UNIVERZITA KARLOVA V PRAZE

FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

Katedra analytické chemie

ANALÝZA STATINŮ V BIOLOGICKÉM MATERIÁLU

Disertační práce

2011 Mgr. Hana Vlčková

PODĚKOVÁNÍ

Ráda bych poděkovala svému školiteli Prof. RNDr. Petru Solichovi, CSc. a školiteli specialistovi Doc. PharmDr. Lucii Novákové, Ph.D. za odborné vedení po dobu mého postgraduálního studia, za poskytování cenných rad a zkušeností, za inspiraci a připomínky jak při tvůrčí praktické práci, tak při psaní odborných publikací. Také děkuji za podporu prezentace výsledků na zahraničních a tuzemských konferencích.
Děkuji také kolegům z katedry analytické chemie, hlavně PharmDr. Petru Chocholoušovi, Ph.D., Doc. RNDr. Daliboru Šatínskému, Ph.D. za předání praktických a teoretických zkušeností a pomoc při řešení technických nesnází. Nesmím také opomenout na poděkování všem členů, katedry analytické chemie za příjemnou atmosféru na našem pracovišti.
Mé poděkování patří také UHPLC-MS/MS týmu, kolegyni PharmDr. Marcele Seifrtové, Ph.D., mnoha pregraduálním studentům. Mgr. Martině Rabatinové bych chtěla zvláště poděkovat za spolupráci na vývoji metody pro stanovení pravastatinu a pravastatinu laktonu v potkaní plazmě a moči a za vytvoření některých obrázků.
Nemohu zapomenout také na poděkování externím pracovníkům z Fakultní nemocnice v Hradci Králové, Prof. MUDr. Vladimíru Bláhovi, CSc., Prof. MUDr. Milanovi Bláhovi, CSc. a Doc. RNDr. Dagmar Solichové, Ph.D. za spolupráci na tématice statinů. Poděkání patří také kolegům z Lékařské fakulty v Hradci Králové, Mgr. Gabriele Kolouchové a Doc. MUDr. Stanislavu Mičudovi, Ph.D. za spolupráci na zajímavé studii.

Práce byla podporována projektem SVV/2011/263002. Ráda bych také poděkovala grantovým agenturám FRVŠ, GA UK, IGA MZ ČR za finanční podporu.

Na závěr děkuji také mé rodině a příteli za trpělivost a každodenní podporu.

Prohlašuji, že tato práce je mým původním autorským dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu.

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ABSTRAKT

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Název disertační práce: Analýza statinů v biologickém materiálu

Disertační práce se zabývá analýzou statinů v biologickém materiálu. Statiny patří do skupiny léčiv snižující hladinu cholesterolu a jedná se o nejpoužívanější léčiva v léčbě Familiární hypercholesterolémie. Z důvodu velmi nízkých koncentrací statinů (ng/ml) v krevní plazmě je výběr vhodné analytické metody značně omezen. Díky své citlivosti a selektivitě se ukázalo spojení kapalinové chromatografie s hmotnostně spektrometrickou detekcí jako nejvhodnější technika. V případě analýzy složitých matricí, jako jsou biologické vzorky, musí chromatografické separaci vždy předcházet úprava vzorku.
Teoretická část disertační práce nejprve shrnuje jednotlivé farmakologické vlastnosti a účinky statinů. Dále je věnována teoretickým poznatkům z oblasti UHPLC a hmotnostní spektrometrie. Nejrozsáhlejší díl teoretické části je věnován popisu jednotlivých technik úpravy vzorků.
Praktická část se zabývá nejen vývojem UHPLC-MS/MS metodiky pro stanovení statinů v biologickém materiálu, ale i volbou a vývojem vhodné extrakční techniky. Extrakce na tuhou fázi (SPE) a mikroextrakce tuhým sorbentem (MEPS) jsou používány jako metody úpravy vzorků před chromatografickou analýzou. Výsledky praktické části jsou dokumentovány pěti originálními články uvedenými v přílohách I-V. Stručný komentář k jednotlivým článkům je sepsán v kapitole „Výsledky a diskuse“. S ohledem na použitou extrakční metodu je praktická část disertační práce rozdělena na dva tematické celky. První oddíl praktické části se zabývá využitím SPE jako kroku úpravy vzorků. Metoda pro stanovení atorvastatinu, simvastatinu a jejich metabolitů za použití SPE-UHPLC-MS/MS technik v lidském séru byla validována a následně aplikována na reálné vzorky pacientů s familiární hypercholesterolémií. Naměřené výsledky přispěly k úpravě dávkovacího schématu. Druhá část praktické práce je zaměřena na aplikaci MEPS techniky, která umožňuje užití velmi malých objemů vzorku. SPE postup pro stanovení atorvastatinu a jeho metabolitů byl převeden na mikroextrakci tuhým sorbentem. MEPS-UHPLC-MS/MS metoda byla validována a následně nahradila dříve vyvinutou SPE metodiku. Další metoda užívající MEPS jako krok úpravy vzorků byla vyvinuta pro stanovení pravastatinu a pravastatinu laktonu v potkaní plazmě a moči.

ABSTRACT

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Title of Doctoral Thesis: Analysis of statins in biological samples

The doctoral thesis deals with analysis of statins in biological samples. Statins are the most widely used drugs for the treatment of Familiar hypercholesterolemia. Due to very low concentration of statins (ng/ml) in human plasma a choice of suitable analytical methods is limited. Liquid chromatography with mass spectrometry detection is the most suitable technique due to high selectivity and sensitivity. Sample preparation step has to be used before chromatographic determination of analytes in complex matrices, such as biological samples.

Primarily theoretical part of doctoral thesis summarizes individual pharmacological properties and effects of statins. Another part is devoted to theoretical knowledge of UHPLC-MS/MS. The widest section deals with description of the individual sample preparation techniques. The practical part contends with development of UHPLC-MS/MS methods for the determination of statins in biological samples and selection and development of suitable sample preparation techniques. Solid phase extraction (SPE) and microextraction by packed sorbent (MEPS) are used as the sample preparation step. The results of experiments are summarized in five original articles appended in the supplement I-V. Brief comments of articles are documented in part “results and discussion”. Doctoral thesis is divided into two sections with regard to use of sample preparation methods. The first part deals with use of solid phase extraction. Method for the determination of atorvastatin, simvastatin and their metabolites using SPE-UHPLC-MS/MS techniques in human serum was developed. Validated method was applied to patients with Familiar hypercholesterolemia. The second part of practical thesis is focused on application of MEPS which enables use of very small volume of sample. SPE procedure of determination of atorvastatin and their metabolites in human serum was transferred to microextraction by packed sorbent. MEPS-UHPLC-MS/MS method was validated and replaced earlier developed SPE extraction. Another method using MEPS as the sample preparation step was developed for determination of pravastatin and pravastatin lactones in rat plasma and urine

SEZNAM POUŽITÝCH ZKRATEK

APCI	chemická ionizace za atmosferického tlaku, atmospheric pressure chemical ionization
API	ionizace za atmosferického tlaku, atmospheric pressure ionization
APPI	fotoionizace za atmosferického tlaku, atmospheric pressure photoionization
BA	biologická dostupnost, biological availability
BEH	hybridní technologie silikagelu a polymeru, bridged ethyl hybrid
CE	kapilární elektroforéza, capillary electrophoresis
CFME	mikroextrakce kontinuálního toku, continuous-flow microextraction
CI	chemická ionizace, chemical ionization
CID	kolizně indukovaná disociace, collision-induced dissociation
CRP	C- reaktivní protein, C- reactive protein
CSH	hybridní stacionární fáze s nabitým povrchem, charged surface hybrid
CYP	cytochrome P450, cytochrome P450
DART	přímá analýza v reálném čase, direct analysis in real time
DESI	desorpční ionizace elektrosprejem, desorption electrospray ionization
DI-SDME	mikroextrakce jednou kapkou pomocí přímého ponoření, direct immersion single drop microextrakcion
DLLME	disperzní extrakce z kapaliny do kapaliny, dispersive liquid liquid microextraction
DPX	extrakce pomocí naplněných špiček pipet, disposable pipette tip extraction
EMEA	European Medicines Agency
ESI	ionizace elektrosprejem, electrospray ionization
GC	plynová chromatografie, gas chromatography
GTP	Guanin-nukleotid-trifosfát, guanine-nucleotide-triphosphate
HF-LPME	mikroextrakce do kapalné fáze s využitím dutého vlákna, hollow-fiber liquid phase microextraction
HMG- CoA	3- hydroxy- 3- methylglutaryl koenzym A, 3-hydroxy-3methylglutaryl coenzyme A
HPLC	vysokoúčinná kapalinová chromatografie, high performance liquid chromatography
HRMS	hmotnostní spektrometrie s vysokým rozlišením, high resolution mass spectrometry
HS-SDME	mikroextrakce jednou kapkou pomocí head space, head space single drop microextrakcion

ICH	International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
ICR	iontová cyklotronová rezonance, ion cyclotrone resonance
IT	iontová past, ion trap
LC	kapalinová chromatografie, liquid chromatography
LD	kapalná desorpce, liquid desorption
LDL	lipoprotein nízké hustoty, low density lipoprotein
LDLR	LDL receptor, LDL receptor
LIT	lineární iontová past, linear ion trap
LLE	extrakce kapalina- kapalina, liquid- liquid extraction
LLLME	mikroextrakce z kapaliny do kapaliny a zpět, liquid liquid liquid microextraction
LLME	mikroextrakce z kapaliny do kapaliny, liquid liquid microextraction
MALDI	ionizace laserem za účasti matrice, matrix assisted laser desorption ionization
MEPS	mikroextrakce tuhým sorbentem, microextraction by packed sorbent
MEPS	mikroextrakce tuhým sorbentem, microestraction by packed sorbent
MIP	molekulárně vtištěné polymery, molecular imprinted polymer
MMP	metaloproteináza, methaloproteinase
MS/MS	tandemová hmotnostní spektrometrie, tandem mass spectrometry
PLE	vysokotlaká extrakce kapalinou, pressurized liquid extraction
PP	srážení proteinů, protein precipitation
Q	kvadrupól, quadrupole
QqQ	trojitý kvadrupól, triple quadrupole
RAM	materiál s omezeným přístupem, restricted access material
SALLE	extrakce z kapaliny do kapaliny pomocí vysolování, salting-out liquid liquid extraction
SAX	silný měnič aniontů, strong anion exchanger
SBSE	sorpční extrakce míchátkem, stir bar sorptive extraction
SCX	silný měnič kationtů, strong kation exchanger
SDME	mikroextrakce jednou kapkou, single drop microextrakcion
SIM	monitorování vybraného iontu, single ion monitoring
SPE	extrakce na tuhou fázi, solid phase extraction
SPME	mikroextrakce na tuhou fázi, solid phase microextraction
SRM	sledování vybrané reakce, selected reaction monitoring
SST	test způsobilosti systému, system suitability test

