojem (electrospray ionization, ESI) pracujícím v pozitivním módu [18]. Jaterní *in vitro* metabolismus AB byl studován Albertím a kol. Ke stanovení AB a jeho metabolitů byl využit HPLC-MS systém. Bylo použito ESI v pozitivním nebo negativním módu v závislosti na tom, jaké hydrolytické metabolity byly sledovány. Metabolity byly identifikovány pomocí trojitého kvadrupólu jako analyzátoru [19]. Stanovení aclidinia a jeho hydrolytických metabolitů v inkubačních vzorcích ze stabilitních plazmatických studií bylo provedeno pomocí HPLC-MS metody. Pro detekci byl využit hybridní trojitý kvadrupól s lineární iontovou pastí, kvantifikace byla provedena pomocí MRM [20].

Terapeutické účinky má (*R*)-enantiomer AB, a proto je potřeba mít k dispozici screeningovou metodu pro stanovení nízkých koncentrací (*S*)-AB jako optické nečistoty. Pro kontrolu kvality výrobního procesu a pro farmakologické a farmakokinetické studie jsou požadované metody pro chirální separaci (*R*)-AB a (*S*)-AB enantiomerů. Z výše uvedeného je patrné, že v literatuře lze nalézt především metody kapalinové chromatografie pro separace AB s využitím pro klinické studie.

2.1.1 Kapilární elektroforéza v analýzách léčiv

Kapilární elektroforéza je účinnou analytickou metodou se vzrůstajícím využitím ve farmaceutickém průmyslu. Kapilární elektroforéza a HPLC v reverzním módu (RP-HPLC) představují komplementární techniky pro analýzy léčiv v 21. století [21]. Specifickými výhodami CE jsou vysoká separační účinnost, rychlý vývoj metody a malá spotřeba chemikálií a léčiv [10]. Ve srovnání s ostatními technikami je instrumentace CE jednoduchá. Hlavní výhodou CE je dostupnost různých módů, které umožňují separaci a analýzu různých látek od anorganických látek až po biopolymery [21].

Pro detekci léčiv, což jsou většinou malé organické molekuly s chromoforem, který absorbuje UV záření v oblasti 195 – 254 nm, mají nejširší využití UV detektory.

Analyty, které nemají chromofor ani fluorofor, mohou být detektovány po předchozí derivatizaci nebo nepřímými detekčními technikami [9]. Ačkoliv je UV detekce (pro přímé nebo nepřímé měření) v CE nejvíce používaná, její citlivost je relativně nízká, protože velikost signálu je přímo úměrná délce optické dráhy, tedy vnitřnímu průměru kapiláry [22]. Citlivější detekční techniky představují fluorimetrická detekce, především laserem indukovaná fluorescenční detekce (LIF), která je vysoce citlivá, a MS detekce, poskytující jak citlivost, tak informaci o molekulární hmotnosti analytu a jeho struktuře (v případě MS-MS nebo MSn uspořádání) [9]. Pro zvýšení citlivosti v CE byly vyvinuty různé přístupy, zahrnující techniky dávkování vzorku, derivatizace, mikro SPE (extrakce tuhou fází), již zmiňované specifické detektory (LIF, MS) a kapiláry s prodlouženou optickou dráhou [21].

Kapilární elektroforéza se ukázala být vhodnou metodou pro testování enantiomerní čistoty, pro enantioselektivní analýzy léčivých přípravků a také pro analýzy chirálních léčiv a jejich metabolitů v biologických vzorcích [23]. Chirální separace pomocí CE mohou být provedeny buď nepřímo za použití chirálního derivatizačního činidla, tvořícího ireverzibilní diastereomerní páry, které mohou být rozděleny za achirálních podmínek, nebo přímo za použití chirálních selektorů v základním elektrolytu, kde vznikají reverzibilní diastereomerní páry enantiomeru a chirálního selektoru a následně mohou vyústit v rozdílnou mobilitu jednotlivých stereoizomerů [24, 25]. Většina separací představuje přímou enantioseparaci v přítomnosti chirálního selektoru. Vzhledem k tomu, že časy separace jsou relativně krátké, je možné vyzkoušet během krátké doby široké spektrum různých chirálních selektorů [7]. Pro přímé metody chirální analýzy v CE lze využít následující chirální selektory: cyklodextriny (CD; neutrální, nabité a duální systémy), lineární polysacharidy, proteiny, makrocyklická antibiotika, crown ethery, chirální kovové komplexy a další [21].

Nejčastěji používanými chirálními selektory jsou CD kvůli široké škále velikostí kavity, postranních řetězců, stupně substituce a náboje [26]. Od jejich zavedení byly nativní CD chemicky modifikovány derivatizací nabitými skupinami, hydrofilními segmenty nebo hydrofobními zbytky pro následnou úpravu jejich fyzikálně-chemických vlastností a schopnosti molekulárního rozpoznávání [27]. V roce 1995 byly jako chirální selektory představeny sulfatované CD a tyto aniontové CD vynikají univerzální využitelností. Lze je použít pro enantioseparace kyselých, bazických, neutrálních

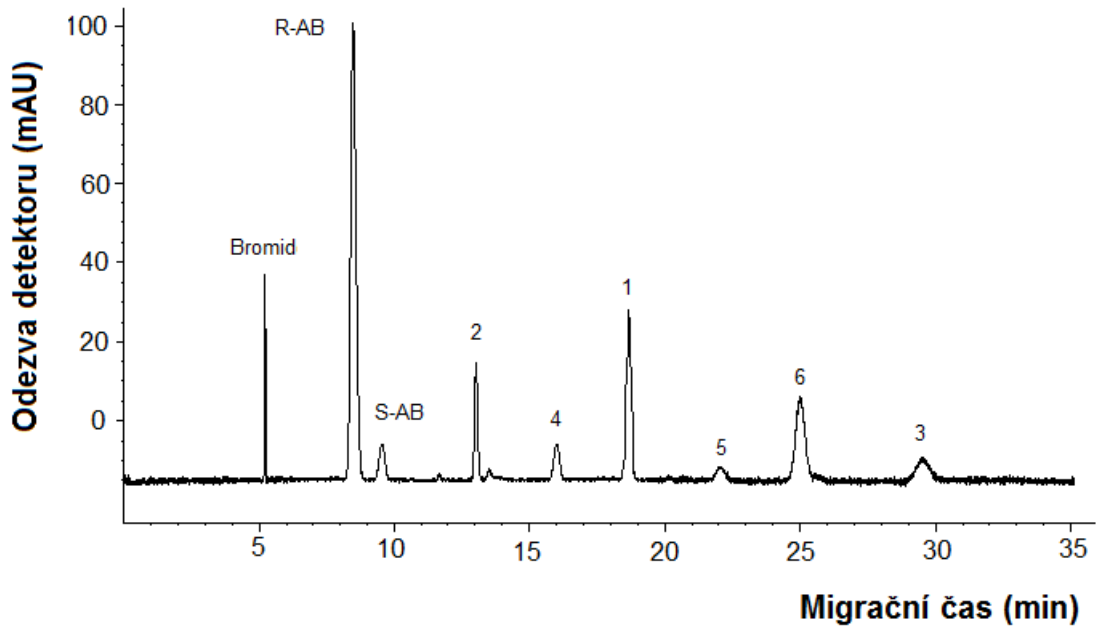
i amfoterních látek, čehož je využíváno při analýzách léčiv, herbicidů, alkaloidů, fungicidů a dalších. Literatura zmiňuje minimální potřebu optimalizace pro dosažení úspěšné separace. Díky těmto vlastnostem jsou sulfatované CD vhodné pro vývoj separací řady chirálních molekul [28]. Teoretický stupeň úplné sulfatace CD je 18, 21, a 24 pro α-, β- a γ-CD. Kompletní sulfatace zatím nebylo dosaženo, pravděpodobně z důvodu velkého elektrostatického a stérického bránění. Pro CE separace byly úspěšně zařazeny CD s rozdílným stupněm částečné sulfatace. Termín „vysoce sulfatovaný" se běžně používá pro označení CD s více než polovinou možných míst sulfatovaných. Při porovnání rozpustnosti neutrálních a sulfatovaných CD ve vodě, je rozpustnost sulfatovaných CD zhruba desetkrát vyšší, což poskytuje možnost vyzkoušet širší koncentrační rozmezí selektoru při optimalizaci a jeho vyšší rozsah pro dávkování vzorku. Permanentní náboj také způsobuje iontový charakter těchto aditiv, což je činí nezávislými na pH. Tyto aniontové CD umožňují separaci většího množství analytů [28]. Výhody použití CD v chirálních separacích jsou následující: dobré enantiomerní rozpoznávací schopnosti, dobrá rozpustnost ve vodě, nízká absorpce UV záření při vlnových délkách běžně používaných v CE a široký výběr neutrálních, kationtových a aniontových CD s rozdílnými funkčními skupinami [29]. Mechanismus chirálního rozpoznávání CD je založen na inkluzi lipofilních skupin analytu do hydrofobní kavity CD. V případě nabitých CD mohou přispívat i iontové interakce, které mohou dokonce převyšovat nad komplexačním mechanismem [27]. V případě nativních CD je to vodíková vazba mezi OH skupinami CD a vhodnou skupinou v analytu. CD již byly úspěšně použity pro separace API [30, 31].

2.1.2 Enantiomerní separace (*R,S*)-aclidinium bromidu negativně nabitým γ-cyklodextrinem pomocí CE – komentář k publikaci V

Všechna měření byla prováděna na HP3D CE systému (Agilent Technologies, Waldbronn, Německo) s DAD detektorem. Během vývoje metody byly vyzkoušeny různé CD (sulfatované α-CD, β-CD a γ-CD a hydroxypropyl β-CD a γ-CD) jako potenciální selektory. Byl sledován vliv základního elektrolytu (tris(hydroxymethyl)aminomethanu a dihydrogenfosforečnanu draselného) na tvar píku. Pro dosažení vyšší citlivosti byla po úvodních měřeních zvolena pro další separace kapilára s rozšířenou optickou dráhou. Byly studovány a optimalizovány hodnoty

vloženého napětí a tlaku, dále pak doby nástřiku a teploty kapiláry. Hodnotícím kritériem bylo rozlišení mezi separovanými (*R*)- a (*S*)-enantiomery. Změny v hodnotách vloženého napětí a tlaku měly na separaci větší vliv než změny v době nástřiku či v teplotě kapiláry. S rostoucím vloženým napětím bylo rozlišení větší, s rostoucím tlakem byl pozorován opačný trend. S delší dobou nástřiku bylo pozorováno větší rozlišení a s nižší teplotou kapiláry bylo rozlišení mezi enantiomery menší. Byly vyzkoušeny také různé molární koncentrace (5 – 120 mM dihydrogenfosforečnanu draselného) a hodnoty pH základního elektrolytu (2,0 – 4,0). S rostoucí koncentrací základního elektrolytu rostla také hodnota rozlišení. Byl optimalizován obsah chirálního selektoru v separačním elektrolytu; koncentrace sulfatovaného γ-CD byly testované v rozsahu 2,4 – 7,0 % (*w/v*) a s rostoucí koncentrací CD klesalo rozlišení mezi separovanými enantiomery.

Enantioseparace, umožňující stanovení 0,4 % (*S*)-AB jako optické nečistoty v syntetickém vzorku AB bylo dosaženo za použití sulfatovaného γ-CD 4,8% (*w/v*) v kyselém základním elektrolytu dihydrogenfosforečnanu draselného (100 mM, pH = 3,0) na nepokryté křemenné kapiláře (s celkovou délkou kapiláry 48,5 cm a efektivní délkou kapiláry 40,0 cm o vnitřním průměru 50 μm) s rozšířenou optickou dráhou. Metoda byla validována jako limitní test. Mezi validované parametry u limitního testu patří selektivita a LOD. Cílem limitního testu je ověření, že testovaná látka nepřesahuje stanovenou hodnotu limitu. Tento test není zcela kvantitativní ale pouze semikvantitativní. Selektivita vyvinuté metody je demonstrována na obrázku 2.2, ukazujícího oddělení následujících nečistot: nečistoty 1 (3-fenoxypropyl-chinuklidinolu), nečistoty 2 (esteru chinuklidinylu), nečistoty 3 (di(2-thienyl) glykolové kyseliny), nečistoty 4 (methyl 2,2-dithienylglykolátu), nečistoty 5 (3-fenoxypropyl bromidu) a nečistoty 6 (3-chinuklidinolu) od (*R,S*)-AB enantiomerů. Tato metoda může být použita pro screening enantiomerní čistoty během syntézy API, pro kontrolu finálního produktu bude potřeba vyvinout citlivější metodu. Detaily vývoje a validace metody jsou popsány v publikaci V [32].



Obr. 2.2 Enantioseparace (*R,S*)-AB v přítomnosti nečistot 1 – 6: 1 (3-fenoxypropyl-chinuklidinol), 2 (ester chinuklidinylu), 3 (di(2-thienyl) glykolová kyselina), 4 (methyl 2,2-dithienylglykolát), 5 (3-fenoxypropyl bromid) a 6 (3-chinuklidinol) za podmínek: 100 mM KH_2PO_4 pH = 3,0 s 4,8 % (*w/v*) sulfatovaného γ-CD.

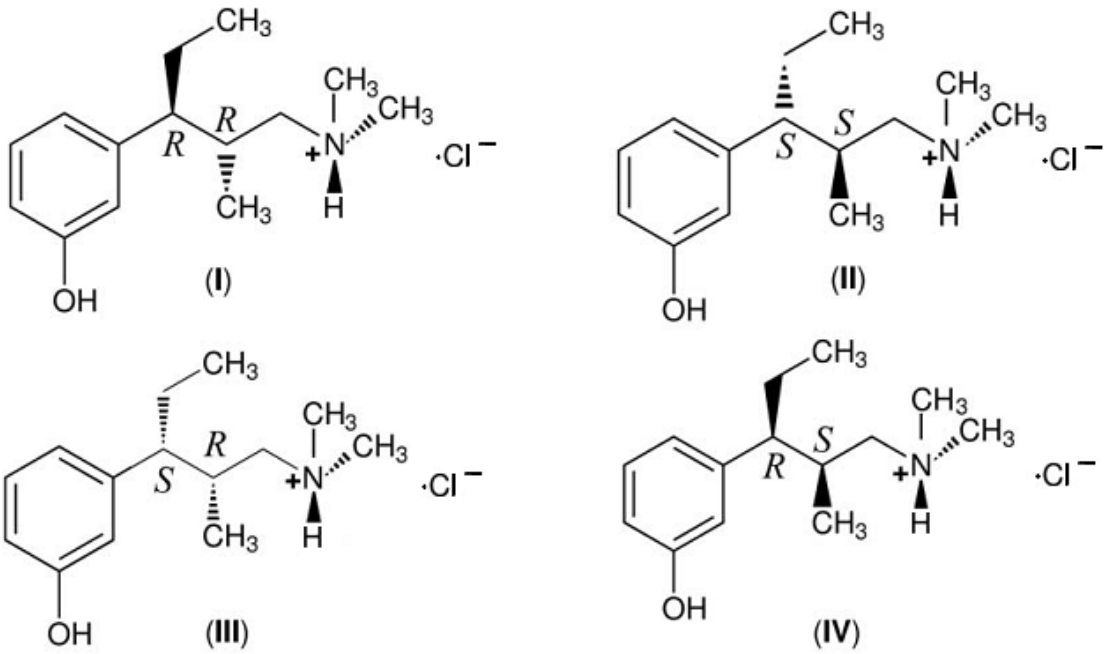
2.2 Tapentadol

Tapentadol hydrochlorid (3-[(1*R*,2*R*)-3-(dimethylamino)-1-ethyl-2-methylpropyl]fenol hydrochlorid) je centrálně působící analgetikum pro léčbu středně těžké až těžké akutní bolesti nebo chronické bolesti, jako je bolest po úrazu nebo chirurgickém zákroku, bolestivá diabetická periferní neuropatie a chronická bolest zad. Tapentadol hydrochlorid je schválen FDA ve dvou formách jako tablety s okamžitým nebo prodlouženým uvolňováním pro chronické bolesti, kde je požadován kontinuální analgetický účinek [33]. Chronická bolest vzniká působením různých faktorů a z různých příčin. To znamená, že může být zřídkakdy kontrolována pouze jedním léčivem. Kombinace léků s různými analgetickými účinky sice zvyšuje pravděpodobnost přerušení bolestivého signálu, ale často je spojena se zvýšeným rizikem interakcí mezi léky a vedlejšími účinky [34].