TFC	turbulentní průtoková chromatografie, turbulent flow chromatography
TOF	analyzátor doby letu, time of flight
UHPLC	ultra vysokoúčinná kapalinová chromatografie, ultra high performance liquid chromatography

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1. ÚVOD

Statiny patří do skupiny léčiv nazývaných hypolipidemika. Jedná se o inhibitory 3-hydroxy-3-methylglutaryl koenzym A reduktázy, který představuje klíčový enzym endogenní syntézy cholesterolu. Používají se nejen k léčbě hypercholesterolémie, ale i jiných onemocnění jako je Alzheimerova choroba nebo diabetes mellitus. V současné době jsou v klinické praxi používány zejména atorvastatin, simvastatin a rosuvastatin. Přestože jsou statiny považovány za bezpečná a účinná léčiva, jejich podávání je spojeno se vznikem nežádoucích účinků, jako je myopatie a rabdomyolýza. Vyšší výskyt vedlejších účinků je typický pro lipofilní statiny, jako například atorvastatin, simvastatin, a pro zvyšující se léčebnou dávku. Monitorování hladin statinů v průběhu terapie by mohlo přispět k objasnění farmakokinetiky a k vytvoření individuálního dávkovacího schématu.

Protože zavedení monitorování lékových hladin do klinické praxe znamená analýzu velkého počtu vzorků, hlavními požadavky na výběr a vývoj vhodné bio-analytické metody jsou miniaturizace a automatizace společně s dostatečnou spolehlivostí a přesností. Miniaturizace vede ke zkrácení doby analýzy, ke snížení potřebného objemu vzorku či rozpouštědel a tím ke snížení nákladů na analýzu. V současné době je vysokoúčinná kapalinová chromatografie (HPLC) nejrozšířenější separační technikou v klinické praxi. Vývoj této metody směřuje v posledních letech k urychlení vlastní doby analýzy, zvýšení účinnosti systému, rozlišení a citlivosti, což vedlo ke vzniku nových přístupů tzv. rychlé chromatografie. Jedním z těchto přístupů je ultra vysokoúčinná kapalinová chromatografie (UHPLC), která v posledních letech získává stále širší uplatnění v oblasti rutinní analýzy. K detekci se nejčastěji používá detektor diodového pole, ale v mnohých laboratořích je již dostupný citlivější a selektivnější detektor, a to hmotnostní spektrometr, který umožňuje sledování lékových hladin řádově v nmol/l.

Protože biologické vzorky jsou ve většině případů komplexní a velmi složité matrice, vzorek nemůže být aplikován přímo do chromatografického systému, ale musí předcházet krok úpravy vzorku. Přestože vzniklo mnoho nových extrakčních technik, dosud nebyla nalezena vhodná metoda splňující požadavky dnešní klinické praxe, jako jsou rychlost, miniaturizace, automatizace a vysoká účinnost společně s dostatečnou přesností, správností a reprodukovatelností výsledků. Na rozdíl od chromatografických technik, kde došlo k významnému zkrácení doby analýzy, ve většině případů na 2 až 6 minut podle počtu stanovovaných analytů a složitosti matrice, krok úpravy vzorků trvá řádově několik desítek minut, což je zhruba 70% celkové doby analýzy vzorku. Jedná se tedy o časově nejnáročnější a nejproblematičtější část bio-analytické metody.

2. CÍL PRÁCE

Cílem této disertační práce byl vývoj a validace metodik pro stanovení statinů v biologickém materiálu s využitím nových trendů v oblasti kapalinové chromatografie a moderních technik úpravy vzorků před analýzou. Dané metody byly po jejich validaci aplikovány na reálné vzorky, které byly získány z Fakultní nemocnice v Hradci Králové anebo z Lékařské fakulty Univerzity Karlovy v Hradci Králové. Z důvodu aplikace metod do klinické praxe bylo nutné, aby jednotlivé validační parametry splňovaly požadavky norem určených pro bio-analytické metody. Hlavními předpisy v této oblasti jsou normy zabývající se monitorováním léčiv, zejména směrnice ICH řady Q (Quality) a směrnice EMEA (Guideline on validation of bioanalytical methods).

Experimentální práce byla zaměřena na využití UHPLC-MS/MS techniky pro analýzu jednotlivých statinů a extrakci na tuhou fázi jako kroku úpravy vzorků. Z důvodu dostupnosti pouze malého objemu vzorku bylo nutné najít vhodnou miniaturizaci této extrakce. Mikroextrakce tuhým sorbentem (MEPS) byla tedy zvolena jako druhá technika úpravy vzorků využitá při analýze statinů.

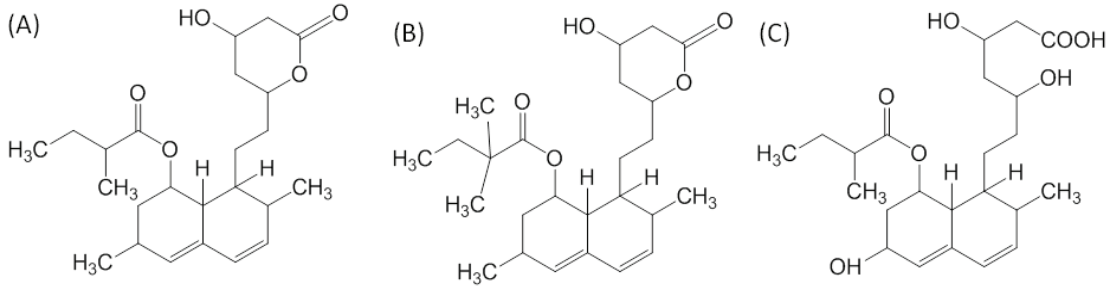
Experimenty byly rozděleny podle typu použité úpravy vzorků do dvou tematických celků. První část zabývající se využitím konvenční metody extrakce na tuhou fázi v bio-analytických stanovení statinů se věnovala vývoji a validaci SPE-UHPLC-MS/MS metodiky pro kvantitativní analýzu atorvastatinu, simvastatinu a jejich metabolitů v lidském séru a jednotlivých frakcích cholesterolu. Po validaci sloužila hlavně k monitorování hladin atorvastatinu a simvastatinu u pacientů s familiární hypercholesterolémií léčených kombinovanou terapií statiny a extrakorporální eliminační procedůrou. Na základě získaných výsledků bylo upraveno dávkovací schéma. V druhé části práce jsem se věnovala využití moderní techniky mikroextrakce tuhým sorbentem. Dříve vyvinutý SPE postup pro analýzu atorvastatinu v biologických tekutinách byl převeden na MEPS a následně aplikován na reálné vzorky pacientů. MEPS umožnila použití velmi malých objemů vzorků, a tudíž představovala nižší zátěž pro pacienty. Dále byla MEPS uplatněna při stanovení pravastatinu a pravastatinu laktonu v potkaním séru a moči.

3. TEORETICKÁ ČÁST

Statiny patří do skupiny léčiv snižující hladinu cholesterolu. Jedná se o inhibitory klíčového enzymu endogenní syntézy cholesterolu, 3-hydroxy-3-methylglutaryl koenzym A (HMG-CoA) reduktázy. Používají se zejména k léčbě familiární hypercholesterolémie, ale ovlivňují i další onemocnění jako Alzheimerova choroba a diabetes mellitus. Jsou považována za bezpečná léčiva, ale přesto jsou spojeny s výskytem nežádoucích účinků, jako je rabdomyolýza a myopatie.

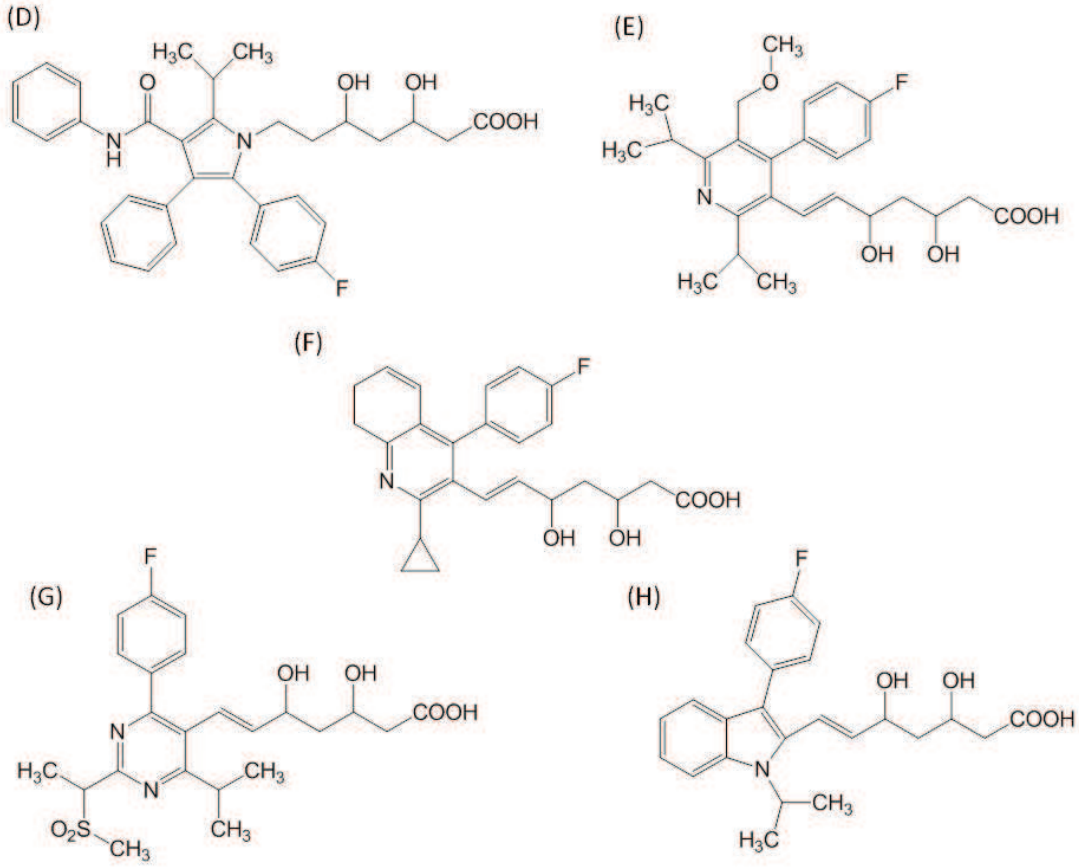
3.1. OBECNÁ CHARAKTERISTIKA STATINŮ

První molekula statinů, mevastatin, byla izolována z hub *Penicillium citrinum* (Ascomycota) roku 1976. Jeho molekula nebyla nikdy používána jako léčivo značných nežádoucích účinků. Postupem času došlo k izolaci nebo umělé syntéze dalších statinů, které již byly v klinické praxi používány. Statiny zahrnují nejen přirozené (lovastatin) nebo částečně modifikované houbové extrakty (simvastatin, pravastatin), Obr. 1, ale i syntetické sloučeniny (fluvastatin, atorvastatin, cerivastatin, rosuvastatin a pitavastatin), Obr. 2 [1].



Obr. 1: Struktury přirozených a polosyntetických statinů - (A) lovastatin, (B) simvastatin, (C) pravastatin.

Molekula statinu existuje ve dvou formách – laktonové a otevřené hydroxy formě kyseliny. In vivo je hydroxy forma vlastní účinnou látkou snižující hladinu cholesterolu, zatímco lakton je neaktivní formou, tzv. proléčivem. Laktonová forma je tedy in vivo přeměněna v játrech na aktivní hydroxy kyselinu. Jako proléčivo je podáván lovastatin a simvastatin, zatímco pravastatin a všechny synteticky odvozené statiny jsou podávány v aktivní formě. Všechny plně syntetické statiny obsahují navázaný fluorofenylový substituent. Jednotlivé druhy se od sebe liší také svou hydrofilitou. Nejvíce lipofilní sloučeniny jsou simvastatin a lovastatin, zatímco pravastatin a rosuvastatin jsou nejvíce hydrofilní. Většina statinů s výjimkou pravastatinu je z velké části vázaná na bílkoviny. Jednotlivé vlastnosti včetně hodnot log P, které vypovídají o lipofilitě daného statinu, jsou shrnuty v Tab. 1 [2][3].



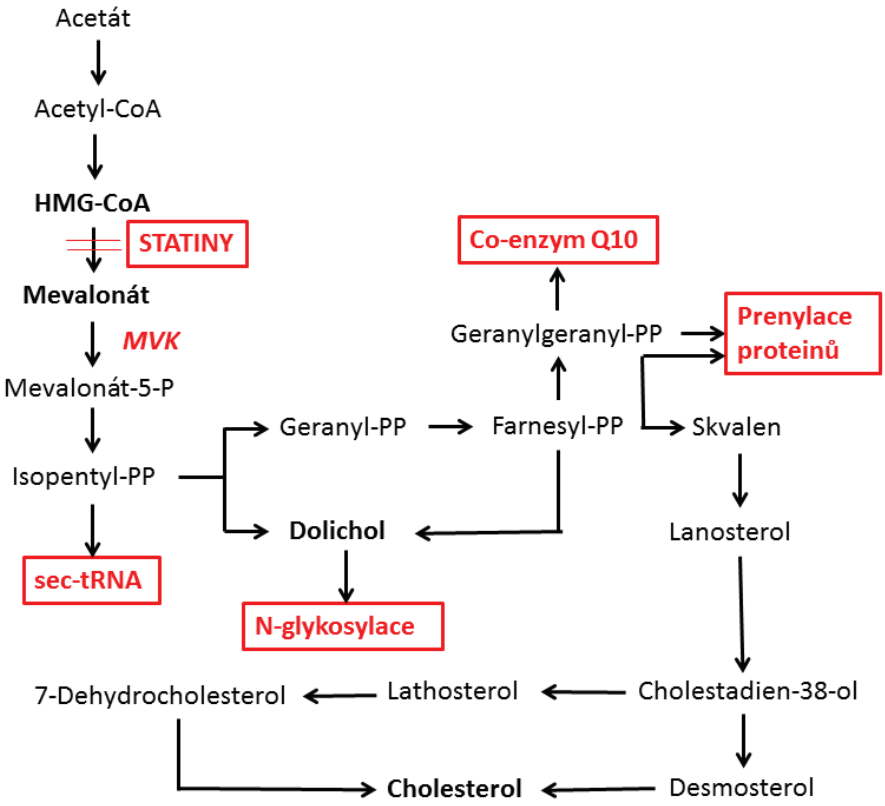
Obr. 2: Struktury syntetických statinů – (D) atorvastatin, (E) cerivastatin, (F) pitavastatin (G) rosuvastatin, (H) fluvastatin.

	původ léčiva	rozpustnost	podávaná forma	log P
atorvastatin	syntetický	lipofilní	hydroxy kyselina	3,85
fluvastatin	syntetický	slabě hydrofilní	hydroxy kyselina	4,57
rosuvastatin	syntetický	hydrofilní	hydroxy kyselina	0,89
pitavastatin	syntetický	hydrofilní	hydroxy kyselina	1,92
cerivastatin	syntetický	lipofilní	hydroxy kyselina	3,18
pravastatin	semi-syntetický	hydrofilní	hydroxy kyselina	2,21
simvastatin	semi-syntetický	lipofilní	lakton	4,72
lovastatin	přírodní	lipofilní	lakton	4,31

Tab. 1: Vlastnosti jednotlivých statinů. Převzato ze zdroje [1][3].

3.2. FARMAKOLOGICKÁ ČÁST

Statiny patří mezi nejčastěji používaná léčiva snižující hladinu cholesterolu v lidském těle, nazývaná hypolipidemika. Působí jako specifické a kompetitivní inhibitory HMG-CoA reduktázy, klíčového enzymu vnitřní syntézy cholesterolu, Obr. 3. Snižují hladinu nejen celkového cholesterolu, ale i LDL cholesterolu, hlavního rizikového faktoru vzniku aterosklerózy. Jsou používány zejména jako vysoce efektivní léčba familiární hypercholesterolémie (FH) a snižují mortalitu a morbiditu spojenou se srdečním onemocněním [1][2][3].

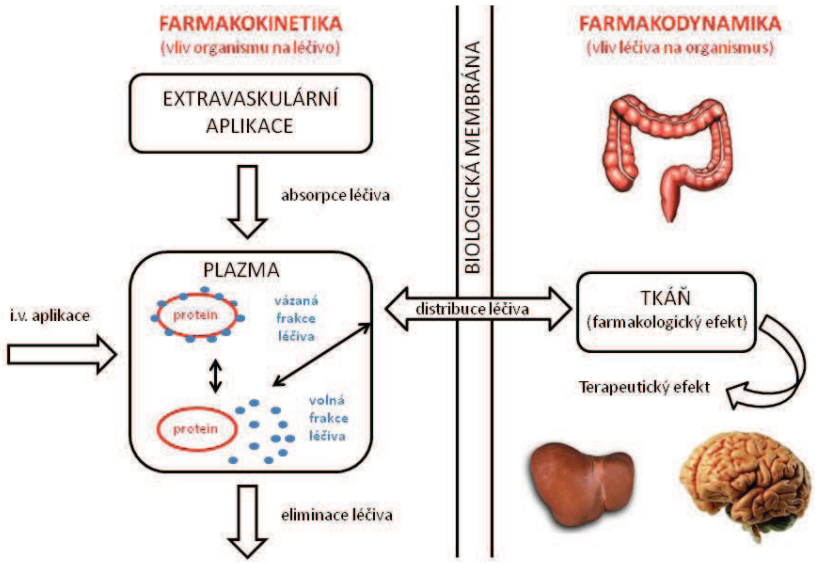


Obr. 3: Vliv inhibice HMG-CoA reduktázy na biosyntézu isoprenoidů a cholesterolu. Překresleno ze zdroje [4].

3.2.1. FARMAKOKINETIKA STATINŮ

Farmakokinetika je ovlivněna několika faktory, jako například fyzikálně-chemickými vlastnostmi léčiva (lipofilita a ionizace), fyziologickými či patologickými vlastnostmi místa aplikace a působení (prokrvení, pH) a zejména vazbou na bílkoviny plazmy. Pouze volná léčiva (nevázaná na proteiny) mohou difundovat přes kapilární stěnu k efektorovým či eliminačním orgánům. Farmakokinetika zahrnuje zejména absorpci, distribuci, eliminaci. Množství léčiva, které se dostane do systémového řečiště, určuje tzv. biologická dostupnost (BA) léčiva. Distribuce léčiva spočívá v jeho přechodu ze systémového řečiště do cílových tkání. Další oblastí farmakokinetiky je eliminace léčiva z organismu. Je charakterizováno dvěma procesy, biotransformací (metabolismem) a exkrecí. Metabolismus zvyšuje polaritu léčiva, čímž usnadňuje jejich exkreci. Mezi hlavní exkreční mechanismy patří exkrece močí, žlučí a plícemi. Biliární exkrece je uskutečněna cestou: játra → žluč → střevo → vyloučení stolicí. Tato cesta

může být přerušena štěpením glukuronidů a následnou reabsorbcí střevní sliznicí zpět do systémové cirkulace, čímž dochází k prodloužení účinku a oddálení eliminace léčiva. Eliminace léčiva z organismu je popsána zejména dvěma hlavními parametry, a to systémová clearence, tj. objem plazmy očištěný od daného léčiva za jednotku času, a eliminačním poločasem, který udává čas potřebný ke snížení koncentrace léčiva v plasmě na polovinu.