Tapentadol hydrochlorid kombinuje v jediné molekule dva mechanismy působení: agonismus μ-opioidního receptoru a inhibici zpětného vychytávání noradrenalinu, přičemž oba mechanismy přispívají k jeho analgetickým účinkům. Tyto mechanismy

poskytují úlevu od bolesti srovnatelnou s oxykodonem a morfinem, přičemž TAP má nižší výskyt nežádoucích účinků, jako jsou nevolnost, zvracení a zácpa [33, 35].

Molekula TAP se skládá z metasubstituovaného fenolového kruhu majícího ethyl na C7 a aminopropyl na C10, díky kterým mohou vznikat čtyři možné stereoizomery (obr. 2.3), avšak pouze (*R,R)*-izomer je v současné době klinicky používanou formou [36]. Pro stanovení TAP bylo vyvinuto několik metod. Byly publikovány postupy pro stanovení TAP a jeho hlavního metabolitu *N*-desmethyltapentadolu v moči a slinách za použití různých separačních technik jako je SPE-LC-MS/MS [37] nebo UPLC-MS-MS [38]. Pro kvantifikaci TAP v psí plazmě byla použita HPLC metoda s fluorimetrickou detekcí [39]. Pro separaci dvou enantiomerů (*R,R*)-TAP a (*S,S*)-TAP byla publikována CE metoda za použití CD jako chirálního selektoru [40]. Všechny čtyři izomery TAP byly odděleny za použití CD duálního systému popsaného v [31]. Pro sledování chirální čistoty TAP během enantioselektivní syntézy byly popsány dvě nevalidované chirální metody. V těchto metodách byl chirální profil 4 stereoizomerů TAP určen za použití kolony Chiralpak AD-H (s navázaným 3,5-dimethylfenylkarbamátem amylózy) a mobilní fáze hexan–tetrahydrofuran (85:15, *v/v*) [41] nebo hexan–diethylamin (100:0,2, *v/v*) [42].



Obr. 2.3 Struktury enantiomerů TAP.
I – (*S,S*)-TAP; II – (*R,R*)-TAP; III – (*R,S*)-TAP; IV – (*S,R*) – TAP.

2.2.1 Chirální HPLC v analýzách léčiv

Separace enantiomerů pomoci kapalinové chromatografie na chirálních stacionárních fázích (CSPs) se ukázala jako nejúčinnější a nejvhodnější způsob stanovení enantiomerního složení mnoha chirálních sloučenin, včetně různých chirálních léčiv [43]. Chirální separace se provádí v různých separačních módech: v normálním (NP), RP nebo v polárně-organickém módu [44]. Vysokoúčinná kapalinová chromatografie je široce využívána díky své, přesnosti, vysoké reprodukovatelnosti, robustnosti a různým stacionárním a mobilním fázím, které lze použít [45]. Byla vyvinuta řada CSPs založených na makrocyklických antibiotikách, polysacharidech, crown etherech, CD, glykoproteinech nebo na principu ligandové výměny [46-50].

Chirální stacionární fáze, založené na derivátech polysacharidů, jsou široce aplikovatelné na mnoho různých typů sloučenin [51] a umožňují rychlou ekvilibraci a separace [52]. Deriváty amylózy a celulózy patří mezi nejčastěji používané chirální selektory pro HPLC. Tři z těchto derivátů: tris(3,5-dimethylfenylkarbamát) celulózy

(OD), tris(3,5-dimethylfenylkarbamát) amylózy (AD) a tris(4-methylbenzoát) celulózy (OJ) vykazují komplementární selektivitu. Tyto CSPs jsou známé pod obchodními názvy Chiralcel OD, Chiralpak AD a Chiralcel OJ [44, 53]. Tyto CSPs mohou být použity v NP i RP módu [50]. Enantioselektivita se může měnit v závislosti na přítomnosti buď esterové, nebo karbamátové skupiny. Tyto skupiny mohou selektivně interagovat s chirálními analyty. Vzhledem k tomu, že polysacharidové polymery mají velký počet funkčních skupin, jsou schopny při separacích v NP módu vázat širokou škálu látek vodíkovými vazbami nebo π-π interakcemi; hydrofobní interakce jsou důležité pro separace v RP módu [54].

Chirální diskriminace je velmi složitý jev. Proto je téměř nemožné předpovědět, která kombinace CSP a mobilní fáze zajistí nejlepší enantioseparaci [55]. Pro zrychlení a vyšší efektivitu výběru vhodného selektoru jsou potřeba jednoduché přístupy s rychlým screeningem kombinací CSPs a mobilních fází, poskytujících chirální separaci požadované látky. Cílem této strategie je rychle analyzovat velké množství rozličných molekul. Proto je prvním krokem tohoto přístupu výběr omezeného počtu chirálních selektorů s širokým rozsahem chirálně rozpoznávacích schopností, takže většina enantiomerů bude moci být rozdělena při použití jednoho z nich. Dále je důležité definovat malé množství experimentálních podmínek jako je např. několik mobilních fází, které mají být vyzkoušeny s vybranými selektory [45].

Ve strategii pro NP-HPLC, definované Matthijsem a kol. [53] sestává první krok ze sekvenčního screeningu tří CSPs na bázi polysacharidů v kombinaci se dvěma mobilními fázemi za účelem dosažení chirální separace testovaných látek. První mobilní fáze je hexan–propan-2-ol, druhá hexan–ethanol. Každá mobilní fáze obsahuje jako aditivum buď diethylamin nebo trifluoroctovou kyselinu, v závislosti na povaze testované látky. Bazická a kyselá aditivita mají vliv na kvalitu separace, jak ve smyslu selektivity, tak tvaru píku [56, 57]. Hexan je vhodné v mobilní fázi nahradit heptanem, protože je méně toxický a retence a rozlišení analytů se velmi nemění [53]. Jak již bylo zmíněno výše, v tomto přístupu jsou obě mobilní fáze použity v kombinaci se třemi CSPs v následujícím pořadí Chiralpak AD-H, Chiralcel OD-H and Chiralcel OJ-H. Detaily screeningu záleží na povaze testované látky, zda jde o bázi, nebo o kyselou či neutrální látku. U bazických sloučenin se testují dva faktory: typ CSP (pouze dvě kolony Chiralpak AD-H a Chiralcel OD-H) a typ organického modifikátoru (propan-2-

ol, ethanol). Pro získání většího rozlišení, selektivity a účinnosti může následovat optimalizace separace [44].

2.2.2 Základní studie enantioselektivní HPLC separace enantiomerů tapentadolu za použití chirální stacionární fáze na bázi celulózy v NP módu – komentář k publikaci VI

Pro separaci stereoizomerů TAP bylo vyzkoušeno 10 chirálních polysacharidových kolon (Chiracel OD-H, Chiralpak AY-H, Chiralcel OZ-H, Chiralcel OA, Chiralcel OG, Chiralcel OK, Chiralcel OC a Chiralcel OF). Dostatečnou chirální selektivitu poskytla kolona Chiralpak AD-H na bázi amylózy, která byla použita pro další vývoj a optimalizaci metody. Byl studován vliv složení mobilní fáze na retenci a enantioseparaci jednotlivých izomerů. Rozlišení a retence všech izomerů byly velice citlivé na množství propan-2-olu v mobilní fázi, s větším množstvím propan-2-olu se snižovala retence i selektivita. Uspokojivé enantioseparace a přijatelné retence bylo dosaženo při použití mobilní fáze s 2% obsahem propan-2-olu. Pro zjištění vlivu alkoholu jako modifikátoru mobilní fáze na enantioseparaci a retenci bylo vyzkoušeno 8 zástupců alkoholů: ethanol, *n*-propanol, *n*-butanol, 2-butanol, 2-methylpropan-2-ol, 2-methylpropan-1-ol a 2-methyl-2-butanol. Dostatečné enantioseparace všech čtyř enantiomerů bylo dosaženo za použití 2-butanolu. Při použití ostatních alkoholů buď k separaci nedošlo vůbec, nebo nebyly rozděleny všechny enantiomery, případně byly analyty rozděleny s nedostatečným rozlišením. Vliv *n*-alkanu na enantioseparaci byl studován použitím směsi alkan (*n*-hexan, *n*-heptan, *n*-oktan, 2,2,4-trimethylpentan) a 2% propan-2-ol a 0,02% diethylamin. Různé alifatické alkany v mobilní fázi měly zanedbatelný dopad na retenci, zatímco účinnost separace rostla se zkracující se délkou alifatického řetězce. Pro optimalizaci tvaru píku byl vyzkoušen vliv čtyř aminů (diethylaminu, triethylaminu, ethylendiaminu a dibutylaminu) přidaných do mobilní fáze obsahující 2% propan-2-ol. Vliv diethylaminu nebyl příliš viditelný, nedošlo ke vzniku experimentálních problémů jako při použití triethylaminu, nebo ke ztrátě selektivity jako u ethylendiaminu a dibutylaminu. S rostoucím obsahem diethylaminu docházelo ke snižování retence (v testovaném rozsahu 0,02 – 0,2 %), množství 0,1 % diethylaminu bylo nalezeno jako optimální. Dále byl zkoumán vliv teploty kolony na rozlišení při enantioseparaci v rozmezí 15 – 45 °C. Pokles teploty se projevil nižší účinností a selektivitou, na druhou stranu se s poklesem teploty výrazně zvětšilo

rozlišení zejména mezi (*R,R*)-TAP a (*S,S*)-TAP. Jako vhodná teplota kolony pro robustní enantioseparaci, nezávislou na malých teplotních změnách, byla zvolena teplota 35 °C. Byl také zkoumán vliv přítomnosti vody v mobilní fázi na enantioseparaci, ke komerčně dodávanému propan-2-olu s obsahem vody 0,01 % bylo přidáno 0,5 % vody (*v/v*). Přídavek malého množství vody do propan-2-olu vedl ke zhoršení enantioseparace. Vliv obsahu ethanolu v rozpouštědle vzorku byl vyzkoušen v rozmezí 4 – 20 %, zvyšování obsahu ethanolu vedlo ke zborcení píku, optimalní koncentrace ethanolu v rozpouštědle vzorku byla určena jako 4 – 5 %. Enantioseparace s rozlišením větším než 2,5 pro všechny enantiomery bylo dosaženo při použití kolony Chiralpak AD-H s mobilní fází heptan/propan-2-ol/diethylamin (980:20:1, *v/v/v*). Vyvinutá metoda byla validována dle požadavků směrnice ICH Q2(R1) zahrnující linearitu, LOD, limit kvantifikace (LOQ), přesnost, správnost a selektivitu. Praktická využitelnost metody byla demonstrována analýzou reálných vzorků. Kompletní výsledky a další detaily z vývoje, optimalizace a validace metody jsou popsány v publikaci VI [58].

2.3 Studie užívání alkoholu a drog na vybraných pracovištích v Norsku

Užívání alkoholu a psychotropních látek může ovlivňovat bezpečnost práce a její produktivitu [59, 60]. Testování užívání drog a léčiv na pracovišti (workplace drug testing; WDT) je jednou z metod pro zjištění rozšíření používání drog a vybraných léčiv mezi zaměstnanci. Tato metoda je v Norsku méně častá než v jiných státech. Aby bylo popsáno rozšíření používání psychofarmak mezi zaměstnanci na pozicích spojených s bezpečnostním rizikem v pobřežním ropném průmyslu, přepravě a leteckém průmyslu ve firmách, které mají zavedený systém WDT, byla v letech 2000-2006 Norským institutem veřejného zdraví realizována WDT studie [61]. Vzhledem k tomu, že WDT není v Norsku široce rozšířené, je používané pouze v rámci několika oblastí, zejména těch výše zmiňovaných. Tyto firmy nejsou reprezentativním vzorkem zaměstnanců a pracovišť obecně. Bylo potřeba zjistit rozsah používání drog mezi zaměstnanci, a proto byla provedena průřezová epidemiologická studie [62].

Sliny představují jednoduše dostupné médium, které může být odebráno neinvazivními metodami bez narušení soukromí [63]. Vzorky slin mohou být získány pod pečlivým dohledem, aby nedošlo k jejich nahrazení nebo falšování, což může být

problémem při odběru vzorků moči [64]. Přítomnost drog nebo jejich metabolitů ve slinách svědčí o jejich velmi nedávné konzumaci [65]. Pozitivní nález ve slinách typicky ukazuje na užití drog během posledních 24 – 48 hodin [66] a zohledňuje lépe než nález v moči, zda by objekt mohl být pod vlivem drog nebo alkoholu v době odběru vzorku [63]. Ethylglukuronid je markerem nedávného požití alkoholu, který může být relevantní například v testovaní na pracovištích. Pro tyto účely byly dříve používány vzorky moči [67]. Ethylglukuronid je obvykle stanovován ve vzorcích moči, nebo krve, ale může být detekován také ve slinách [68]. Vysoké koncentrace EtG ve slinách mohou odrážet velmi nedávnou konzumaci velkého množství alkoholu dokonce i když není žádný alkohol stanoven v krvi. Nicméně koncentrace EtG ve slinách, krvi nebo moči neukazuje na konzumaci alkoholu během delší doby [69].