Obr. 4: Schéma interakce léčiva v organismu.

V závislosti na odlišné chemické struktuře mají jednotlivé statiny odlišnou afinitu k HMG-CoA reduktáze, což způsobuje odlišné farmakologické účinky a farmakokinetické vlastnosti. Statiny jsou po perorálním podání rychle absorbovány ve střevech s maximální koncentrací (T_{max}) po 4 hodinách. Rozsah absorpce jednotlivých statinů je značně odlišný, od 30 % do 98 %. Všechny statiny jsou podávány v aktivní formě, kromě lovastatinu a simvastatinu, které jsou podávány jako proléčiva a v játrech musí být hydrolyzovány na odpovídající aktivní hydroxy kyselinu. Pro statiny je typická neúplná biologická dostupnost. Pouze okolo 5-30 % z podané dávky se dostane do systémového řečiště, což odpovídá koncentraci řádově v ng/ml. Rozsah BA je individuální pro každý statin a je dán jejich odlišnými farmakokinetickými vlastnosti, jako jsou například vazba na složky potravy, lékové interakce, vazba na plazmatické bílkoviny a metabolismus při prvním průchodu játry (first pass efect). Velkou roli hraje také míra vaznosti molekuly léčiva na bílkoviny krevní plazmy, protože pouze volné léčivo je terapeuticky aktivní. Všechny statiny jsou z velké části vázány na plazmatické bílkoviny, více než 90 %, pouze pravastatin je na bílkoviny vázán z 50%. Distribuce statinů do tkání je dána schopností podléhat pasivní difúzi přes buněčnou membránu, což souvisí s lipofilitou jednotlivých statinů. Lovastatin a simvastatin, podávané ve formě laktonu, jsou lipofilní, a proto snadno prochází buněčnou membránou, na rozdíl od ostatních statinů podávaných ve formě hydroxy kyseliny. Pravastatin je z této skupiny léčiv nejhydrofilnější, a proto není umožněn transport do tkání pomocí pasivní difúze a musí být uskutečněn pomocí specifických transportních proteinů. S nespecifickým průchodem buněčnou membránou souvisí také hodnota IC50, tj. koncentrace statinu, při které za daných podmínek dochází k 50 % poklesu aktivity HMG-CoA reduktázy. IC50 je u hydrofilních léčiv, tedy pravastatinu také podstatně vyšší. Statiny, s výjimkou pitavastatinu, rosuvastatinu a pravastatinu, procházejících pouze částečným metabolismem prvního průchodu játry, jsou v játrech plně metabolizovány a jinak způsobují nízkou

systémovou biologickou dostupnost. Cytochrom P450 je zodpovědný za metabolizaci a eliminaci většiny léčiv a je lokalizován zejména v jaterních buňkách. Největší podíl na metabolismu statinů má isoforma CYP 3A4, která se z 30 % vyskytuje v jaterních mikrosomech a ze 70 % ve sliznici tenkého střeva. Další tři isoformy jako CYP2C8, CYP2C9 a CYP2D6 hrají také klíčovou roli v metabolismu statinů. Zatímco rosuvastatin podléhá glukuronizaci, simvastatin a lovastatin jsou metabolizovány pomocí CYP3A4, fluvastatin pomocí CYP2C9 a na metabolizaci cerivastatinu se podílejí dvě formy CYP systému, CYP3A4 a CYP2C8. Velmi unikátní je mezi statiny pravastatin, který je minimálně metabolizován CYP systémem, ale podléhá řadě metabolických reakcí, jako je izomerace, sulfonace, oxidace a konjugace s glutathionem. Podobně jako pravastatin jsou metabolizovány i rosuvastatin a pitavastatin. Laktonizace je hlavní metabolická cesta pitavastatinu v lidském těle, protože jeho laktonová forma může být zpětně neenzymaticky přeměněna na hydroxy kyselinu. Pitavastatin má také dlouhý eliminační poločas, okolo 11 hodin, pravděpodobně zapříčiněný enterohepatální cirkulací [1][2][5][6][7].
Statiny jsou vylučovány močí a žlučí na základě jejich fyzikálně-chemických vlastností. Pravastatin a cerivastatin podléhají značné renální exkreci, zatímco ostatní jsou vylučovány zejména žlučí. Podíl eliminace močí se u jednotlivých statinů značně liší. Shrnutí nejdůležitějších farmakokinetických parametrů je v Tab. 2.

	AT	CV	FV	LV	PTV	PV	RV	SV
Absorpce								
AF [%]	30	98	98	30	-	34	-	60-80
T_{max} [hod]	2-3	2,5	0,5-1	2-4	1,2	1-1,6	5	1,3-2,4
BA [%]	14	60	19-29	5	80	18-34	20	5
Distribuce								
PB [%]	98	99	98	> 95	90	50	95	95
Metabolismus								
biotransf. pomocí CYP	3A4	2C8, 3A4	2C9	3A4	min.	min.	min.	3A4
Exkrece								
$t_{1/2}$ [hod]	15-30	2-3	0,5-2,3	3	11	1,3-2,7	18-20	2-3
močí [%]	2	30	5	10	5	20	10	13
játry [%]	90	60	90	83	90	70	90	60

AF – absorbovaná frakce, BA – biologická dostupnost, PB – vazebnost na proteiny, $t_{1/2}$ - biologický poločas, CYP – cytochrom P450, min. – minimální biotransformace pomocí CYP, AT – atorvastatin, CV – cerivastatin, FV – fluvastatin, LV – lovastatin, PTV – pitavastatin, PV – pravastatin, RV – rosuvastatin, SV - simvastatin

Tab. 2: Parametry farmakokinetiky statinů [1][3][5][9].

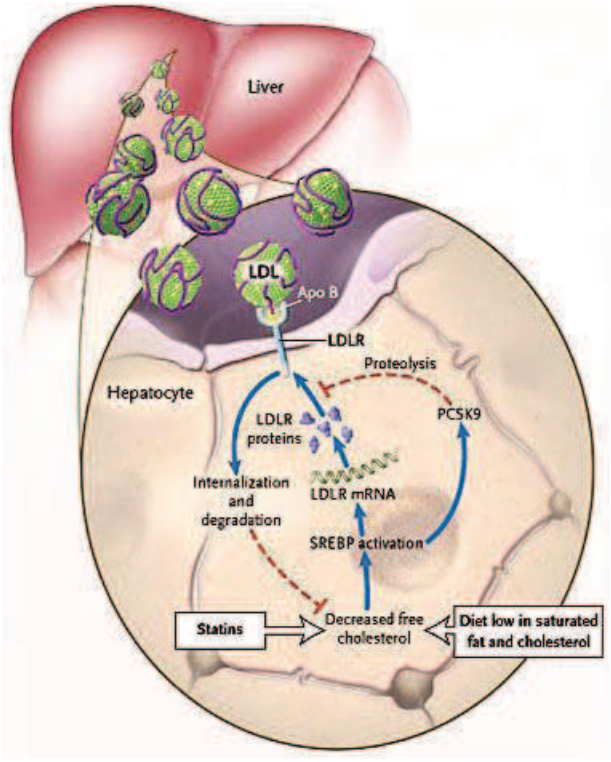
3.2.2. HYPOLIPIDEMICKÉ ÚČINKY STATINŮ

Statiny jako specifické, kompetitivní inhibitory HMG-CoA reduktázy jsou vysoce účinné ve snížení hladiny celkového a LDL cholesterolu v lidském těle, čímž snižují riziko kardiovaskulárních onemocnění a aterosklerózy. Afinita jednotlivých druhů statinů je odlišná v závislosti na jejich struktuře. Novější statiny obsahující ve své molekule HMG složku, poskytují vyšší afinitu k HMG-CoA reduktáze a tím vyšší inhibiční účinek [1][2][3].

Cílovým orgánem účinku statinů jsou zejména játra, která hrají důležitou roli v biosyntéze cholesterolu, ale i katabolismu LDL. Více než 50 % celkového cholesterolu je vnitřního původu. Statiny působí na lipidový metabolismus nejen potlačením biosyntézy cholesterolu, ale i zvýšeným vychytáváním a degradací LDL cholesterolu, potlačením lipoproteinové sekrece a endocytózy modifikovaných LDL. Na základě inhibice HMG-CoA reduktázy je blokován vznik mevalonátu v biosyntetické dráze cholesterolu, čímž dochází ke snížení hladiny cholesterolu. V důsledku snížené hladiny cholesterolu dochází ke zvýšení exprese LDL receptoru na povrchu hepatocytů, ale i ostatních buněk. To má za následek zvýšené vychytávání LDL cholesterolu, následnou degradaci a pokles intracelulárního cholesterolu, Obr. 5. Cytochrom P450 7A, specifický pro játra, přeměňuje intracelulární cholesterol na žlučové kyseliny, což vede ke snížení cholesterolu v játrech. Dávka 1 mg statinů denně sníží hladinu LDL cholesterolu o 10 %. Platí zde pravidlo „5 – 7 %“, které říká, že dvojnásobená dávka způsobí 5 - 7 % zvýšení účinnosti LDL redukce. Statiny mají velmi omezenou účinnost u homozygotních pacientů s familiární hypercholesterolémií, která je typická zkrácením nebo delecí alely pro LDL receptor, proto nemůže dojít ke zvýšení jeho exprese.

Jak bylo zmíněno výše, statiny snižují zejména koncentraci celkového a LDL cholesterolu, ovšem v menší míře inhibují i hladinu triglyceridů, což souvisí s vlivem na apoprotein B a LDL redukci. Intracelulární působení zahrnuje snížení velikosti zásob cholesterolu, které jsou začleněny do hydrofobní kapsy pomocí genu pro apolipoprotein B. Tyto intracelulární lipidové zásoby obsahují nejen cholesterol, ale i triglyceridy. Vlivem statinů je apolipoprotein B produkován v nadbytku, není stabilizován dodávkou cholesterolu, je konjugován pomocí ubiquitinu a následně konjugát podléhá destrukci v proteasómu. Účinek statinu na plasmatickou koncentraci triglyceridů souvisí také s celkovou koncentrací triglyceridů. Jestliže je menší než 2,8 mmol/l, vliv statinů je zanedbatelný. Vhodnější a efektivnější léčbou izolované hyperlipidémie je kyselina nikotinová a statiny se nejeví jako racionální léčba [3].