Do této epidemiologické studie bylo pozváno 44 pracovišť bez implementovaného WDT programu a polovina z nich souhlasila s účastí. Zaměstnanci vyplnili dotazník a poskytli vzorek slin, ve kterém byla zjišťována přítomnost alkoholu, EtG, vybraných léčiv (zejména psychofarmak) a drog. Jako drogy byly definovány následující látky: amfetamin, metamfetamin, extáze, kokain, tetrahydrokanabinolová kyselina a 6-acetylmorfin. Účast byla dobrovolná a anonymní. Jedním z cílů studie bylo zjistit užívání zvolených léčiv, drog a alkoholu zaměstnanci na vybraných pracovištích pomocí kombinace dotazníků a analýz slin. Ve studii byla zahrnuta tato odvětví: zdravotnictví (917 zaměstnanců), finanční sektor (457 zaměstnanců), výroba (254 zaměstnanců), přeprava/skladování (233 zaměstnanců), restaurace/bary (131 zaměstnanců), veřejná správa (211 zaměstnanců), media (152 zaměstnanců) a výzkumné ústavy (82 zaměstnanců) [62].

2.3.1 Analýzy vzorků slin norských zaměstnanců

Vzorky slin byly odebírány pomocí komerčně dostupné sady Statsure Saliva Sampler™ (Statsure Diagnostic Systems, Framingham MA, USA). Čas potřebný pro odběr vzorku a vyplnění dotazníku byl zhruba 5 minut. Vzorky slin byly zmraženy během jednoho dne po odběru a rozmraženy před analýzou [62]. Alkohol byl analyzován automatizovanou enzymatickou metodou [70].

Příprava vzorku pro analýzu EtG byla následující: ke 200 µL směsi slin a konzervačního pufru bylo přidáno 50 µL EtG-d5 a 1 mL vody. Zamíchaný vzorek byl prekoncentrovaný přes Hyper-SEP SAX kolonku (Thermo Fisher Scientific Inc.,

Boston, MA, USA), která byla prekondicionovaná methanolem (2 mL) a vodou (2 mL) a vymyta vodou (2 mL). Analyt byl eluován 2 mL směsi methanol/mravenčí kyselina (98:2, *v/v*). Eluát byl odpařen do sucha pod proudem dusíku (50 °C), rozpuštěn v 60 μL vody a umístěn na 30 minut do mrazáku (–20 °C). Vzorek byl následně rozmražen a centrifugován (5251 × g, 15 min 4 °C) a čirá fáze byla odpipetována do vialek pro analýzu. Ethylglukuronid byl analyzován pomocí UPLC-MS/MS systému (Acquity UPLC – Quattro Premier XE, Waters, Milford, MA, USA) na HSS T3 koloně (1,8 μm 2,1 × 100 mm) s gradientovou elucí mobilní fází (A) 0,1% mravenčí kyselina ve vodě a (B) methanol [68]. Kolona UHPLC C18 HSS T3 od firmy Waters obsahuje vysoce odolné silikagelové (high strength silica; HSS) částice určené pro aplikace kolem 1000 bar; T3 (tribonding) označuje technologii navázání C18 skupin.

Vzorky pro zjištění přítomnosti vybraných léčiv a drog (tabulka 2.1) byly připraveny podle následujícího postupu. 0,5 mL vzorku slin bylo smícháno s 50 μL příslušného vnitřního standardu a 250 μL 0,2 mol/L uhličitanu amonného pH = 9,3. Vzorky byly extrahovány 1,3 mL ethylacetát:heptan (4:1) mícháním po dobu 10 minut. Po centrifugaci (1400 × g, 5 min) byla organická fáze pipetována do vialek pro UPLC analýzu a odpařena do sucha pod proudem dusíku (40 °C) (Zymark Turbovap). Usazenina byla následně rozpuštěna v 60 μL směsi acetonitril/voda (10:90, *v/v*) [64]. Vzorky byly analyzovány pomocí UPLC-MS/MS systému (Acquity UPLC – Quattro Premier XE, Waters, Milford, MA, USA) s BEH C18 kolonou (1.7 μm 2.1 × 50 mm) s gradientovou elucí mobilní fází (A) 5 mM hydrogenuhličitan amonný pH = 8,5 a (B) methanol, s gradientem od 20 % do 90 % B během 9 minut. Hmotnostní spektrometrie byla prováděna v ESI+ módu s tandemovou hmotnostní detekcí [71]. Kolonu UHPLC BEH C18 od firmy Waters (ethylene bridged hybrid; BEH) od firmy Waters lze vzhledem k vnitřní chemické stabilitě díky technologii hybridního sorbentu použít pro širší rozsah hodnot pH (1 – 12).

Tab. 2.1 Vybraná léčiva a drogy* analyzované ve vzorcích slin

Látka	Popis
3,4-Methylendioxy-metamfetamin (extáze)*	ilegální psychedelická halucinogenní droga
6-Acetylmorfin*	metabolit heroinu
7-Aminoflunitrazepam	metabolit flunitrazepamu
7-Aminoklonazepam	metabolit klonazepamu
7-Aminonitrazepam	metabolit nitrazepamu
Alprazolam	benzodiazepin, anxiolytikum
Amfetamin*	stimulant
Benzoylekgonin	metabolit kokainu
Buprenorfin	opioid používaný zejména pro opioidní závislosti, také analgetikum
Diazepam	benzodiazepin, anxiolytikum, antikonvulzivum, sedativum
Fenazepam	benzodiazepin, anxiolytikum
Fentanyl	opioidní analgetikum
Flunitrazepam	benzodiazepin, anxiolytikum
Klonazepam	benzodiazepin, antikonvulzivum, anxiolytikum
Kodein	opioidní analgetikum, antitusikum
Kokain*	stimulant
Kotinin	metabolit nikotinu

Kyselina tetrahydrokanabinolová*	kannabis
Metamfetamin*	stimulant
Methadon	opioid používaný zejména pro opioidní závislosti, také analgetikum
Morfin	opioidní analgetikum, také metabolit kodeinu a heroinu
Nitrazepam	benzodiazepin, anxiolytikum
Nordiazepam	metabolit diazepamu
Oxazepam	benzodiazepin, anxiolytikum, antikonvulzivum a metabolit diazepamu
Oxykodon	opioidní analgetikum
Tramadol	opioidní analgetikum
Zolpidem	krátce působící hypnotikum
Zopiklon	krátce působící hypnotikum

2.3.2 Užívání alkoholu a drog zaměstnanci na vybraných pracovištích v Norsku: studie za použití testování slin a dotazníků – komentář k publikaci VII

Publikace je stručně zmíněna v této disertační práci, protože některá data použitá v tomto článku byla získána během mé stáže na Norském institutu veřejného zdraví v roce 2014. Byla zjišťována přítomnost EtG, vybraných léčiv a drog ve vzorcích slin pomocí metod popsaných výše v kapitole 2.3.1.

Bylo analyzováno 14 sérií pro EtG se zhruba 532 reálnými vzorky, dohromady i s kontrolami 672 vzorků. Ani v mých měřeních, ani v rámci celé studie nebyly nalezeny žádné pozitivní výsledky pro EtG. Podle studie z roku 2010 [69] je nutná

konzumace velkého množství alkoholu, aby bylo dosaženo dostatečné koncentrace EtG pro stanovení ve vzorcích slin následující den.

Co se týče analýz léčiv a drog, bylo změřeno 12 sérií se zhruba 456 reálnými vzorky, dohromady i s kontrolami 576 vzorků. Dohromady se tohoto projektu zúčastnilo 2437 zaměstnanců, projekt byl realizován jako pilotní a navazující studie, která byla ukončena v roce 2014. Léčiva byla detekována častěji než drogy. Nejčastěji detekovanými látkami byly: lék proti nespavosti zopiklon (43 zaměstanců), sedativum diazepam (16 zaměstnanců), kannabis (16 zaměstnanců) a analgetikum kodein (6 zaměstnanců). Léčiva byla detekována častěji a drogy méně často ve vzorcích slin u zaměstnanců ve zdravotnictví než u zaměstnanců v ostatních odvětvích. Drogy byly častěji stanoveny ve vzorcích u pracovníků v restauracích/barech než ve vzorcích z ostatních odvětví. Souhrnně lze říci, že pouze malá část zaměstnanců byla pozitivních pro přítomnost drog a žádný zaměstnanec nebyl pozitivní pro EtG. Detailní výsledky zahrnující statistické zpracování a výsledky dotazníků lze nalézt v publikaci [62].

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PUBLIKACE V

ENANTIOMERIC SEPARATION OF (*R,S*)-ACLIDINIUM BROMIDE WITH NEGATIVELY CHARGED GAMMA-CYCLODEXTRIN BY CE

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SHORT COMMUNICATION

Enantiomeric Separation of (*R*,*S*)-Aclidinium Bromide with Negatively Charged Gamma-Cyclodextrin by CE

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Abstract A new CE method for separation of (*R*,*S*)-aclidinium bromide enantiomers has been developed and validated. In this method, sulphated γ-CD 4.8% (w/v) in acidic background electrolyte based on potassium dihydrogen phosphate (100 mM, pH = 3.0) buffer was used for the determination in an uncoated fused silica capillary with extended capillary light path. The effect on the resolution of the chiral selector concentration, of the pH of the background electrolyte and also of other parameters was studied. (*R*)-Aclidinium bromide has therapeutic properties and, thus, a screening method is required for determination of low concentrations of (*S*)-aclidinium bromide as an optical impurity. The proposed method enables determination of 0.4% (*S*)-aclidinium bromide as an optical impurity in a synthetic laboratory sample.

Keywords CE · Chiral separation · Sulphated CD · Aclidinium bromide

Introduction

The significance of the stereochemistry of pharmaceuticals is well defined as stereoisomers and, by extension, enantiomers often differ in their pharmacological, toxicological and/or pharmacokinetic profiles [1]. The CE technique has proven to be suitable for enantiomeric purity testing, for enantioselective analysis of drug products and also for the analysis of chiral drugs and their metabolites in biological samples [2]. The majority of separations employ direct enantioseparation in the presence of a chiral selector (CS). Since the separation times are rather short, a broad spectrum of CSs can be screened within a short time. The unsatisfactory injection precision and the lack of detection sensitivity are drawbacks [3]. The most commonly used chiral selectors are CDs due to their wide variety of cavity size, side chain, degree of substitution and charge [4]. CDs have already been successfully used for active pharmaceutical ingredients (APIs) enantioseparations [5, 6]. Since the introduction of CDs, native CDs have been chemically modified by the inclusion of charged groups, hydrophilic segments, hydrophobic moieties or both hydrophobic and hydrophilic units to further modulate their physicochemical properties and molecular recognition capability [7]. CDs have the following advantages for use in chiral separations: good enantiorecognition abilities, good water solubility, low absorption at the UV wavelengths commonly used in CE and the availability of a wide assortment of various neutral, cationic and anionic CDs with different functional groups [8]. The chiral recognition mechanism of CDs is very often believed to occur via the inclusion of lipophilic moieties of the analyte into the hydrophobic cavity of the CD. In the case of charged CDs, ionic interactions will also contribute to or may even dominate the complexation mechanism [9].

Aclidinium bromide (AB) has a single stereoisomer with the (3*R*) configuration with the structure shown in Fig. 1: ((3*R*)-3-{[hydroxy(di-2-thienyl)acetyl]oxy}-1-(3-phenoxypropyl)-1-azoniabicyclo[2.2.2]octane bromide).

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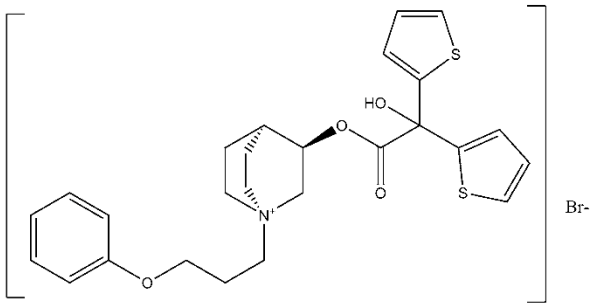


Fig. 1 The structure of aclidinium bromide

AB is a quite recently approved (2012 in both Europe and the USA) long-acting inhalable muscarinic antagonist to relieve the symptoms of chronic obstructive pulmonary disease (COPD) [10, 11]. COPD is characterised by airflow limitation that is not fully reversible. It is associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases [12].

To our knowledge, no analytical method for enantioseparation of AB is described in the literature. A number of clinical studies have been published comparing inhalatory drugs such as tiotropium, ipratropium, glycopyrronium and aclidinium [13–15]. The literature also contains HPLC/UHPLC methods for determination of AB and its metabolites in plasma. The determinations of aclidinium, tiotropium and ipratropium in incubation samples from plasma stability studies were conducted using UHPLC with MS detection. Detection was performed on a triple quadrupole mass spectrometer with electrospray ion source operating in the positive ion mode [16]. Literature is also available related to AB for possible COPD treatment [12, 13]. In vitro liver metabolism of AB was studied by Alberti et al. An LC–MS system was used for determination of AB and its metabolites. MS was conducted with an electrospray ion source operating in either the positive or negative ion mode depending on the hydrolysis metabolites observed, coupled to a triple quadrupole analyser [17]. The determination of aclidinium and its hydrolysis metabolite in incubation samples in plasma stability studies was conducted by LC–MS. Hybrid triple quadrupole linear IT-MS was used for detection; quantification was performed using multiple reaction monitoring [18].

Fast chiral separation methods for (*R*,*S*)-AB enantiomers are required for QC of the synthesis procedure and for pharmacological and pharmacokinetic studies. (*R*)-AB has therapeutic properties and, thus, it is necessary to have a screening method for determination of low concentrations of (*S*)-AB as an optical impurity. Therefore, this work was carried out to develop and validate a routine control method for enantioseparation of (*R*,*S*)-AB in a synthetic sample.

Experimental

Apparatus

All the separations were performed on the HP3D CE system (Agilent Technologies, Waldbronn, Germany) with DAD operating in the range 190–400 nm. Uncoated fused silica capillaries (Agilent Technologies) 50 μm ID, total capillary length 48.5 cm, and effective capillary length 40.0 cm either with or without extended capillary light path (bubble factor 3) were used. The capillary was thermostated from 25 to 35 °C; the separation voltage range was selected from 4 to 15 kV to the anode, the applied pressure was tested within the range 0–15 mbar. Samples were injected by a pressure of 50 mbar for 4 s. The capillary was washed daily before the experiments with deionized water for 5 min, 0.1 M NaOH solution for 5 min, deionized water for 5 min and then with background electrolyte (BGE) containing CS also for 5 min. The capillary was flushed with BGE containing CS at an appropriate concentration for 2 min before each analysis set.

Chemicals

Phosphoric acid and potassium dihydrogen phosphate (KH_2PO_4) were purchased from Sigma (Czech Republic). (*R*)- and (*S*)-AB enantiomers were obtained from Zentiva (Prague, Czech Republic). TRIS was obtained from Merck (Czech Republic) and sodium hydroxide was purchased from Penta (Czech Republic). Methanol was purchased from J. T. Baker (BDL, Czech Republic). Impurities, namely: impurity 1 (3-phenoxypropyl-quinuclidinol), impurity 2 (quinuclidinyl ester), impurity 3 (di(2-thienyl) glycolic acid), impurity 4 (methyl 2,2-dithienylglycolate), impurity 5 (3-phenoxypropyl bromide) and impurity 6 (3-quinuclidinol) were obtained from Zentiva (Prague, Czech Republic).