Statiny ovlivňují v malé míře také HDL cholesterol. Vliv statinů na zvýšení HDL cholesterolu se liší u jednotlivých statinů. Na příklad u atorvastatinu se při dávce 80 mg za den hladina HDL cholesterolu a apolipoproteinu (a) snížila, naopak u simvastatinu došlo při stejné dávce k naprosto opačnému efektu. HDL zvyšující efekt nebyl dosud plně objasněn. Byly však prokázány značné inter-individuální rozdíly v účinnosti jednotlivých statinů, které mohou být způsobeny na příklad genetickou variabilitou transportního proteinu pro estery cholesterolu (CETP) a jaterní lipázy [3].

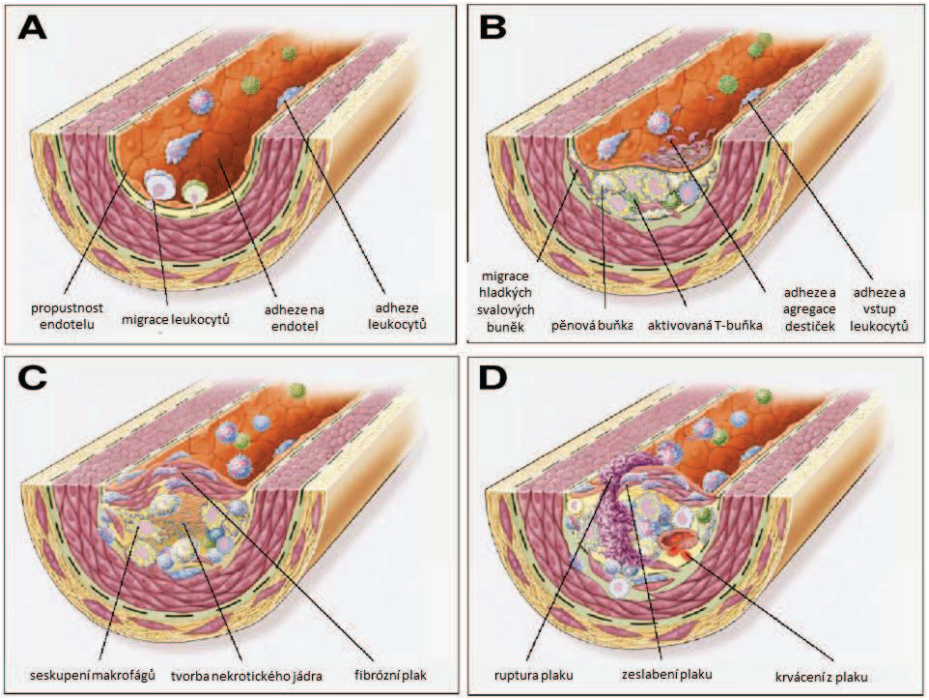


Obr. 5: Znázornění vlivu statinů na produkci LDL receptorů (LDLR). Obrázek vysvětluje vliv stravování, genetických faktorů a léčby statiny na hladinu LDL cholesterolu zejména na jeho syntézu LDL receptoru. Modré šipky na obrázku znázorňují aktivaci a červené čáry inhibici daného kroku. Snížením nitrobuněčné syntézy cholesterolu vlivem statinu dochází ke zvýšené transkripci genu pro LDL receptor. Vzniklé LDLR jsou transportovány do buněčné membrány, kde vychytávají LDL cholesterol z krevního řečiště. Pomocí endocytózy dochází k internalizaci a degradaci LDL cholesterolu. Převzato ze zdroje [8].

3.2.3. OSTATNÍ ÚČINKY STATINŮ

Přestože se statiny podílejí zejména na snížení hladiny cholesterolu, mají nepřímý vliv také na aterosklerózu a srážecí proces. Statinům jsou přičítány nejen účinky na lipidový metabolismus, ale i tzv. pleiotropní účinky, tedy nezávislé na lipidovém metabolismu. Mohou ovlivnit zejména endotelové funkce, zánětlivou reakci, krevní srážení, funkci krevních destiček, buněčnou proliferaci, imunologické odpovědi, kostní metabolismus a některé další procesy. Přestože nelze zcela rozlišit účinky na lipidech závislé a nezávislé, zvyšující se počet studií potvrzuje hypotézu, že nikoli hypolipidemické, ale i pleitropní účinky hrají hlavní roli ve stabilizaci aterosklerotického poškození. V mnoha studiích bylo demonstrováno, že vlivem účinků došlo u pacientů užívajících statiny ke snížení výskytu kardiovaskulárních, cerebrovaskulárních onemocnění, ale také ke snížení rizika Alzheimerovy choroby[1][9][10][12].

Vliv statinů na aterosklerotický proces je způsoben zvýšením stability aterosklerotického plaku a s tím souvisejícím snížením rizika trombózy. Statiny mohou tento proces modifikovat hned několika cestami. Jednak snižují citlivost LDL cholesterolu na oxidaci a snižují syntézu cholesterolu v makrofázích, což vede ke zvýšení LDL degradace a snížení tvorby pěnových buněk. Tímto vzniká stabilnější plát, který obsahuje méně lipidů. Snížení reaktivity cévní stěny vede ke zlepšení endoteliální dysfunkce, prevenci prasknutí plaku a snížení trombogenicity [12].



Obr. 6: Zobrazení procesu aterosklerózy. (A) Vlivem LDL cholesterolu a jeho oxidací dochází k poškození endotelu, následně ke zvýšení propustnosti endotelu, migraci a adhezi leukocytů a krevních destiček. (B) Monocyty a T buňky infiltrují do intimy cév a vyvolávají zánětlivou reakci. Makrofágy pohlcují LDL cholesterol a vznikají tzv. pěnové buňky a hladké svalové buňky podléhají značné proliferaci. Tímto procesem vzniká aterosklerotický plát složený zejména z lipidů. (C) Aktivací leukocytů je odstartována produkce cytokinů, chemokinů a růstových faktorů vedoucí k dalšímu poškození a tvorbě nekrotického ložiska. Proliferací hladkých svalových buněk vzniká vrstva fibrózní tkáně na povrchu plátu. (D) Tento fibrózní aterosklerotický plát je velmi náchylný prasknutí. Ruptura plaku umožní kontakt s krevními komponentami a dochází k aktivaci krevních destiček, ukládání fibrinu a ucpání lumen cévy. Převzato ze zdroje [13].

Inhibicí HMG-CoA reduktázy statiny blokují nejen syntézu cholesterolu, ale i dalších metabolitů vznikajících z mevalonátu, například **isoprenoidy**, které se podílejí na prenylaci biologicky aktivních proteinů, zejména malých GTP vázajících proteinů (G proteinů). Tyto proteiny se vyskytující v buněčné membráně a zasahují do mnoha procesů v buňkách, jako je karcinogeneze, apoptóza, angiogeneze, zánět a buněčná proliferace. Isoprenoidy jako prekursory koenzymu Q10 ovlivňují také funkci mitochondrií a jako prekursory dolicholu jsou nezbytné pro glykoproteinovou syntézu, Obr. 3. Statiny proto inhibicí syntézy isoprenoidů ovlivňují výše zmíněné funkce a dochází ke vzniku mnoha pleiotropních účinků [2][9].

Mezi pleiotropní účinky statinů jsou řazeny [9][12][14]:

- regulace endoteliální syntézy oxidu dusnatého
- obnova endoteliálních funkcí
- protizánětlivý účinek způsobený sníženou syntézou cytokinů, chemokinů, adhezivních molekul a prozánětlivých molekul jako například C reaktivního proteinu (CRP). Inhibice aktivity metaloproteináz (MMP) produkovaných makrofágy hraje důležitou roli ve stabilizaci aterosklerotického plaku
- antiagregační a antitrombotické účinky jsou způsobeny jednak ovlivněním aktivace destiček, ale také zásahem do procesu srážení a fibrinolýzu
- účinek na angiogenezi je velmi diskutován a závisí na typu orgánu, procesu onemocnění, na dávce a lokální koncentraci léčiva
- antikarcinogenní efekt je ovlivněn antiproliferačním, proapoptickým, antiinvazivním a radiosenzitivním účinkem statinů
- vliv na regulaci metabolismu β-amyloidu byl prokázán v některých studiích a vedl k inhibici vývoje Alzheimerovy choroby

	AT	CV	FV	LV	PV	SV
endoteliální funkce	-	↑	-	↑	↑	↑
anti-aterosklerotický účinek						
cholesterol v makrofázích	-	↓	-	↓	↓	↓
resistence LDL cholesterolu	-	↑	-	↑	↑	↑
proliferace hladkých svalových buněk	↓	↓	↓	↓	→	↓
antitrombotický účinek						
tkáňový faktor	-	↓	↓	-	→	↓
aktivita inhibitoru tkáňového faktoru	-	-	-	-	↓	↓
agregace destiček	-	-	-	↑	↓	↓
Fibrinogen	↑	-	-	↑	↓	→
viskozita krve	-	-	-	↑	↓	→
inhibitor aktivátoru destiček	↑	-	↑	↑	↓	↑
lipoprotein (a)	-	↑	→	↓	↑	↑

AT – atorvastatin, CV – cerivastatin, FV – fluvastatin, LV – lovastatin, PTV – pitavastatin, PV – pravastatin, RV – rosuvastatin, SV – simvastatin

Tab. 3: Pleiotropní účinky a jejich rozdíly mezi jednotlivými statiny. Převzato ze zdroje [12].