Sulphated α-CD (S-α-CD) degree of substitution ≈12, sulphated β-CD (S_1-β-CD) degree of substitution ≈13, sulphated γ-CD (S-γ-CD) degree of substitution ≈14, all as the sodium salts and hydroxypropyl-β-CD (HP-β-CD) were obtained from Cyclolab (Budapest, Hungary). Hydroxypropyl-γ-CD (HP-γ-CD) and sulphated β-CD sodium salt (S_2-β-CD) were obtained from Sigma (Czech Republic). All the chemicals were of analytical grade purity.

BGEs were prepared by dissolution of the appropriate amounts of TRIS or KH_2PO_4 individually (TRIS solution at a concentration of 31 mM, KH_2PO_4 solution at concentrations of 5, 20, 50, 70, 80, 100 or 120 mM) in deionized water (18 MΩ cm^{-1}). The pH of the BGE solutions was adjusted to the desired pH values using phosphoric acid.

Charged CDs were added individually to the various BGEs solutions. The standard stock solutions of (*R*)- and (*S*)-AB were prepared at concentration of 1 mg mL^{-1} by dissolving the standard compound in 10% methanol. The working solutions of limit concentrations were prepared by proper dilution of stock standard solutions in deionized water. The limit concentration to determine the presence or absence of the impurity was set at 0.4% (*S*)-AB. Therefore, the concentration of standard solution for this limit test was 0.4% (*R*)-AB.

Results and Discussion

Method Development and its Optimisation

First, S-α-CD, S_1-β-CD, S_2-β-CD and S-γ-CD were tested as potential selectors for resolution (R_S) of (*R*,*S*)-AB. In as much as they are negatively charged, these CDs also undergo electrostatic interactions as another interaction mechanism in addition to inclusion. In the preliminary experiments, 31 mM TRIS pH = 2.5 buffer was used as the BGE of first choice in our laboratory. An uncoated fused silica capillary 50 µm ID, total capillary length 48.5 cm, and effective capillary length 40.0 cm were used for these separations. S-α-CD, S_1-β-CD and S_2-β-CD yielded insufficient R_S values. The values were equal to approx. 1, whereas R_S values higher than 1.5 are required for accurate quantification of individual enantiomers. The use of S-γ-CD yielded high R_S with a value of 6.8. HP-β-CD and HP-γ-CD were also tested but without any success in the separation. Despite the high R_S value when using 31 mM TRIS pH = 2.5 buffer, the peak shape was not satisfactory. Therefore, a 20 mM KH_2PO_4 pH = 2.5 solution was studied as the BGE. In the comparison of BGEs TRIS versus KH_2PO_4, the latter yielded better peak shapes and it was, therefore, used as the BGE in the following experiments.

To obtain higher sensitivity, a capillary with the same parameters as mentioned above, but with extended light path (bubble factor 3), was chosen for further experiments. Its application yielded three times higher peak areas and, therefore, it enabled the use of lower working solution concentrations. The influence of the applied voltage (in the range from 4 to 15 kV to the anode) and pressure (from 0 to 15 mbar) values was optimised under the following conditions: 20 mM KH_2PO_4 pH = 2.5 as the BGE, S-γ-CD as the CS, capillary with the extended light path (bubble factor 3). With increasing applied voltage, the R_S value between two enantiomers increased; the opposite trend was observed with increasing applied pressure. The best results were obtained for 8 kV to the anode and 10 mbar. The migration time was about 8 min and R_S = 4.7. During optimisation of the procedure, the injection time (from 3 to 5 s) and capillary temperature (from 25 to 35 °C) were also studied. It was found that the impact of these parameters on R_S is smaller than the influence of changes in the applied voltage or pressure. However, the trends were as follows: as the injection time increased, R_S also increased; as the capillary temperature increased, R_S decreased. The time of injection was chosen as 4 s and the capillary temperature as 30 °C.

In the subsequent experiments, the influence of BGE on the resolution was studied. Different molar concentrations of KH_2PO_4 buffer were investigated (in the range 5–120 mM). With increasing BGE concentration, R_S increased. Finally, a concentration of 100 mM was chosen, yielding $R_S \geq 6.0$. It was also important to study the influence of the pH value on R_S in the 100 mM KH_2PO_4 buffer. The values were tested in the range of 2.0–4.0 with a step of 0.5 pH. All the tested pH levels yielded sufficient resolution; R_S = 5.3 was the lowest value for pH = 2.0 and pH = 3.0 with the highest obtained resolution (R_S = 6.1) was chosen as the most suitable.

Once having optimised the BGE parameters, it was necessary to choose the composition of the separation electrolyte. Therefore, various concentrations of S-γ-CD were examined in the range 2.4–7.0% w/v. It was observed that R_S decreased with increasing S-γ-CD concentration from 9.1 to 5.2. 4.8% w/v of S-γ-CD was chosen as a compromise amongst optimal migration times, R_S values, peak heights and CD consumptions. The migration time of (*R*)-AB was 8.2 min and R_S = 6.4 which is depicted in Fig. 2.

Validation of the Method

The method was validated in terms of repeatability, selectivity and limit of detection (LOD) as a limit test. This assay could be used for screening of enantiomer purity during the API synthesis procedure; a more sensitive method will be needed for API control.

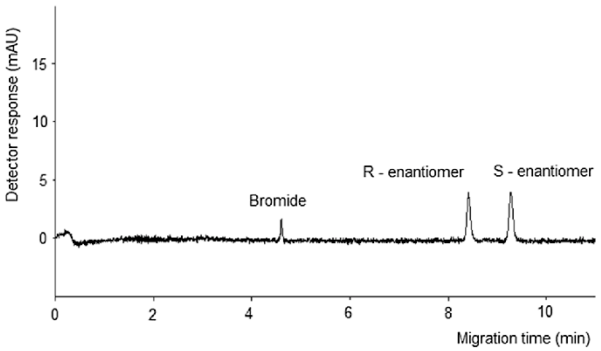


Fig. 2 Electropherogram of the solution for resolution of (*R*)- and (*S*)-AB on the limit concentration in 100 mM KH_2PO_4 pH = 3.0 with 4.8% (w/v) S-γ-CD

System Suitability

The system suitability test was performed before each run to ensure that the analytical method can be used with satisfactory performance with the employed electrophoretic system. Repeatability of injections expressed as RSD % of (*R*)-AB corrected peak area and R_S between (*R*)- and (*S*)-enantiomers for five consecutive injections of the system suitability test solution were limited to ≤5.0% and $R_S \geq 2.0$, respectively. R_S values of more than 2.0 (6.4) and RSD of corrected peak areas of ≤5.0 (4.1) % were achieved for all the measurements performed during the validation. The corrected peak area is the respective area (area units) divided by its migration time in second.

Validated Parameters

Limit of Detection LOD was calculated for (*S*)-AB based on signal-to-noise ratio (*S/N*). The baseline noise was measured in a blank experiment in the region of migration times of (*S*)-AB using chromatographic software (Chem-station Agilent software). LOD was computed using the following equation:

$$\mathrm{LOD} = 3.3\frac{\sigma \times c_{\mathrm{lim}}}{H_{c_{\mathrm{lim}}}},$$

where $H_{c_{\mathrm{lim}}}$ is the peak height of (*S*)-AB at the limit concentration, c_{lim} is the limit concentration of (*S*)-AB and σ is the baseline noise in the electropherogram of the placebo solution or sample solvent (for medicinal products or APIs) in the time interval corresponding (or close) to the migration time of relevant analyte. LOD value was 0.15% (*S*)-AB.

Robustness The solution for resolution at the limit concentration of 0.4% (*R*)- and (*S*)-AB was used for robustness measurements. The influence of the following parameters was verified during the validation: time of injection, applied voltage, applied pressure, temperature of the capillary space, concentration of CS and type of capillary. Trends observed during method development were confirmed during the method validation.

Figure 3 depicts an electropherogram of synthetic sample with 0.4% (*S*)-AB determined as an optical impurity.

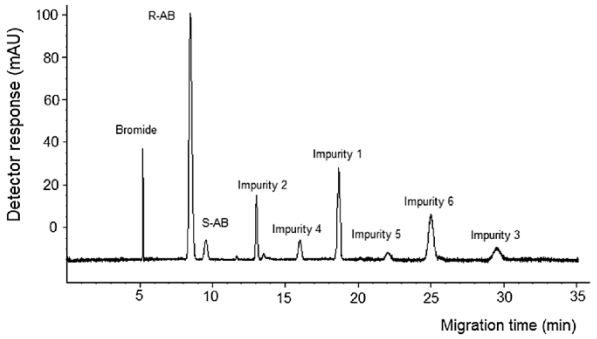


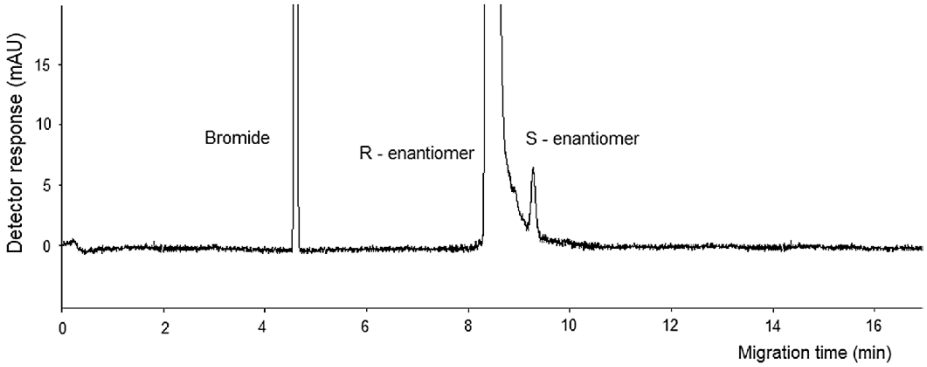
Fig. 4 Electropherogram of relevant AB impurities demonstrating method selectivity. The conditions are described in Fig. 3

Selectivity All following impurities: impurity 1 (3-phenoxypropyl-quinuclidinol), impurity 2 (quinuclidinyl ester), impurity 3 (di(2-thienyl)glycolic acid), impurity 4 (methyl 2,2-dithienylglycolate), impurity 5 (3-phenoxypropyl bromide) and impurity 6 (3-quinuclidinol) were successfully separated from the (*R*)- and (*S*)-AB enantiomers under the conditions of the CE method (Fig. 4).

Conclusion

Various CDs such as S-α-CD, S-β-CD, S-γ-CD, HP-β-CD and HP-γ-CD were investigated to resolve AB enantiomers with higher resolution than 2.0 (necessary for accurate quantification of individual enantiomers). The best results were obtained for S-γ-CD. Each selector employed revealed different affinity for the analytes and resulted in various migration times. The use of S-γ-CD resulted in

Fig. 3 Electropherogram of synthetic sample of AB demonstrating determination of 0.4% (*S*)-AB in 100 mM KH_2PO_4 pH = 3.0 with 4.8% (w/v) S-γ-CD



a favourable system in which the physiologically active compound migrated as the last compound. 2.4% (w/v) concentration of CD provided baseline separation for AB enantiomers. The method was validated as a limit test. This method enables determination of 0.4% of the (*S*)-AB in a synthetic laboratory sample. This assay could be used for screening of enantiomer purity during the API synthesis procedure but a more sensitive method is required for API control. The final conditions were set at 100 mM KH_2PO_4 at pH 3.0 applying 4.8% (w/v) S-γ-CD.

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Compliance with Ethical Standards

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest All the authors declare that they have no conflict of interest.

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PUBLIKACE VI

FUNDAMENTAL STUDY OF ENANTIOSELECTIVE HPLC SEPARATION OF TAPENTADOL ENANTIOMERS USING CELLULOSE-BASED CHIRAL STATIONARY PHASE IN NORMAL PHASE MODE

Douša, M.; Lehnert, P.; Adamusová, H.; Bosáková, Z

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Short communication

Fundamental study of enantioselective HPLC separation of tapentadol enantiomers using cellulose-based chiral stationary phase in normal phase mode

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Chiral HPLC separation
Chiralpak AD-H
Validation
Mobile phase optimization

ABSTRACT

A sensitive and specific high performance liquid chromatography method for the separation and determination of tapentadol enantiomers has been developed and validated. Ten different chiral columns were tested in a normal phase system. Excellent enantioseparation with the resolution more than 2.5 for all enantiomers was achieved on Chiralpak AD-H using mixture of heptane–propan-2-ol–diethylamine (980:20:1, v/v/v). The detection was carried out using fluorescence detector at excitation wavelength of 295 nm and emission wavelength of 273 nm. The influence of mobile phase composition, namely organic modifiers, additives, aliphatic alkanes and water content in mobile phase, on retention and enantioseparation was studied. Validation of the developed method including linearity, limit of detection, limit of quantification, precision, accuracy and selectivity was performed according to the International Conference on Harmonization guidelines. The advantage of the method is a good enantioseparation, short analysis time (less than 20 min) and therefore this method is suitable for routine determination of chiral purity of (*R,R*)-tapentadol in enantiopure active pharmaceutical ingredient.

1. Introduction

A range of analgesic drugs is currently available, but recent guidelines recommend that NSAIDs and COX-2 inhibitors should be prescribed cautiously. Chronic pain is multifactorial and this approach ignores the fact that different causative mechanisms may be involved. The presence of more than one causative mechanisms means that chronic pain can seldom be controlled by a single agent. This leads to combining different drugs together, which raises the risk of drug interactions and possible side effects [1]. Tapentadol hydrochloride (TAP) (3-[(1*R*,2*R*)-3-(dimethylamino)-1-ethyl-2-methylpropyl]phenol hydrochloride), has been approved in 2008 by Food and Drug Administration as an immediate release oral tablet for the relief of moderate to severe acute pain. TAP molecule consists of a meta-substituted phenol ring possessing an ethyl and aminopropyl residue at C7 and C10, respectively which results in four possible stereoisomers (Fig. 1) and only the *R,R* isomer is currently the clinically used form [2].

Several analytical methods have been developed for determination of TAP. A procedure to determinate TAP and its main metabolite (*N*-desmethyltapentadol) in urine and oral fluid using different separation technique such as SPE–LC–MS–MS [3] or UPLC–MS–MS method [4] has been published. The spectrofluorimetric detection coupled with HPLC was used for quantification of TAP in canine plasma [5]. Different types of chiral stationary phases have been developed and used; derivates of amylose and cellulose belong to the most widely used chiral selectors in high-performance liquid chromatography (HPLC). The polysaccharide derivate-based chiral stationary phases (CSPs) are broadly applicable to many compound types [6] providing rapid equilibration and fast separations [7]. Various combinations of CSPs with different chiral recognition mechanisms can be tested to reach enantioseparation of compounds of interest [8–10].

Two non-validated chiral methods have been described to monitor chiral purity during TAP enantioselective synthesis. In these methods, chiral profiles of the four TAP stereoisomers was determined using a Chiralpak AD-H column and hexane–tetrahydrofuran (85:15, v/v) [11] or hexane–diethylamine (100:0.2, v/v) [12]. Hence the aim of the presented work is evaluation of the influence of some experimental parameters on chiral recognition of *R,R*-TAP from their enantiomers. The development and validation of a fast chromatographic method for the determination of enatiometric purity of *R,R*-TAP is described. The applicability of the new method was successfully verified by analysis of commercial samples of active pharmaceutical ingredients (API).

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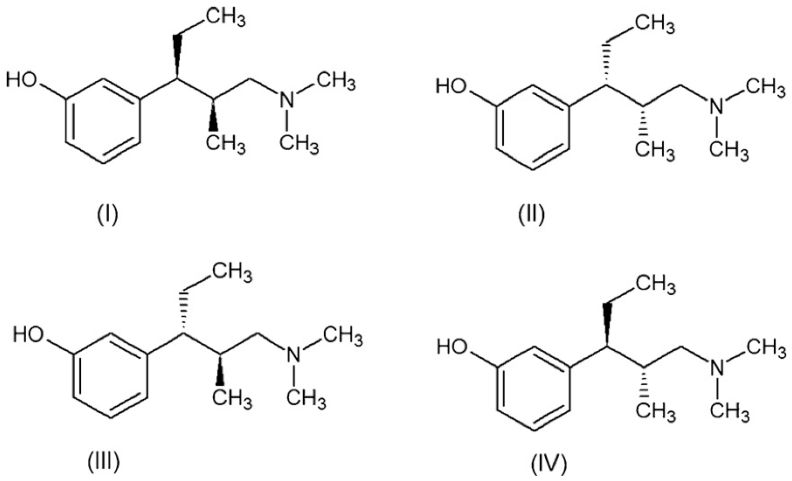

Fig. 1. Structural formula of TAP enantiomers. I – *S,S*-TAP; II – *R,R*-TAP; III – *R,S*-TAP; IV – *S,R*-TAP.

2. Experimental

2.1. Reagents and chemicals

Heptane, hexane, cyclohexane and propan-2-ol for HPLC (Sigma–Aldrich, Czech Republic) were used for preparation of samples, solutions and mobile phases. All other chemicals used were of analytical grade or pure grade quality (Sigma–Aldrich, Czech Republic).

2.2. Sample and standard solution preparation

The standard of (*R,R*)-TAP (in house standard, purity 99.8%, enantiomeric excess more than 99.9%) and (*S,R*)-TAP, (*R,S*)-TAP and (*S,S*)-TAP (in house standard, purity more than 99.5%, enantiomeric excess more than 99.8%) were dissolved in ethanol (EtOH) and diluted using hexane in ratio 4:96 (v/v) to obtain standard stock solutions (concentration of 0.1 mg/ml). 10 mg of TAP sample was weighed into a 100 ml volumetric flask and 4 ml of EtOH was added. The sample was dissolved by sonication for 2 min in an ultrasonic bath UCC4 (TESON, Slovakia). The flask was filled to the mark by hexane to obtain TAP preparation solution.

The system suitability test solution was prepared by spiking of (*R,R*)-TAP standard stock solution with all relevant TAP enantiomers to obtain concentration of 0.15% with respect to (*R,R*)-TAP. All reference solutions were prepared by diluting of relevant standard stock solutions to obtain final concentration of 0.15 μg/ml (concentration of 0.15% with respect to (*R,R*)-TAP sample solution).

2.3. Instrumentation and chromatographic conditions

All chromatographic experiments were carried out using a liquid chromatographic system consisting of Alliance 2695 separation module and fluorescence detector 2475 (all Waters, USA). The system was controlled by data station using Empower software (Waters, USA).

Ten chromatographic columns (all Daicel, Japan) were employed, namely Chiralcel OD-H (tris-[3.5-dimethylphenyl] carbamoyl cellulose, 250 mm × 4.6 mm, 5 μm), Chiralpak AD-H (tris-[3.5-dimethylphenyl]carbamoyl amylose, 250 mm × 4.6 mm, 5 μm and 150 mm × 4.6 mm, 3 μm), Chiralpak AY-H (tris-[5-chloro-2-methylphenyl]carbamoyl amylose, 250 mm × 4.6 mm, 5 μm) Chiralcel OJ-H (tris-[4-methylphenyl]carbamoyl cellulose, 250 mm × 4.6 mm, 5 μm), Chiralcel OA (cellulose acetate, 250 mm × 4.6 mm, 10 μm), Chiralcel OG (tris-[4-methylphenyl]carbamoyl cellulose, 250 mm × 4.6 mm, 10 μm), Chiralcel OK (cellulose tricinnamate, 250 mm × 4.6 mm, 10 μm), Chiralcel OC (tris-[phenyl]carbamoyl cellulose, 250 mm × 4.6 mm, 10 μm), Chiralcel OF (tris-[4-chlorophenyl]carbamoyl cellulose, 250 mm × 4.6 mm, 10 μm) and Chiralcel OZ-H (tris-[3-chloro-4-methylphenyl] carbamoyl cellulose, 250 mm × 4.6 mm, 5 μm). The mobile phases were prepared by mixing of appropriate components in corresponding volume ratios. The flow rate was changed in range from 0.2 to 1.4 ml/min, the injection volume was 5 μl in all the experiments. The temperature of the column was controlled with the precision ±0.5 °C over narrow temperature range (15–45 °C). The fluorescence detection was carried out at wavelength of 295 nm and 273 nm for excitation and emission, respectively.

2.4. Evaluation and validation of method

The efficiency (expressed as plate number N), resolution R, retention factor k and selectivity α were used for the evaluation of separation quality. The final chromatographic conditions were optimized to obtain retention factor $k \geq 2$, efficiency $N \geq 4000$ and to achieve baseline separation (resolution $R \geq 2.0$). The hold-up time was determined from the first perturbation of the baseline. All chromatographic data were calculated according to the European Pharmacopeia (*Ph. Eur.*) [13]. The method was fully validated in compliance with International Conference on Harmonization Q2(R1) guideline (linearity, limit of detection, limit of quantification, accuracy, precision, selectivity and robustness) [14].

3. Results and discussion

3.1. Development and optimization of the method

3.1.1. Mobile phase composition study

The strategy of chiral method development in the normal-phase liquid chromatography defined by Matthijs et al. [15] was applied. In the current study, hexane was replaced by heptane because of its lower toxicity and because retention and resolution data are very similar. Initial investigations using previously described CSPs was focused on a heptane mobile phase with propan-2-ol (*i*-PrOH) as the polar alcoholic modifier. Diethylamine (DEA) in initial concentration of 0.02% was added to improve the peak shape of

TAP, which is a tertiary amine. Mobile phase *i*-PrOH concentrations less than 3% were necessary to provide adequate retention and baseline separation of all isomers.

No chiral separations for *S,R*-TAP/*R,S*-TAP and *R,R*-TAP/*S,S*-TAP were obtained on Chiralcel OD-H, Chiralpak AY-H, Chiralcel OZ-H, Chiralcel OA, Chiralcel OG, Chiralcel OK, Chiralcel OC and Chiralcel OF column. No chiral separations for *S,R*-TAP/*R,S*-TAP and partial separation between *R,R*-TAP and *S,S*-TAP was observed on Chiralcel OJ-H column. The Chiralpak AD-H column showed satisfactory chiral selectivity therefore this column was used for following experiments.

The resolution and retention of all isomers were very sensitive to the amount of *i*-PrOH in the mobile phase. The retention and selectivity decreased with higher amount of *i*-PrOH in the mobile phase which is in agreement with theory [16]. The satisfactory enantioseparation and acceptable retention time ($k \geq 2$ and $R \geq 2.0$) were achieved using mobile phase containing 2.0% *i*-PrOH. In order to investigate the influence of the organic modifier on enantioseparation and retention, eight aliphatic alcohols have been used namely EtOH, *n*-propanol (*n*-PrOH), *n*-butanol (*n*-BuOH), 2-butanol (2-BuOH), 2-methylpropan-2-ol (*tert*-BuOH), 2-methyl-propan-1-ol (*iso*-BuOH) and 2-methyl-2-butanol (*tert*-AmylOH). These modifiers are tested because it is difficult to predict the optimal organic modifier for a given column and it can affect also the enantioseparation [17]. The study indicates that with the use of different alcohol modifiers in mobile phase, the retention factors (k) showed in the order of $k_{EtOH} < k_{n\text{-PrOH}} < k_{i\text{-PrOH}} < k_{n\text{-BuOH}} < k_{iso\text{-BuOH}} < k_{tert\text{-BuOH}} < k_{2\text{-BuOH}} < k_{ter\text{-AmylOH}}$. The elution order of all enantiomers remained the same. The results also show that no enantioseparation in presence of EtOH and *n*-PrOH was obtained. When *n*-BuOH, *iso*-BuOH and *tert*-AmylOH were used, no enantioseparation between *R,R*-TAP/*S,S*-TAP was observed. The poor enantioseparation between *S,R*-TAP/*R,S*-TAP ($R = 0.89$) and *R,R*-TAP/*R,S*-TAP ($R = 1.53$) was obtained in case of *tert*-BuOH use. The acceptable enantioseparation for all enantiomers was obtained using 2-BuOH but the resolution between *S,R*-TAP/*R,S*-TAP was somewhat lower for 2-BuOH ($R = 1.99$) compared to *i*-PrOH ($R = 2.64$). This probably was the result of poorer column efficiency ($N = 6373$ for 2-BuOH; $N = 9003$ for *i*-PrOH).

The impact of the aliphatic alkanes nature on the enantioseparation was studied using mixture of alkanes, namely *n*-hexane, *n*-heptane, *n*-octane and 2,2,4-trimethylpentane (*iso*-octane), with 2.0% *i*-PrOH and 0.02% DEA. The use of various aliphatic alkanes in the mobile phase had negligible impact on the retention while the efficiency increased with shortening of the aliphatic chain length ($N = 8567$ for *n*-octane; $N = 11{,}191$ for *n*-hexane). Also, it resulted in a decrease of selectivity when *n*-octane and *iso*-octane were used (*S,R*-TAP/*R,S*-TAP resolution < 1.75).

The additive nature can have a significant influence on the peak shape [18,19]. To optimize peak shape, the effect of four amines (diethylamine (DEA), triethylamine (TEA); ethylendiamnie (EDA); dibutylamine (DBA)), added to the mobile phase containing 2% of *i*-PrOH was examined. TEA, one of the most commonly used amine additives, had very little effect on retention and selectivity compared to DEA. The use of TEA showed experimental problems related with stability of the baseline and the negative peaks appeared on chromatogram [20]. On the other hand, the addition of EDA or DBA in the mobile phase led to loss of selectivity and the efficiency drastically decreased (N(*R,R*-TAP) = 1564 for EDA; N(*R,R*-TAP) = 3470 for DBA). Higher concentration of additives can also have positive influence on enantioselectivity and efficiency [17]. The influence of DEA concentration in the range from 0.02% to 0.2% was investigated. The effect of DEA content on the enantioseparation was inconspicuous. The retention decreased with DEA content increase (k (*R,R*-TAP) = 5.36 for 0.02% DEA; k (*R,R*-TAP) = 4.55 for 0.2% DEA). Optimal concentration of DEA was found to be 0.1%.

The influence of flow rate on selected chromatographic data (α, N and R) was investigated. The mobile phase flow rate was varied from 0.2 to 1.4 ml/min. The flow rate has significant influence on efficiency which is in agreement with theory [21]. Hence the contribution of efficiency has dominant influence on resolution. The highest efficiency corresponds to N (*R,R*-TAP) = 13,000 at 0.6 ml/min. However, sufficient efficiency (N (*R,R*-TAP) = 10,557) and resolution is achieved for 1.0 ml/min flow rate which results in shorter analysis time.

3.1.2. Temperature study

It has reported improved enantioresolution on polysaccharide-based CSP when column was operated at sub-ambient temperature [22,23]. Study of the effect of temperature can also provide insight into separation mechanism and enable the design of optimum conditions for analysis. In this study, the effect of temperature on the retention factor and the resolution was studied in the range of 15–45 °C. The temperature dependence of retention can be expressed using van't Hoff's equation [24,25]. The van't Hoff plots ($\ln k$ against $1/T$) showed nonlinear behavior for tapentadol stereoisomers (correlation coefficients were between 0.14 and 0.96, data not shown) which indicates the multiple types of interaction were making significant contributions to either the overall interaction or the chiral recognition [26,27]. The above mentioned situation does not allow simple prediction of tapentadol stereoisomers retention depending on temperature in studied chromatographic system. The decrease of temperature resulted in lower efficiency and selectivity as well. On the other hand, the resolution significantly increased with column temperature decrease, especially between *R,R*-TAP/*S,S*-TAP from $R = 4.75$ (at 15 °C) to $R = 3.95$ (at 40 °C). The results are shown in Fig. 2. In fact, column temperature 35 °C represented a good choice for robust enantiometric separation insensitive to small temperature variations.

The final composition of mobile phase was a mixture of: heptane–*i*-PrOH–DEA (980:20:1, v/v/v). The mobile phase flow rate was 1.0 ml/min and column temperature was maintained at 35 °C. The separation of TAP enantiomers is shown in Fig. 3.

3.1.3. Effect of water content and injection solvent on separation

It was described that the presence of trace amounts of water in the mobile phase of normal phase chromatography does have positive impact on the separation of enantiomers [28,29]. The effect of trace amounts of water on chromatographic characteristics was investigated by deliberate addition of controlled, trace amounts of water into the mobile phase for the separation of TAP and its enantiomers. Commercial *i*-PrOH (water content of 0.01%) was premixed with 0.5% (v/v) water and then used for preparation of the mobile phase. Unfortunately, low amount of water in *i*-PrOH led to a deterioration of chromatographic performance. The influence of trace amounts of water in the mobile phase is demonstrated in Fig. 3.

The influence of the injection solvent on production of chromatographic artifacts has been reported [30,31]. The effect of amount of EtOH in sample solvent (hexane) in the range from 4 to 20% was examined. Increasing of EtOH amount in sample solvent resulted in peak distortion. This peak distortion becomes dramatic when a concentration of EtOH is more than 6%. Relative to low solubility of TAP in hexane, the optimal concentration of EtOH in sample solvent is 4–5%. It is a critical step in method with aregard to separation and efficiency.