Statiny se samy o sobě vyznačují tedy nejen účinky v lipidovém metabolismu, ale také tzv. účinky pleiotropními. Účinky jednotlivých statinů nejsou shodné, Tab. 3. Rozdílná selektivita tkání byla pozorována zejména mezi lipofilními statiny (simvastatin, lovastatin) a hydrofilním pravastatinem. Zatímco lipofilní statiny mohou vstupovat do buněk pomocí pasivní difúze, pravastatin je závislý na specifickém transportním mechanismu. Specifické transportní proteiny pro pravastatin se vyskytují pouze na hepatocytech nikoli na ostatních buňkách, a proto je slabším inhibitorem syntézy mevalonátu v mimojaterních tkáních na rozdíl od lipofilních sloučenin. Tento jev byl prokázán v in vivo studiích, ovšem v klinických studiích byl účinek pravastatinu a ostatních statinů srovnatelný [12].

3.2.4. NEŽÁDOUCÍ ÚČINKY

Přestože statiny jsou vcelku bezpečná léčiva, mohou se během užívání vyskytnout některé nežádoucí účinky, které jsou prokázány u 0,1 - 5 % pacientů. Jsou popisovány nejen méně závažné zažívací problémy (zvracení, průjem, nadýmání), ale mohou také způsobit zvýšenou hladinu transamináz a myopatii, která se vyznačuje lehkou myalgií nebo dokonce až nebezpečnou rabdomyolýzou, což je hlavním symptomem limitujícím užívání statinů [1][3].

Myopatie bývá doprovázena zvýšenou koncentrací kreatin kinázy nad desetinásobek limitní hodnoty. Rabdomyolýza je velmi vážným typem myopatie, která se vyznačuje svalovým selháním. Dochází k uvolnění myoglobinu do cirkulace, což způsobí hnědé zbarvení moči a následné ledvinné selhání. Bývá diagnostikována na základě zvýšené hladiny kreatin kinázy nad 40ti násobek horní limitní hodnoty anebo akutním orgánovým selháním. Myalgie je pouze svalová bolest, při které dochází ke zvýšení koncentrace kreatin kinázy na méně než 10-ti násobek horní limitní hodnoty. Všechny statiny mohou způsobit myotoxicitu i rabdomyolýzu, ale jejich riziko se liší u jednotlivých druhů statinů. U všech ale platí vyšší riziko nežádoucích účinků při užívání vysokých dávek statinů. Vznik vedlejších účinků nesouvisí s účinností snižování LDL frakce. Například cerivastatin není mimořádně účinný, avšak v porovnání s ostatními statiny způsoboval častěji rabdomyolýzu [14].

Během terapie statiny může dojít také ke zvýšení hladiny jaterních transamináz vedoucí až k jaternímu poškození. Přestože nebyl ještě spolehlivě vysvětlen mechanismus vedlejších účinků statinů, zejména jejich myotického potenciálu, bylo popsáno několik možných principů jejich vzniku – nedostatek sekundárních metabolických intermediátů, navození buněčné apoptické smrti a změna průchodnosti chloridových kanálů v myocytech.

Vysoké dávky statinu vyvolávají poruchu metabolické cesty cholesterol – žlučové kyseliny, což má za následek vznik akutní fáze chemické hepatitidy. Protože statiny snižují hladinu oxidovaného cholesterolu a cholesterolu jako substrátu pro hydrolýzu, mohou ovlivnit i jiné metabolické cesty a to s pozitivními i negativními výsledky. Cholesterol je syntetizován nejen v jaterní tkáni, ale i v ostatních tkáních lidského těla. Je velmi důležitý pro normální buněčnou funkci a pro biosyntézu steroidních hormonů. Působení na mimojaterní tkáně potlačuje jejich buněčnou funkci, což vede k nežádoucím účinkům s minimálním farmakologickým účinkem. Ve svalových buňkách dochází inhibicí biosyntézy cholesterolu ke snížení jeho obsahu v plasmatické membráně, což způsobí její nestabilitu a následné poškození buněk.

Vlivem inhibice HMG-CoA reduktázy dochází k redukci biosyntézy intermediátního metabolitu ubiquitinu (koenzymu Q10), farnesyl pyrofosfátu. Koenzym Q10 jako steroidní isoprenoid hraje klíčovou roli ve tvorbě buněčné energie v mitochondriálním transportním systému. Některé klinické studie prokázaly, že snížená hladina koenzymu Q10 může být tedy jednou z příčin vzniku myotoxicity, protože více než 50% koenzymu Q10 pochází z jeho biosyntézy [15].

Dalším vlivem inhibice HMG-CoA je snížení syntézy farnesyl pyrofosfátu a geranylgeranyl pyrofosfátu vedoucí k redukci prenylace GTP-vázající protein, což může mít za následek snadnější apoptózu hladkých svalových buněk [15]. Tento děj je typický zejména pro atorvastatin, lovastatin a simvastatin. Statiny ovlivňují také průchodnost chloridových kanálů, které jsou zodpovědné za hyperpolarizaci svalových buněk a tím jejich relaxaci. Mechanismus tohoto vlivu statinů nebyl zcela objasněn. Vliv změny poměru mezi cholesterolem a fosfolipidy vede ke změně ve vlastnostech membrány, zejména v jejich propustnosti, což umožní např.

simvastatinu proniknout membránou a způsobit blokádu kalciových kanálu, následně svalovou kontrakci, křeč a bolest [5]. Bylo popsáno také několik rizikových faktorů spojených se vznikem myotoxicity jako nežádoucího účinku léčby statiny. Jedná se zejména o vyšší věk, nadměrné cvičení, multi-systémová onemocnění jako ledvinná a jaterní nedostatečnost, diabetes, či snížená funkce štítné žlázy a osobní a rodinná anamnéza [16].

3.2.5. STATINY A JEJICH POUŽÍVANÍ V KLINICKÉ PRAXI

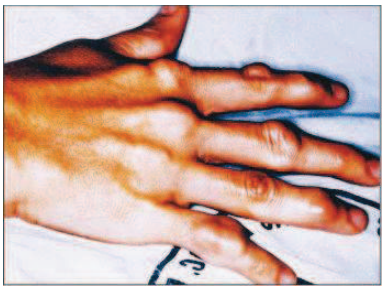
Statiny jsou nejvíce používané léky snižující hladinu cholesterolu. V roce 1987 byl pro klinické použití schválen první statin, lovastatin. Od této doby se studiem účinku statinů zabývalo velké množství vysoce kvalitních randomizovaných studií, které prokázaly vliv na snížení rizika kardiovaskulárních onemocnění (infarkt myokardu, mozková mrtvice, atd.). V současné době je pro klinickou praxi dostupných šest statinů, lovastatin (1987, nedostupný v USA), simvastatin (1988), pravastatin (1991), fluvastatin (1994), atorvastatin (1997), rosuvastatin (2003) a pitavastatin (2003, dostupný pouze v USA, Japonsku a Indii). Cerivastatin, schválený roku 1998, byl kvůli vysokému výskytu nežádoucích účinků, zejména rabdomyolýzy, zakázán v klinické praxi [14]. Lovastatin a simvastatin se vyznačují zejména tím, že se jedná o lipofilní léčiva snadno prostupná plasmatickými membránami, a proto mohou být velmi snadno absorbována ze střeva a zároveň snadno prochází například hematoencefalickou bariérou. Jsou metabolizovány ve střevě, a proto je jejich exkrece velmi vysoká. Většina statinů se vyznačuje velmi nízkou biologickou dostupností, < 5 % pro simvastatin a 5 % pro lovastatin. V České republice jsou dosud nejčastěji používané atorvastatin, simvastatin a rosuvastatin.

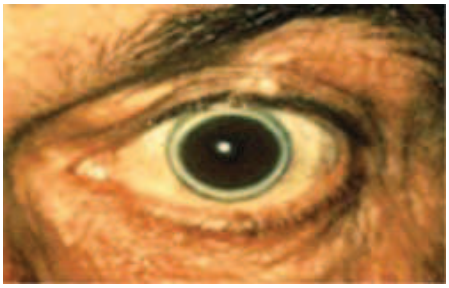
Rosuvastatin v poslední době nahrazuje dříve velmi široce používaný simvastatin. Jako jediný obsahuje polarizovanou methyl sulfonovou skupinu, pomocí které poskytuje více vazebných interakcí s enzymem HMG-CoA reduktázou, a proto má vyšší inhibiční schopnost. Tato hydrofilní skupina také způsobuje nižší pasivní difúzi přes buněčnou membránu a selektivní vychytávání v játrech. Vlivem minimální aktivity hydrofilních statinů v mimojaterní tkáni dochází ke snížení nežádoucích účinků, jako je myalgie a rabdomyolýza. Díky rychlému vstřebávání a dlouhodobé eliminaci rosuvastatinu (19h) je jeho farmakokinetika nezávislá na době podání. Rosuvastatin je minimálně metabolizován pomocí systému cytochromu P-450 (CYP), což snižuje riziko interakce s dalšími léčivy.

Pitavastatin je léčivo, které bylo dosud registrováno pouze v Japonsku, Indii a USA. V ČR je stále ještě ve fázi testování. Představuje nejvhodnější statin s ohledem na jeho farmakokinetiku. Jedná se o léčivo s minimálním metabolismem v játrech a nízkou pravděpodobností lékových interakcí prostřednictvím CYP3A4, a proto se zařazuje do stejné skupiny jako pravastatin, rosuvastatin a fluvastatin. Ovšem na rozdíl od těchto léčiv je pitavastatin lipofilnější a zároveň se vyznačuje vyšší biologickou dostupností. Dlouhý biologický poločas umožňuje variabilitu v časovém užívání léčiva. Statiny s krátkým biologickým poločasem musí být vždy striktně užívány ve večerních hodinách, protože endogenní syntéza cholesterolu je nejintenzivnější během noci.

3.2.6. FAMILIÁRNÍ HYPERCHOLESTEROLÉMIE

Jedná se o onemocnění s autosomálně dominantním typem dědičnosti. Nastává v důsledku mutace genu kódujícího LDL receptor, který hraje velmi důležitou roli v udržení homeostázy cholesterolu v lidském těle. Mutace tohoto genu způsobuje vznik defektů ve vysoce afinitním LDL receptoru, což zabraňuje vstupu LDL cholesterolu do buněk a dochází ke zvýšení koncentrace LDL cholesterolu v krvi. Na druhou stranu v mimojaterní tkáni dochází ke zvýšené syntéze endogenního cholesterolu, protože zde odpadá tlumivý vliv HMG-CoA reduktázy v důsledku sníženého příjmu LDL cholesterolu do buněk. Charakteristickými rysy FH je tedy zvýšená hladina celkového a LDL cholesterolu v krvi a předčasná manifestace ischemické choroby srdeční. Klinicky je onemocnění představováno xantolesmy víček, šlachovými xantomy a šedavým prstencem na okraji duhovky (arcus lipoides corneae), Obr. 7 [17][18][19].