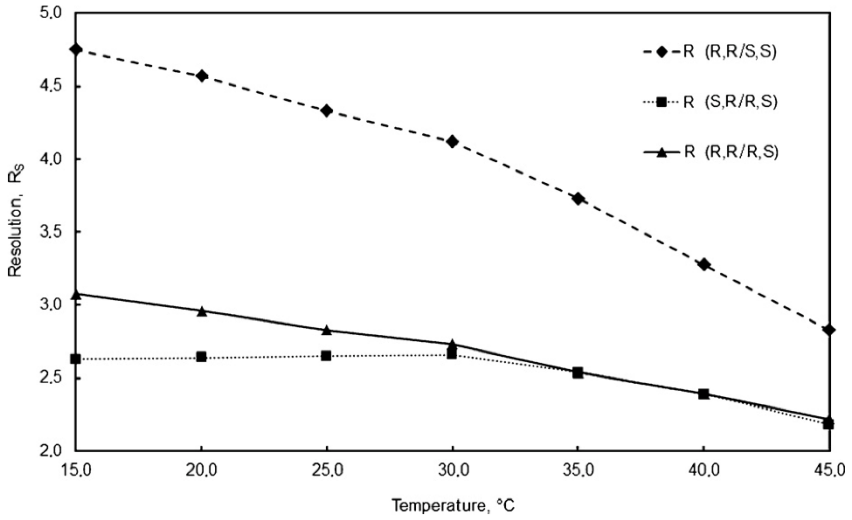

Fig. 2. The influence of temperature on resolution of TAP stereoisomers.

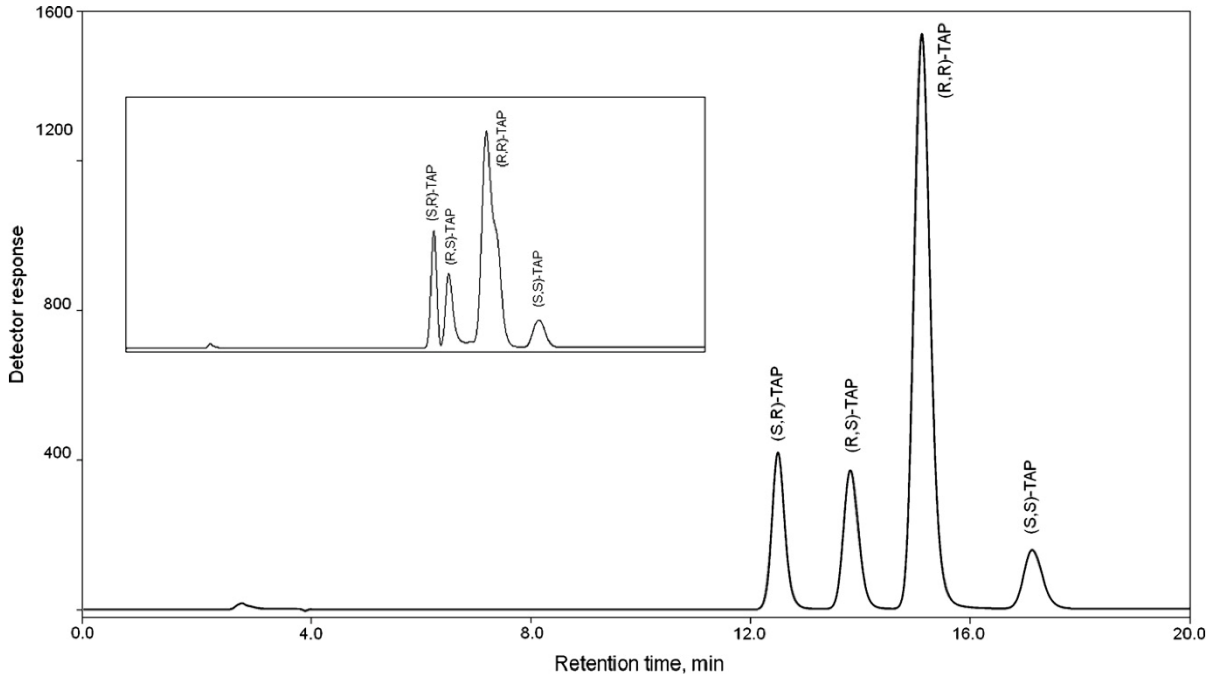

Fig. 3. The chiral separation of TAP stereoisomers. Mobile phase: heptane–*i*-PrOH–DEA (980:20:1, v/v/v); column: Chiralpak AD-H; column temperature: 35 °C; flow rate: 1 ml/min; fluorescence detection at 295 nm (excitation wavelength of 273 nm). The chromatogram of the influence of trace amounts of water in the mobile phase on separation is inserted.

3.2. Validation of the method

3.2.1. System suitability

The system suitability test was performed before each run to assure that the analytical method can be used within the employed chromatographic system with a satisfactory performance. Repeatability of injections expressed as relative standard deviation (RSD%) of (*R*,*R*)-TAP peak area and resolution between (*R*,*R*)-TAP/(*S*,*S*)-TAP for the five consecutive injections of a system suitability test solution were limited to $\leq$1.0% and $R \geq 3.0$ respectively. For all the measurements performed during the validation, the resolution more than 3.0 and RSD of peak areas $\leq$0.9% was achieved.

3.2.2. Limit of detection (LOD), limit of quantification (LOQ) and linearity

LOD and LOQ were calculated for all TAP enantiomers based on signal-to-noise ratio (*S*/*N*). The baseline noise was measured in a blank experiment in the region of retention time of tapentadol enatiomers using chromatographic software. The LOD ($S/N = 3$) and LOQ ($S/N = 10$) are summarized in Table 1.

A set of five TAP enantiomers solutions at the concentration range from LOQ to 120% of the general specification limit (LOQ – 0.18 μg/ml) was prepared. The calibration curves were constructed by plotting the peak area of the given analyte against its concentration and the calibration equations were calculated using linear

Table 1
The validation parameters of methods.

Validation parameter	TAP stereoisomers		
	R,S-	*S,R-*	*S,S-*
LOD (μg/ml)	0.004	0.003	0.010
LOQ (μg/ml)	0.011	0.010	0.031
Linearity			
Intercept	282,739	135,159	55,157
Slope	11,808,693	10,808,623	6,093,107
Correlation coefficient *r*	0.9998	0.9999	0.9996
Precision (%, RSD)			
API batch 1	<0.011	<0.010	0.048 (2.2)
API batch 2	<0.011	<0.010	0.051 (1.9)
API batch 3	<0.011	<0.010	0.042 (2.5)
Accuracy			
Recovery (%)	98.7	98.8	98.2
RSD (%) across all level	1.4	1.2	0.7

regression analysis. The results show a satisfactory linearity of all TAP enantiomers (Table 1).

3.2.3. *Precision, accuracy and determination in commercial preparations*

The precision of TAP enantiomers determination was evaluated by analysis of six independent preparations of the same homogenous sample under the prescribed conditions. Table 1 shows the results of TAP enantiomers content in three API batches of tapentadol. RSDs between 1.9 and 2.5% for a content of TAP enantiomers close to 0.05% demonstrate good precision.

Accuracy of the all TAP enantiomers was evaluated in triplicate at three concentration levels (0.05, 0.15 and 0.18 μg/ml which is equivalent to 0.05, 0.15 and 0.18% with respect to tapentadol) in (*R,R*)-TAP. The average total recoveries of TAP enantiomers are summarized in Table 1. It can be concluded that the developed analytical method gives accurate and precise results for determination of TAP enantiomers.

3.2.4. *Selectivity of the method*

The starting materials, intermediates and related substances of TAP are not detected due to use of selective fluorescence detection. This fact demonstrates selectivity of the developed method.

4. Conclusion

All enantiomers of tapentadol were successfully separated using one of ten tested CSPs in normal phase mode. Firstly, the method was thoroughly optimized and fully validated. The selectivity of method was demonstrated for all available known impurities. Secondly, the applicability of chiral method for routine enantiopurity control was demonstrated using analysis of API samples of routine production.

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PUBLIKACE VII

USE OF ALCOHOL AND DRUGS BY EMPLOYEES IN SELECTED BUSINESS AREAS IN NORWAY: A STUDY USING ORAL FLUID TESTING AND QUESTIONNAIRES

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RESEARCH Open Access

Use of alcohol and drugs by employees in selected business areas in Norway: a study using oral fluid testing and questionnaires

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Abstract

Background: Alcohol or drug use and associated hangover may reduce workplace safety and productivity and also cause sickness absence. The aims of this study were to examine (i) the use of alcohol and drugs, and (ii) reduced efficiency at work and absence due to such use among employees.

Methods: Forty-four companies were invited; half of them agreed to participate. Employees filled in a questionnaire and provided a sample of oral fluid, which was analysed for alcohol, 12 psychoactive medicinal drugs and 6 illicit drugs. Participation was voluntary and anonymous.

Results: Two thousand four hundred thirty-seven employees in eight business areas agreed to participate (92 % of those invited). By combining questionnaires and oral fluid testing, we found that 5.2 % had used psychoactive medication during the last couple of days, 1.4 % had used illicit drugs, 17.0 % had used alcohol during the last 24 h but only one person (0.04 %) was positive for alcohol in oral fluid. About 25 % reported reduced efficiency at work, and 5 % reported absence from work due to alcohol use during the past 12 months. The use of illicit drugs and binge drinking resulting in reduced efficiency and absence was most common among restaurant and bar workers and more common among men than women, whereas use of psychoactive medication was most common among healthcare, transportation and storage workers.

Conclusion: Impairment at work due to alcohol or drugs was rare, whereas reduced efficiency due to drinking was reported by a fairly large proportion. There were marked differences between some business areas, and across gender.

Keywords: Alcohol drinking, Illicit drugs, Prescription drugs, Workplace, Prevalence

Background

Use of alcohol or drugs and associated hangover effects may reduce workplace safety and productivity [1–3] and also cause sickness absence [4, 5]. Little is known about the use of alcohol and drugs that may affect safety and efficacy at work in Norway. A study published in 2004 found that 4 % reported having been under the influence of alcohol or drugs at work and 4 % had been absent from work because of alcohol and drug use [6]. Of the participants, 2.6 % reported having used illegal drugs and 18 % prescribed drugs during the past 12 months.

While few studies have addressed the prevalence of both alcohol and drug use and the consequences of such use in a work setting, the alcohol use-sickness absence association have been addressed in a number of studies. A recent review of 28 studies applying individual-level survey data to study a total of 48 associations between various measures of alcohol use and sickness absence, showed that there is strong empirical evidence for an association between alcohol use and both short- and long-term sickness absence [7]. One of the studies included in that review was conducted among young employees in Norway, where 8.1 % reported that they had been absent from work due to alcohol use the past 12 months [8]. This study found that the proportion of young male employees who reported having alcohol-related sickness absence was nearly twofold that of women, i.e., 10.5 and

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5.7 %, respectively. This finding is consistent with results in other studies [7]. However, less is known about gender differences with respect to drug use and consequences of such use in a workplace setting.

Results from workplace drug testing (WDT) in Norway for the period 2000–2006 showed that 2.9 % of the analysed samples were positive for drugs; only 1.0 % for illicit drugs [9]. However, WDT is used only within few business areas in Norway, primarily in shipping, oil industry and transportation, and it is likely that random workplace drug testing reduces the incidence of drug use among employees. Therefore, WDT findings do not accurately reflect the incidence of drug use among Norwegian employees in general.

The use of alcohol and drugs varies between countries and different business areas. American studies found that heavy alcohol use and illicit drug use was most prevalent among employees within the construction industry, arts, entertainment, recreation, mining, accommodation and food services and least prevalent among healthcare, social assistance and educational services [10, 11]. Differences between business areas have previously not been studied in Norway.

Research on the use of alcohol or drugs in relation to work has in most cases been performed using questionnaires or interviews. However, the use of alcohol and drugs is commonly under-reported [12–14]. A number of studies have found that analysis of biological samples may provide more accurate data than self-reports on alcohol and drug use during the last days or months [15–17]. However, drug testing cannot reveal alcohol and drug using habits and consequences of such use, so a combination of drug testing and questionnaires or interviews provides more data than using a single method alone [18–21].

The aims of this study were: (i) to examine the use of alcohol and drugs using a combination of self-report through questionnaires and testing of oral fluid (mixed saliva), and (ii) self-reported sickness absence and reduced efficiency or hangover at work due to such use among employees in eight business areas in Norway, and across gender.

Methods

We first performed a pilot study that included 526 employees during 2008–2009 [22], and a follow-up study with 1911 employees was conducted during 2011–2014. We present the total findings from both studies in this article.

Ethics

The study was approved by the Regional Committee for Medical and Health Research Ethics. The dataset was completely anonymous.

Consent

Oral informed consent was obtained from the participants for publication of reports.

Study design and setting

The recruitment of companies was performed during 2008–2013. Forty-four companies and business chains were invited to participate, and a general call for participation was published in magazines and on websites. In total, 21 companies agreed to participate, and the Norwegian Public Roads Administration agreed to let us recruit truck drivers at control stations for heavy traffic; thus, altogether 22 businesses participated.

Information about the upcoming study was distributed to all employees except truck drivers several weeks before the recruitment of employees was performed. The date for recruitment was not announced. First, the study days were selected for each company; then, either a random selection or all employees who were present in the building were contacted. Occupational drivers were recruited at a heavy vehicle checking station during scheduled controls.

For all companies except one, and for all occupational drivers, each employee was approached individually by one project assistant from the Norwegian Institute of Public Health (NIPH) and asked to participate. Written and verbal information about the project was given, and oral informed consent was obtained from all participants. Those who agreed to participate filled in a questionnaire in an area shielded from view and provided an oral fluid specimen. The questionnaire and the sample of oral fluid were placed in an unlabelled envelope that was closed and sealed and collected by a project assistant within approximately an hour.

For one company, an envelope containing the questionnaire and sampling device for oral fluid, including instructions for use, was given to random employees when entering the company facilities in the morning. The employees were asked to deliver the questionnaire and the oral fluid sample in closed and sealed, unlabelled envelopes at specified sites before noon.

The recruitment of employees was completed in 2014. In total, 2639 employees were invited and 2437 agreed to participate (92 %). The included business areas were healthcare (917 employees), finance (457 employees), manufacturing (254 employees), transportation/storage (233 employees), restaurants/bars (131 employees), public administration (211 employees), media (152 employees; questionnaires only), and research institutes (82 employees). Participation rates and socio-demographic characteristics for business areas with two or more companies and more than 100 participating employees are presented in Table 1. Data for employees in public administration,

Table 1 Employee participation rates, age and gender of all participants ($N = 2437$) across business areas

	Healthcare	Finance	Industry	Transportation and storage	Restaurants and bars	Other	Total
No. of participants	917	457	254	233	131	445	2437
Participation rate, %	98.3	96.4	91.0	95.9	92.9	78.2	92.3
Gender, % (n)							
Women	79.5 (729)	47.9 (219)	19.3 (49)	6.4 (15)	47.3 (62)	54.6 (243)	54.0 (1317)
Men	18.5 (170)	49.0 (224)	77.2 (196)	77.7 (181)	51.1 (67)	38.9 (173)	41.5 (1011)
Not reported	2.0 (18)	3.1 (14)	3.5 (9)	15.9 (37)	1.5 (2)	6.5 (29)	4.5 (109)
Age distribution, % (n)							
<30 years	14.7 (135)	9.4 (43)	11.8 (30)	23.2 (54)	78.6 (103)	14.6 (65)	17.6 (430)
30–39 years	26.8 (246)	28.2 (129)	15.0 (38)	18.0 (42)	16.8 (22)	28.1 (125)	24.7 (602)
40–49 years	26.7 (245)	24.7 (113)	30.7 (78)	22.7 (53)	3.1 (4)	22.9 (102)	24.4 (595)
50–59 years	22.0 (202)	28.0 (128)	28.7 (73)	21.5 (50)	0.0 (0)	21.6 (96)	22.8 (549)
60+ years	8.2 (75)	8.5 (39)	11.0 (28)	7.3 (17)	0.8 (1)	11.0 (49)	8.6 (209)
Not reported	1.5 (14)	1.1 (5)	2.8 (7)	7.3 (17)	0.8 (1)	1.8 (8)	2.1 (52)

media and research are presented in the column named "Other".