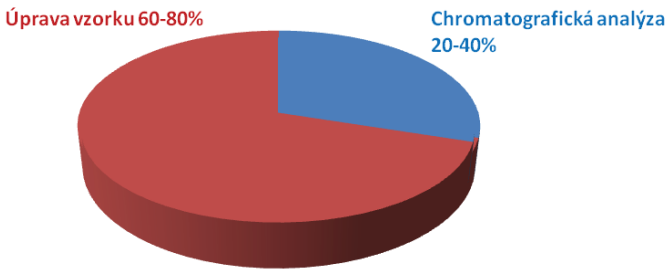
Obr. 7: Příznaky Familiární hypercholesterolémie – xantomy, kruhový lipidový prstenec rohovky. Převzato ze zdroje [20][21].

Toto onemocnění se může vyskytovat ve dvou formách: vzácná homozygotní s četností 1 z milionu, nebo heterozygotní, která je častější s četností 1 z 500. Homozygoti této formy onemocnění jsou těžce postiženi už od dětského věku a zpravidla u nich dochází k srdečním infarktům již v mládí. Heterozygoti často nemusejí mít dlouhá léta žádné klinické projevy a příznaky se zpravidla se objevují ve čtvrté dekádě života u mužů a o deset let později u žen. Zatímco u heterozygotů se setkáváme pouze se sníženým počtem LDL receptorů a zvýšením celkového cholesterolu 2krát, u homozygotů LDL receptory zcela chybí a koncentrace cholesterolu je zvýšena 6krát. Základní terapii hyperlipoproteinémie tvoří dieta s důrazem na vyloučení živočišných tuků. V případě, že změnou životního stylu nedochází k dostatečnému poklesu koncentrace cholesterolu v krvi, přistupuje se k farmakologické léčbě. Jednou z nich je právě užívání statinů, další možností jsou pryskyřice nebo ezetimib. V případě homozygotní formy FH se přistupuje k odstraňování LDL cholesterolu z plazmy plazmaferézou neboli extrakorporální eliminační procedurou, jako je LDL-aferéza či hemorheoferéza [17][18].

3.3. ANALYTICKÁ ČÁST

3.3.1. POUŽITÉ INSTRUMENTÁLNÍ TECHNIKY

Pro analýzu statinů bylo využito nejrozšířenější separační techniky a to kapalinové chromatografie. Protože dané látky byly stanovovány v biologickém materiálu, ve kterém se vyskytují ve velmi nízkých koncentracích, řádově ng/ml, bylo využito citlivé a selektivní detekční techniky, hmotnostní spektrometrie. Nedílnou součástí analýzy biologického materiálu je využití techniky úpravy vzorků před chromatografickou analýzou. Přestože v oblasti kapalinové chromatografie došlo v poslední době k velkému rozvoji ve smyslu urychlení analýzy, úprava vzorků je stále nejproblematičtější a časově nejnáročnější částí analýzy, tvoří až 80% celkového času analýzy.



Obr. 8: Grafické znázornění časové náročnosti úpravy vzorku před analýzou [22].

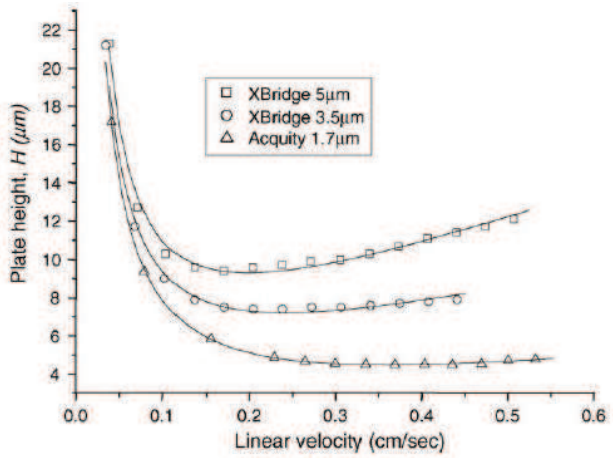
3.3.1.1. ULTRA VYSOKOÚČINNÁ KAPALINOVÁ CHROMATOGRAFIE

V současnosti je hlavním trendem kapalinové chromatografie urychlení separace spolu se zachováním nebo zlepšením účinnosti, rozlišení a tvaru píků. Z důvodů se směr vývoje uchyluje k tzv. rychlé chromatografii, „fast LC“, do které jsou řazeny celkem čtyři přístupy: ultra vysokoúčinná kapalinová chromatografie (UHPLC), využití monolitních kolon, vysokoúčinná kapalinová chromatografie při zvýšené teplotě a povrchově porézní částice. Další část se bude již zabývat pouze UHPLC, která byla využita k analýze studovaných léčiv.

První konvenční UHPLC chromatograf byl představen v roce 2004 společností Waters a v průběhu dalších let byl následován mnoha dalšími výrobci LC systémů. UHPLC systém využívá pro zvýšení účinnosti částice menší než 2 µm neboli částice „sub-2-microne“. Účinnost chromatografického systému je hodnocena na základě výškového ekvivalentu teoretického patra (H). Vztah mezi účinností chromatografické separace, tedy výškovým ekvivalentem teoretického patra a lineární průtokovou rychlostí popisuje van Deemterova rovnice (1) a graficky tuto závislost znázorňuje van Deemterova křivka na Obr. 9 [23].

$$H = A + \frac{B}{u} + C\,u \qquad (1)$$

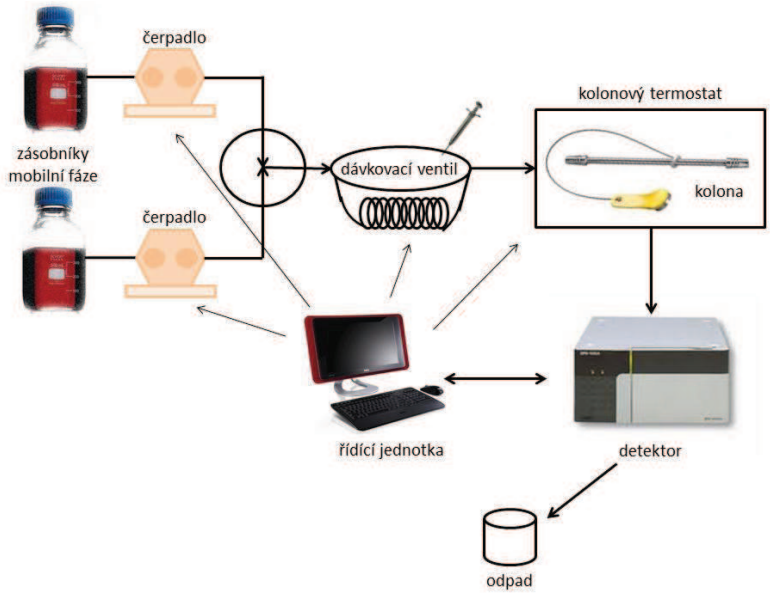
u - lineární průtoková rychlost mobilní fáze
A - příspěvek vířivé difúze analytu v mobilní fázi při průchodu náplní kolony (Eddyho difúze)
B - příspěvek molekulové (podélné) difúze v mobilní fázi
C - příspěvek odporu proti převodu hmoty



Obr. 9: Znázornění průběhu van Deemterových křivek pro částice 1,7 µm, 3,5 µm a 5 µm. Obrázek byl získán ze zdroje [24].

Na Obr. 9 jsou znázorněny křivky odpovídající různým průměrům částic. Je patrné, že se snižující se velikostí částic dochází ke snižování H a tím ke zvyšování účinnosti. Částice s průměrem 1,7 µm umožňují využít vyšší lineární průtokové rychlosti bez ztráty účinnosti. Využití takto malých částic způsobí vyšší zpětný tlak v systému, který bude odpovídat druhé mocnině velikosti částic. Proto je nutné, aby UHPLC systém umožňoval práci za ultra vysokých tlaků až do 120 MPa, zatímco běžná vysokoúčinná kapalinová chromatografie (HPLC)) umožňuje práci pouze do 40 MPa [23].

UHPLC SYSTÉM

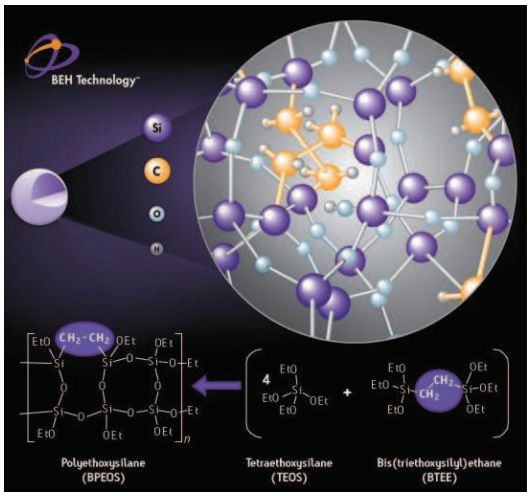


Obr. 10: Zjednodušené schéma uspořádání UHPLC.

Kapalinový chromatograf pracující za ultravysokého tlaku umožňuje kolon s menším vnitřním průměrem (např. 2,1 mm) společně s částicemi menšími než 2 µm. UHPLC systém se skládá ze stejných součástí jako konvenční HPLC systém. Aby byl chromatograf schopný odolávat velmi vysokým tlakům, musí splňovat některé specifické požadavky. Jedná se zejména o požadavky

na hardware. Důležité jsou nejen robustní pumpa, ventily a dávkovací systém, ale také speciální tlaku odolné stacionární fáze a minimalizace veškerých mimokolonových objemů, zejména použití nízkoobjemové detekční cely a kapilár s velmi malým průměrem.