Data collection

For most of the companies, the data collection was performed during weekdays only. Oral fluid was collected using Statsure Saliva Sampler™ (Statsure Diagnostic Systems, Framingham MA, USA). The time required for sample collection and filling in the questionnaire was about 5 min.

The samples of oral fluid were frozen within one day after collection and thawed once before the analysis. Alcohol was analysed by an automated enzymatic method [23]. Medicinal and illicit drugs were analysed by liquid chromatography-tandem mass spectroscopy; two similar analytical methods were used during the project period [24, 25]. The analysed compounds and cut-off concentrations (above which a sample was regarded as positive) are presented in Table 2.

Two versions of the questionnaire were used. The questionnaire used for the pilot study in five businesses within transportation/storage, public administration, media and research did not include questions on drug use during the last 12 months; this question was added based on a request from one of the participating companies and was used for 17 businesses included after the pilot study had been finished.

Statistical methods

Possible differences between the prevalence of medicinal or illicit drugs in oral fluid samples from different business areas were initially assessed using Pearson's chi-square test for categorical data.

Adjusted odds ratios (OR) with 95 % confidence intervals (95 % CI) were calculated using multivariate unconditional logistic regression using SPSS Statistics Version 22 (IBM Corporation, Armonk, NY). Drug findings or self-reported data were included as dependent variable (with 2 categories; 0 = negative; 1 = positive). Independent variables were gender, age group (5 categories) and business areas (6 categories).

Results

The participation rate among invited businesses was 50.0 %. Among the employees in the participating businesses the average participation rate was 92.3 %; when using the regular recruitment procedure 95.6 % (range 80.0–100.0 %) and 67.8 % when the participation when employees were asked to deliver the envelope with questionnaire and oral fluid sample on specified sites.

Oral fluid

Positive alcohol and drug findings are presented in Table 2 and summarized in relation to business area and gender in Table 3. Only one employee (0.04 %) was positive for alcohol; this might be due to alcohol drinking the day before or due to a small alcohol intake during the working day, e.g. at lunch. Few employees were positive for illicit drugs (0.9 %) compared to medicinal drugs (3.0 %). The most frequently detected substances were the sleeping agent zopiclone (1.9 %), the sedative diazepam (0.7 %), cannabis (0.7 %), the analgesic substance codeine (0.3 %) and methamphetamine (0.1 %). The medicinal drugs that were found can in most cases be detected in oral fluid for more than 12 h after using a single dose, perhaps more than 24 h in some cases; amphetamine, cannabis (THC) and the cocaine metabolite benzoylecgonine may also be detected for more than 12 h, rarely longer than 48 h, whereas cocaine can be detected for less than 12 h after use [26].

Table 2 Substances analysed in oral fluid, cut-off concentrations and prevalence above cut-off concentrations

Compound	Description	Cut-off[c] ng/mL	Prevalence % (n)
6-Acetylmorphine	Metabolite of heroin	1.3	0.0 (0)
Alcohol		0.10 g/L	0.04 (1)
Alprazolam	Benzodiazepine; anxiolytic	0.62	0.0 (0)
7-Aminoclonazepam	Metabolite of clonazepam	0.71	0.0 (0)
7-Aminoflunitrazepam	Metabolite of flunitrazepam	0.17	0.0 (0)
7-Aminonitrazepam	Metabolite of nitrazepam	0.63	0.0 (0)
Amphetamine	Stimulant[a]	24	0.09 (2)
Benzoylecgonine	Metabolite of cocaine	9.8	0.04 (1)
Clonazepam	Benzodiazepine; anticonvulsant, anxiolytic	0.63	0.0 (0)
Cocaine	Stimulant[a]	1.8	0.04 (1)
Codeine	Opioid analgesic, antitussive	7.5	0.3 (6)
Diazepam	Benzodiazepine; anxiolytic, anticonvulsant, sedative	0.40	0.7 (16)
Flunitrazepam	Benzodiazepine; anxiolytic	0.31	0.0 (0)
3,4-Methylenedioxy-methamphetamine (MDMA)	Psychedelic hallucinogenic drug[b]	26	0.0 (0)
Methadone	Opioid used mainly for opioid dependence, but also for analgesia	11	0.0 (0)
Methamphetamine	Stimulant[a]	15	0.1 (3)
Morphine	Opioid analgesic, also metabolite of codeine and heroin	7.1	0.2 (4)
Nitrazepam	Benzodiazepine; anxiolytic	0.56	0.0 (0)
Nordiazepam	Metabolite of diazepam	0.68	0.3 (6)
Oxazepam	Benzodiazepine; anxiolytic, anticonvulsant, and metabolite of diazepam	4.9	0.04 (1)
Δ9-Tetrahydrocannabinol (THC)	Cannabis[a]	0.63	0.7 (16)
Zolpidem	Short acting hypnotic	1.2	0.09 (2)
Zopiclone	Short acting hypnotic	1.6	1.9 (43)

[a]Mostly used illegally in Norway
[b]Illegal in Norway
[c]Concentrations in neat oral fluid above which the analytical findings were regarded as positive

Medicinal drugs were detected more frequently (p <0.001) and illicit drugs less frequently (p <0.001) in samples of oral fluid from healthcare workers than in samples from employees in other business areas.

Illicit drugs were detected more frequently (p <0.001) in samples from restaurant/bar workers than in samples from employees in other business areas.

Of the 16 employees who were found to be positive for cannabis, the THC concentrations were above 2 ng/mL in native oral fluid (calculated using sample weight) in 14 cases; this concentration has been proposed as limit in the USA when oral fluid samples are used in workplace drug testing [27]. Four had concentrations between 25 and 300 ng/mL, suggesting cannabis smoking within a few hours before sampling. For two of the three employees who were positive for methamphetamine, concentrations were higher than the proposed limit of 50 ng/mL in the USA [27]. The concentrations were more than 1000 ng/mL, suggesting intake of moderate doses within the last 24 h or large doses 1–3 days ago. Those two individuals had combined methamphetamine with diazepam, which is a commonly used drug combination among problematic drug users. For five of the 43 employees who were positive for the sleeping agent zopiclone, the concentrations were above 50 ng/mL. Those high concentrations suggest that the medication might have been taken less than 6 h before sample collection. One of the diazepam users had 34 ng/mL in oral fluid, which indicates very recent drug intake or high concentration in blood. The other drug findings were of low concentrations that were unlikely to affect safety and efficacy.

Questionnaires

The results from the questionnaire are presented in Table 3. Self-reported use of psychoactive medication was fairly similar across business areas (ranging from 3.5 to 5.3 %). However, large differences were observed for

Table 3 Self-reported alcohol and drug use and results from testing of oral fluid samples

	Healthcare	Finance	Manufacturing	Transportation and storage	Restaurants and bars	Other	Men	Women	Total
Oral fluid samples, % (n)	98.8 (906)	98.2 (449)	99.2 (252)	99.6 (232)	99.2 (130)	65.6 (292)	unk.[a] (928)	unk.[a] (1226)	92.8 (2261)
Questionnaires, % (n)	100.0 (917)	100.0 (457)	100.0 (254)	97.0 (226)	100.0 (131)	100.0 (445)	unk.[a] (1011)	unk.[a] (1317)	99.7 (2430)
Psychoactive medication, % (n)									
A: Detected in oral fluid	4.6 (42)	1.1 (5)	2.0 (5)	3.0 (7)	2.3 (3)	1.7 (5)	2.3 (21)	3.7 (45)	3.0 (67)
B: Self-reported use last 48 h	4.5 (41)	3.7 (17)	3.5 (9)	5.3 (12)	3.8 (5)	3.8 (17)	4.3 (43)	3.9 (52)	4.2 (101)
Either A or B	6.0 (55)	4.2 (19)	4.7 (12)	7.3 (17)	5.3 (7)	3.8 (17)	5.1 (52)	5.2 (69)	5.2 (127)
Self-reported non-therapeutic use last 12 months	0.7 (6)	0.7 (3)	1.2 (3)	nc	1.5 (2)	nc	nc	nc	nc
Illicit drugs, % (n)									
A: Detected in oral fluid	0.0 (0)	0.4 (2)	0.8 (2)	3.0 (7)	6.9 (9)	0.0 (0)	1.7 (16)	0.3 (4)	0.9 (20)
B: Self-reported last 48 h	0.0 (0)	1.1 (5)	0.0 (0)	0.9 (2)	11.5 (15)	0.0 (0)	1.3 (13)	0.7 (9)	0.9 (22)
Either A or B	0.0 (0)	1.3 (6)	0.8 (2)	3.9 (9)	12.2 (16)	0.0 (0)	2.3 (23)	0.8 (10)	1.4 (33)
Self reported use last 12 m	1.9 (17)	3.7 (17)	1.6 (4)	nc	28.2 (37)	nc	nc	nc	nc
Alcohol, % (n)									
A: Detected in oral fluid	0.0 (0)	0.2 (1)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.1 (1)	0.0 (0)	0.0 (1)
B: Self-reported use last 24 h	13.4 (123)	12.3 (56)	9.8 (25)	12.4 (28)	45.0 (59)	27.2 (121)	19.2 (194)	15.3 (201)	17.0 (412)
Either A or B	13.4 (123)	12.5 (57)	9.8 (25)	12.4 (28)	45.0 (59)	27.2 (121)	19.3 (195)	15.3 (201)	17.0 (413)
Drinking habits and consequences, % (n)									
Binge drinking (≥6 units) at least once a month	11.8 (108)	25.6 (117)	23.6 (60)	22.6 (51)	58.0 (76)	27.0 (120)	30.4 (307)	15.7 (207)	21.9 (532)
Reduced efficiency or hangover at work during previous 12 m	12.2 (112)	39.8 (182)	5.5 (14)	9.7 (22)	65.6 (86)	40.9 (182)	27.8 (281)	22.4 (295)	24.6 (598)
Absence from work due to drinking during previous 12 m	0.9 (8)	9.4 (43)	1.2 (3)	3.5 (8)	20.6 (27)	8.8 (39)	7.2 (73)	3.9 (51)	5.3 (128)

[a]Gender was not reported by 109 participants
nc: Data was not collected for all groups of participants

self-reported use of illicit drugs. The proportion who reported using illicit drugs during the last 48 h among restaurant/bar workers was 11.5 % compared to 1 % or less in other business areas.

Employees in four business areas were asked about the use of illicit drugs and the non-therapeutic use of psychoactive medication (recreational use to get intoxicated or high) during last 12 months. More than 25 % of the restaurant/bar workers reported illicit drug use, less than 4 % in other business areas. Less than 2 % reported recreational use of psychoactive medication.

Self-reported alcohol use during the last 24 h, binge drinking, reduced efficiency or hangover and absence from work due to drinking was also most frequently reported by restaurant/bar workers, and was also fairly frequently reported by finance workers and workers in the group called "Other", which included media, research and public administration employees.

Comparing oral fluid and questionnaires

Some under-reporting of drug use was observed when comparing drug findings in oral fluid and self-reported drug use. Of those who were positive for illicit drugs in oral fluid ($n = 20$), 45.0 % reported using illicit drugs during the last 48 h; whereas among those who were positive for medicinal drugs ($n = 67$), 61.2 % reported intake during the last 48 h.

When adding drug findings in oral fluid to self-reported drug used during the last 48 h, the number of medicinal drug users increased by about a quarter and the number of illicit drug users increased by about the half when compared to self-reported use only. When including either analytical findings or self-reported use, recent use of psychoactive medication was most prevalent among transportation and storage workers, whereas recent use of illicit drugs was most prevalent among restaurant/bar workers.

Differences across business areas

The data presented in Table 3 suggest that there were marked differences between business areas. However, there were also significant differences between genders and age groups, making evaluation of the prevalence data more complicated. In order to study differences between business areas while adjusting for differences in the distributions of age and genders, we performed logistic regression analysis using drug findings or self-reported data as dependent variable, and business area, age and gender as covariates. The regression analyses for the detection of medicinal or illicit drugs in samples of oral fluid and self-reported inefficiency or absence due to drinking are presented in Table 4.

Due to the fact that illicit drugs were detected among employees in only some business areas and only one person above 40 years of age, the restaurant business were compared with non-restaurant business employees in total, and the employees were disaggregated into three age groups. Employees in the restaurant business had high odds ratio for being positive for illicit drugs ($p = 0.002$) when adjusting for gender and age group, compared to employees in other business areas.

Employees within the finance business and the group of businesses called "other" had significantly lower odds for being positive for medicinal drugs than healthcare employees. If comparing healthcare workers with employees within non-healthcare businesses in total, they were found to have higher odds ratios for being positive for medicinal drugs ($p = 0.001$) compared to other business areas (results not shown).

When compared with employees in the healthcare business, employees in the finance, restaurant and "other" businesses had significantly higher odds ratios for reporting reduced efficiency or hangover at work at least once during the previous 12 months ($p < 0.001$) and sickness absence ($p < 0.001$) due to drinking. Highest odds ratios were found for restaurant/bar workers.

Gender differences

The results presented in Table 3 show that illicit drugs were detected more frequently among male employees than females, and self-reported binge drinking, reduced efficiency or hangover at work and sickness absence from work due to drinking was also more frequent among males.

Logistic regression analysis was performed adjusting for age group and the six business areas (Table 4). Female employees had statistically significantly lower odds ratios compared to men to report reduced efficiency or hangover at work during previous 12 months ($p < 0.001$) and absence from work due to drinking during previous 12 months ($p = 0.010$). The difference observed between the genders for the detection of medicinal drugs in samples of oral fluid when adjusting for age group and business area were not statistically significant. However, the odds ratio for detection of illicit drugs was statistically significantly lower for females than males when adjusting for age group and business area when disaggregated into restaurant and non-restaurant businesses ($p = 0.004$).

Discussion

In this study, we combined the use of questionnaires and oral fluid testing to compare alcohol and drug use, as well as sickness absence and reduced efficiency due to such use, across eight business areas in Norway, and across gender.

Table 4 Logistic regression analysis

	Univariate analysis			Multivariate analysis		
	OR	95 % CI	*p*	OR	95 % CI	*p*
Detection of illicit drugs in oral fluid						
Non-restaurant/bar employees (referent)						
Restaurant/bar	13.70	5.57 33.69	<0.001	5.00	1.82 13.72	0.002
Females	0.19	0.06–0.57	0.003	0.19	0.06–0.59	0.004
Age < 30 years (referent)						
Age 30 39 years	0.51	0.20 1.28	0.151	0.77	0.28 2.15	0.622
Age 40+ years	0.03	0.00 0.21	0.001	0.05	0.01 0.43	0.006
Detection of medicinal drugs in oral fluid						
Healthcare employees (referent)						
Finance	0.23	0.09–0.59	0.002	0.24	0.09–0.63	0.003
Manufacturing	0.34	0.12–0.95	0.040	0.35	0.11–1.05	0.062
Transportation/storage	0.76	0.34–1.73	0.516	0.92	0.35–2.41	0.872
Restaurant/bar	0.48	0.15–1.57	0.224	0.77	0.20–2.93	0.700
Other lines of business	0.39	0.15–1.00	0.050	0.39	0.15–1.00	0.050
Females	1.66	0.98–2.81	0.058	1.27	0.67–2.42	0.462
Age < 30 years (referent)						
Age 30–39 years	0.71	0.28–1.79	0.464	0.79	0.29–2.11	0.639
Age 40–49 years	1.32	0.58–2.98	0.512	1.50	0.61–3.68	0.375
Age 50 59 years	1.83	0.83 4.05	0.134	2.18	0.91 5.22	0.082
Age 60+ years	2.37	0.95–5.94	0.066	2.83	1.05–7.63	0.040
Reported reduced efficiency or hangover at work last 12 months due to drinking						
Healthcare employees (referent)						
Finance	4.82	3.66–6.35	<0.001	5.10	3.79–6.87	<0.001
Manufacturing	0.37	0.20–0.68	0.001	0.31	0.16–0.58	<0.001
Transportation/storage	0.74	0.44–1.25	0.265	0.45	0.26–0.79	0.006
Restaurant/bar	14.18	9.35–21.52	<0.001	5.75	3.66–9.05	<0.001
Other lines of business	4.85	3.67–6.42	<0.001	4.96	3.69–6.67	<0.001
Females	0.75	0.62–0.90	0.003	0.63	0.50–0.79	<0.001
Age < 30 years (referent)						
Age 30–39 years	0.47	0.36–0.61	<0.001	0.41	0.30–0.56	<0.001
Age 40–49 years	0.30	0.23–0.40	<0.001	0.31	0.22–0.43	<0.001
Age 50–59 years	0.20	0.15–0.27	<0.001	0.18	0.13–0.26	<0.001
Age 60+ years	0.16	0.10–0.26	<0.001	0.13	0.08–0.22	<0.001
Reported sickness absence last 12 months due to drinking						
Healthcare employees (referent)						
Finance	11.93	5.55–25.60	<0.001	11.04	5.06–24.07	<0.001
Manufacturing	0.92	0.19–4.36	0.916	0.80	0.17–3.86	0.778
Transportation/storage	4.22	1.51–11.79	0.006	2.73	0.94–7.97	0.066
Restaurant/bar	29.22	12.93–66.02	<0.001	13.76	5.78–32.77	<0.001
Other lines of business	10.92	5.04–23.67	<0.001	9.92	4.54–21.69	<0.001
Females	0.52	0.36–0.75	0.001	0.59	0.39–0.88	0.010
Age < 30 years (referent)						
Age 30–39 years	0.68	0.44–1.05	0.080	0.75	0.45–1.24	0.259
Age 40–49 years	0.23	0.13–0.42	<0.001	0.31	0.16–0.59	<0.001
Age 50–59 years	0.22	0.12–0.41	<0.001	0.28	0.14–0.55	<0.001
Age 60+ years	0.13	0.04–0.42	0.001	0.14	0.04–0.48	0.002

More detailed results from one business area (healthcare) have been published in an separate article [28]. In this article we present the total findings from the pilot and main studies, which included 2437 employees from eight business areas. Data from the pilot study are included to enable the comparison between all included business areas; this has not been reported for the pilot study previously.

Few employees were impaired by alcohol or drugs at the time of collection of oral fluid. One employee had concentration of alcohol of about 0.2 g/L in oral fluid (the concentration in blood is about the same as in oral fluid), which either may be caused by drinking one glass of beer or wine within the last hour, or residual alcohol after heavy drinking the day before. For drugs, it is impossible to accurately estimate concentrations in blood based on concentrations in oral fluid due to large individual variation [29]. However, about 10 persons (0.4 %) had drug concentrations in oral fluid that might be associated with recent drug use that may affect the performance at work.

In a U. S. survey, 8.1 % reported workplace use of alcohol during the last 12 months, 0.99 % reported weekly use, 0.78 % reported alcohol-related impairment weekly, and 9.23 % reported being hungover at work during the last 12 months [30]. In Europe, the situation varies a lot between different countries; in some countries the use of alcohol during the working day has been very common. The proportion of workers who consumed alcohol during the working day was reportedly 11 % in Austria, 14 % in Denmark, 8.2 % in Poland; whereas in the Netherlands, 4 % of the workers who drank alcohol sometimes drank before going to work or at work [31]. Thus, the use of alcohol in relation to work seemed to be very much less common in our study than in some European countries.

In a study based on data from a U.S. National Survey performed in 2002–2003, a total of 14.1 % of the workforce reported having used illicit drugs during the last 12 months, 3.6 % at least once a week, and 1.25 % reported use 6–7 days a week [32]. Thus, the use of illicit drugs was significantly more common among employees in the USA than in Norway. There is little information about drug use in relation to work for other European countries [31]. However, there is large variation in the use of illicit drugs in general between European countries [33].

The results show large differences between some business areas. Restaurant/bar workers reported more often alcohol use during the last 24 h. This is partly due to the fact that for those businesses the data collection included weekends, when approximately 70 % of drinking situations occur [34]. A larger proportion of restaurant/bar workers also reported binge drinking during the past 12 months compared to employees in other business areas as well as being less efficient at work and absence from work due to alcohol use during the last 12 months. Drug use during the last 48 h was also more common among restaurant/bar workers.

The findings among restaurant/bar employees are partly related to the large proportion of employees below 30 years of age, who are expected to use more alcohol and illicit drugs than older age groups. However, when adjusting for age and gender distributions, significantly more drug use and alcohol-related hangover and absence were found among restaurant/bar employees than among employees in most other business areas. Also the fact that they are working in an environment with high availability of alcohol and work-related norms that are supportive of after-work drinking and hangover at work may have influenced their drinking habits [35].

Also previous studies have found high alcohol consumption or high prevalence of hazardous drinking among restaurant workers, both in Scandinavia and elsewhere [10, 35–38]. Previous American studies have also found that restaurant workers more often reported use of illicit drugs than employees in many other business areas [10, 11].

The proportion of workers within finance and the "Other" category (i.e., media, research and public administration employees) who reported reduced efficiency and sickness absence due to alcohol was also fairly high.

It has previously been reported that problematic alcohol and drug use may be common among health professionals [39], particularly because of their easy access to prescription medication. In our study, binge drinking, reduced efficiency and drinking-related absence was less common among healthcare workers than the other business areas. However, the use of psychoactive medication, both self-reported use and findings in samples of oral fluid, was higher among health professionals. Moreover, we found that use of psychoactive medication was more common among employees within transportation and storage than in other business areas.

Studies in the USA have found that in addition to hotel, restaurant and bar workers, employees within construction, building and grounds maintenance, arts, entertainment, sports and media businesses had higher odds ratios for illicit drug use and illicit drug impairment [10, 11, 32, 40] as well as working under the influence of alcohol and hangover at work [30]. An Australian study found that alcohol use at work was most common among hospitality, construction and financial services, whereas working under the influence of alcohol was most common among hospitality employees; in total, more than 5 % of the Australian workers admitted to having worked under the influence of alcohol and almost 2 % under the influence of drugs [41]. Another Australian study found that the risk of workers frequently drinking at levels

associated with short-term harm was lowest in the education industry and significantly higher in the hospitality, agriculture, manufacturing and construction industries [42]. Alcohol-related absenteeism was also most common among hospitality and manufacturing employees in Australia [43]. Our study did not include the same business areas, but our findings are similar for employees within restaurant/bar and finance industries.

Finally, this study showed that the proportion who reported alcohol-related sickness absence was about twice as large as for men compared to women, a finding which is consistent with results from previous studies [8, 43], whereas reduced efficiency or absence due to drinking was reported about 60–70 % more often among men. A plausible explanation of this finding is that men drink more frequently than women and that they more often drink to intoxication than women, in all societies surveyed [44].

Limitations

The participating employees do not represent a random selection from the total working population or the included business areas. There might have been geographical differences between urban and rural areas as well as between different parts of the country regarding the use of alcohol and drugs. Geographical areas could not be used as covariates in the statistical analysis due to low number of companies within each business area.

It is possible that some employees who had recently used alcohol or drugs refused to participate in the study because this information is regarded as sensitive. As shown above and previously [22, 34], underreporting of alcohol and drug use on the questionnaires also occurred in spite of the fact that the project team members told that the study was anonymous.

A positive drug finding in oral fluid most likely represents drug intake during the last 48 h. However, use of some drugs more than 48 h ago might also give a positive result, particularly repeated use more than a couple of days before sample collection. On the other hand, a negative oral fluid sample does not prove that drugs were not taken during the last 48 h; intake of a single dose of cannabis, cocaine or medication will in most cases cause positive oral fluid sample for less than 24 h.

One of the companies within the finance sector required that the recruitment of employees should occur in the large entrance hall. This made it possible for some employees to deliberately avoid being asked to participate; thus, the selection of participants in this company might have been somewhat biased. For all other companies, it was not possible to avoid being asked for participation.

Conclusions

Overall, a small proportion of employees were positive for alcohol or drugs in samples of oral fluid but a significant proportion of the employees reported absence or hangover at work due to drinking. Of the studies business areas, restaurant/bar workers most often reported frequent binge drinking, reduced efficiency or absence from work due to drinking. Many of them also reported use of illicit drugs. Thus, the restaurant workers comprise a high-risk group regarding alcohol and drug use. Employees within the finance industry often reported reduced efficiency or absence because of drinking. Larger proportions of male than female workers reported binge-drinking each month, reduced efficiency or hangover at work or absence from work due to drinking during the past 12 months.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors participated in designing the study and assessing the data. HMEE had the main responsibility for planning and coordinating the acquisition of data. HMEE and HG had the main responsibility for drafting the manuscript. All co-authors contributed in revising the manuscript critically for intellectual content. All authors read and approved the manuscript.

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3 ZÁVĚR

V první části této disertační práce jsou shrnuty výsledky týkající se vlivu vybraných látek na fertilizační schopnosti spermií. Pro studium vlivu βE2 na kapacitaci myších spermií *in vitro* byla vypracována nová HPLC-MS/MS metoda, pomocí níž se měřily koncentrace celkového nevázaného βE2 ve spermiích. Kapacitace byla prováděna se třemi počátečními koncentracemi βE2 (200, 20 a 2 μg/L). Pokusy byly realizovány se dvěma kmeny laboratorních myší (BALB/c a C57BL/6Nvel). U obou kmenů myší byl pro všechny testované koncentrace pozorován obdobný trend závislosti koncentrace nevázaného βE2 na čase kapacitace. Koncentrace βE2 se snižovala, aby dosáhla svého minima a poté se opět zvyšovala. Pozice minima se lišila pro jednotlivé testované koncentrace βE2. V rámci jednotlivých testovaných koncentrací βE2 byly pozorovány pouze mírně odlišné rozsahy poklesu mezi spermiemi obou kmenů myší.

Experimentální výsledky získané pro koncentrační změny βE2 v různých časech kapacitace byly podrobeny kinetické analýze. Křivky proložené experimentálně stanovenými body vykazovaly autokatalytický charakter. Při hledání různých kinetických modelů bylo zjištěno, že pro shodu mezi křivkami získanými proložením experimentálními body a teoreticky vypočítanými křivkami je nutné předpokládat, že prvním krokem je adsorpce βE2 na povrch spermií řízená Langmuirovou izotermou. Jiné modely (bez adsorpce) vedly ke zcela odlišným výsledkům. Je důležité, že teoretické křivky jsou v dobré shodě s experimentálně získanými body pro oba testované typy myší, což dokládá nalezení teoretického mechanismu, který vyhovuje druhově specifickým modifikacím.

Získané poznatky ukazují, že množství βE2 dostupné pro myší spermie během kapacitace *in vitro*, nezávisle na kmenovém původu, může být kvantifikováno HPLC-MS/MS metodou. Vyvinutá metoda představuje důležitý, obecně použitelný nástroj pro studium množství biologických látek, v tomto případě βE2.

Výsledky kinetické studie použité pro studium působení fluoridů a fluorohlinitých komplexů na kapacitaci myších spermií vykazují velmi dobrou shodu experimentálních dat s teoreticky získanými křivkami. Časové závislosti pTyr vyhovují rovnici, která je platná pro tvorbu nestabilního intermediátu ve dvou následných reakcích 1. řádu. Navržené kinetické rovnice objevují existenci určitých center ve spermiích a jejich nestabilní aktivní formy; pouze tyto nestabilní aktivní formy mohou být fosforylovány a

následně se rozpadají. Čas odpovídající maximální produkci nestabilního intermediátu je pro spermie pravděpodobně nejvhodnější k získání schopnosti oplodnit vajíčko.

Ve druhé části této práce je shrnut vývoj metod pro chirální separaci AB a TAP. Pro enantioseparaci (*R,S*)-AB byla použita metoda CE. Pro rozdělení obou enantiomerů byly vyzkoušeny různé CD (sulfatované α-CD, β-CD a γ-CD a hydroxypropyl β-CD a γ-CD) jako potenciální selektory. Nejlepších výsledků bylo dosaženo při použití sulfatovaného γ-CD. Během vývoje metody byl studován vliv změn v experimentálních parametrech na enantioseparaci (*R,S*)-AB. Pro stanovení (*S*)-AB jako optické nečistoty v syntetickém laboratorním vzorku bylo použito 4,8% (*w/v*) sulfatovaného γ-CD v kyselém základním elektrolytu dihydrogenfosforečnanu draselného (100 mM, pH = 3,0) na nepokryté křemenné kapiláře s rozšířenou optickou dráhou. Metoda byla validována jako limitní test.

Pro chirální separaci čtyř stereoizomerů TAP bylo použito NP-HPLC. Bylo vyzkoušeno 10 různých chirálních kolon, z nichž kolona Chiralpak AD-H poskytla dostatečnou chirální selektivitu. Během vývoje byl studován vliv složení mobilní fáze (konkrétně vliv alkoholu jako modifikátoru, použitého *n*-alkanu, aditiv a obsahu vody v mobilní fázi) a dalších parametrů na retenci a enantioseparaci. Chirální separace čtyř stereoizomerů TAP metodou NP-HPLC s rozlišením větším než 2,5 pro všechny z enantiomerů bylo dosaženo při použití kolony Chiralpak AD-H s mobilní fází heptan/propan-2-ol/diethylamin (980:20:1, *v/v/v*). Vyvinutá metoda byla validována dle požadavků směrnice International Conference on Harmonisaton (ICH).

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