S rozvojem UHPLC musel být spojen také vývoj nových mechanicky a chemicky odolných stacionárních fází. Nové stacionární fáze se vyvíjí třemi hlavními směry, kterými jsou odolnost vůči vysokým tlakům, odolnost v širokém rozsahu pH a vznik nových modifikací pro zvýšení selektivity separace. Částicemi stacionární fáze o velikosti 1,7-2 µm jsou naplněny UHPLC kolony s vnitřním průměrem 1,0 nebo 2,1 mm. Malý průřez kolony minimalizuje teplotní gradient vzniklý při zahřívání mobilní fáze v důsledku vysokých tlaků. Na počátku vzniku UHPLC systému byl velmi omezený výběr stacionárních fází. V době vzniku prvního UHPLC chromatografu firma Waters představila zcela novou technologii hybridních stacionárních fází pro UHPLC, tzv. BEH (bridged-ethyl-hybrid), Obr. 11. V průběhu času se začalo vývojem zabývat mnoho dalších firem, Tab. 4. Firma Waters uvedla na trh další generaci UHPLC stacionárních fází, tzv. Acquity CSH, která patří také do skupiny hybridních stacionárních fází. Přehled dostupných stacionárních fází je uveden v Tab. 4.



Obr. 11: Struktura hybridní stacionární fáze s využitím BEH technologie. Převzato ze zdroje [25].

Výhodami UHPLC přístupu jsou zkrácení doby analýzy, vysoká účinnost, nižší spotřeba rozpouštědel, dostupnost širokého spektra mechanicky a chemicky stabilních stacionárních fází. Nevýhodou je nutnost speciální instrumentace a stacionárních fází, také nižší dávkovací kapacita a větší vliv tepla vznikající třením při zvýšeném průtoku [26][22].

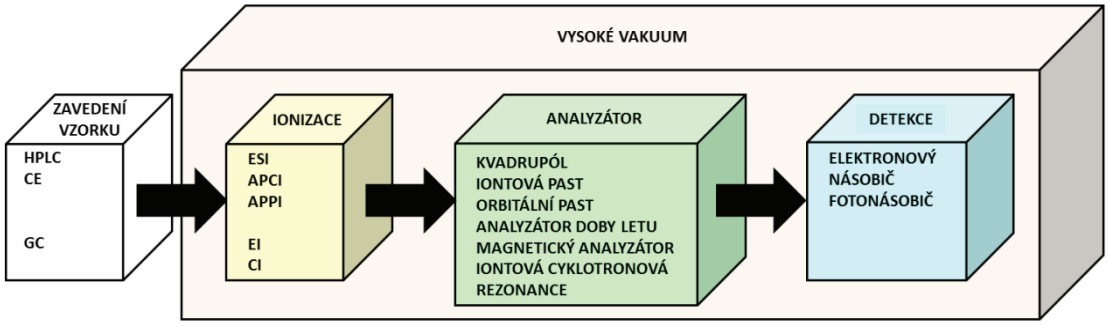
NÁZEV KOLONY	ZÁKLAD STAC. FÁZE	VELIKOST ČÁSTIC (µm)	MODIFIKACE STACIONÁRNÍCH FÁZÍ	LIMITY pH	t (°C)	VÝROBCE
Acquity BEH	hybridní	1,7	C8, C18, fenyl shield RP18 BEH amid, glykan	1-12 2-11 1-8 2-11	20-90	Waters
Acquity HSS	silikagel	1,8	T3, C18 SB, C18	2-8	20-45	Waters
Acquity CSH	hybridní	1,7	C18, fenyl-hexyl fluorofenyl	1-11 1-8	<80 <60	Waters
Alltima HP	hybridní	1,5	HILIC	1-10	20-60	Alltech
Platinum	silikagel	1,5	C18, C8	2-8	20-60	Alltech
GP Series	silikagel	1,8	C18, C8, C4	2-8,5	20-60	Sepax
HP Series	silikagel	1,8	fenyl, CN, NH2, SCX, SAX, silikagel, HILIC	2-8,5	20-60	Sepax
Poly RP	hybridní	1,0/ 1,7	fenyl	1-14		Sepax
HypersilGold	silikagel	1,9	C18 C8, Q pentafluorfenyl	1-11 2-9 2-8	25-60	Thermo-Electron
Nucleodur	silikagel	1,8	C8, C18 C18 isis, Sphinx RP C18 pyramid	1-11 2-9 2-8	<85	Machery Nagel
Pathfinder	hybridní	1,5	AS, AP, PS, MR	1-12	<250	Shimadzu
Pinnacle DB	silikagel	1,9	C18, PFP-propyl, silikagel, acq-C18, CN, C8, PAH, X3-C18	2,5-7,5	<80	Restek
Pronto Pearl		1,8	C18, C8, aminopropyl	2-8	20-60	Bischof
		1,5	C18			
TSKgel Super ODS	silikagel	2,0	C18, C8, PHE	2-7,5	20-60	Tosoh
YMC ultra-fast	silikagel	2,0	C18, Hydro C18	2-8	20-60	YMC
Zorbax	silikagel	1,8	Eclipse plus C8, C18, Eclipse XDB-C18, C8, fenyl Eclipse fenyl-hexyl, PAH, Eclipse XDB-CN Extend-C18 StableBond	2-9 2-8 2-11 1-6	<60 80-100	Agilent

Tab. 4: Přehled dostupných UHPLC stacionárních fází s částicemi menšími než 2 µm. Převzato ze zdroje [22].

3.3.1.2. DETEKCE S VYUŽITÍM HMOTNOSTNÍ SPEKTROMETRIE

Hmotnostní spektrometr patří do skupiny destruktivních detektorů, ale může být také využit jako samostatná analytická technika sloužící k identifikaci neznámých látek. Ve spojení s kapalinovou chromatografií se využívá zejména pro bioanalytické aplikace, dále v potravinářském odvětví, proteomice, metabolomice a mnoha dalších aplikacích. Ještě do nedávné doby se jednalo o detektor, který byl využíván zejména pro výzkumné účely, nikoli pro rutinní analýzy v klinických laboratořích. V posledních letech i v této oblasti došlo ke značnému rozšíření systémů LC-MS a to hlavně pro monitorování lékových hladin, identifikaci návykových látek. Své využití našel díky vysoké citlivosti a selektivitě. Poskytuje nejen údaje z chromatogramu, ale také spektra jednotlivých látek. Hmotnostní spektrometr může být použitý jak pro kvalitativní, tak i kvantitativní analýzy. Poskytuje informace o čistotě vzorku, molekulové hmotnosti, struktuře, identitě a množství jednotlivých analytů.

Proces detekce v hmotnostním spektrometru probíhá ve třech základních krocích. Nejprve dojde k ionizaci analytů, neutrální molekuly jsou v iontovém zdroji převedeny na nabité částice. Následuje rozdělení jednotlivých iontů v plynném stavu na základě poměru molekulové hmotnosti a náboje (m/z) v analyzátoru za vysokého vakua. Nakonec proběhne detekce iontů a zesílení signálu. Hmotnostní spektrometr je řízen sofistikovaným softwarem, který zprostředkovává sběr dat a slouží k vyhodnocování výsledků. Kromě těchto základních součástí se každý hmotnostní spektrometr skládá ještě z vakuové pumpy, zařízení pro dávkování vzorku, počítače a iontové optiky k urychlení a fokusaci iontů. Schematické znázornění hmotnostního spektrometru a nejpoužívanějších typů ionizace, analyzátorů a detekce je uvedeno na Obr. 12 [27][28].

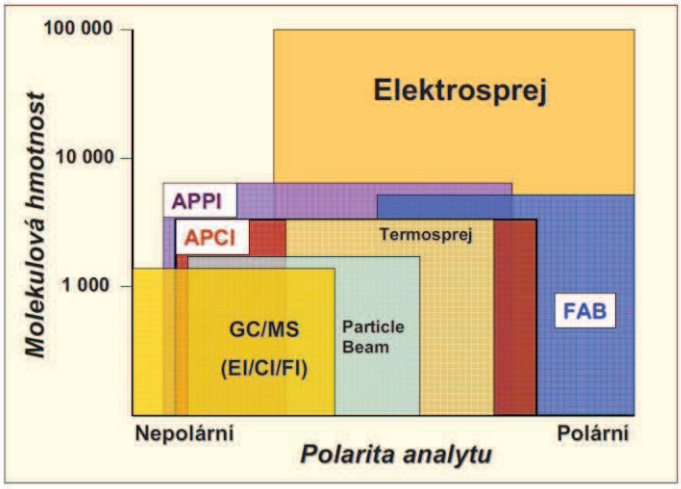


Obr. 12: Schematické znázornění jednotlivých ionizačních technik, základních typů analyzátorů a způsobů detekce.

3.3.1.2.1. IONIZAČNÍ TECHNIKY

Ionizace je prvním a klíčovým krokem MS analýzy. Hlavní funkcí je převedení neutrálních molekul analytů na nabité částice. Existuje mnoho druhů ionizačních technik více, či méně specifických pro určitý typ látek, nicméně neexistuje žádná univerzální ionizační technika. Výběr ionizační techniky se řídí vlastnostmi stanovovaného analytu, zejména jeho těkavostí, molekulovou hmotností, polaritou a tepelnou stabilitou. Ionizační techniky se rozdělují na dvě základní skupiny, na tvrdé a měkké ionizační techniky. Ve spojení s kapalinovou chromatografií se využívají techniky měkké ionizace, při kterých dochází ke vzniku protonovaných molekul $[M+H]^+$ v pozitivním záznamu iontů a deprotonovaných molekul $[M-H]^-$ v negativním záznamu iontů. Na rozdíl od tvrdých ionizačních technik, při kterých dochází k rozsáhlé fragmentaci

vlivem velmi vysoké energie, při ionizaci měkkými ionizačními technikami nedochází k významné fragmentaci molekul analytů. Mezi nejčastěji používané techniky ve spojení s HPLC patří ionizace za atmosférického tlaku (API), mezi které se řadí ionizace elektrosprejem (ESI), chemická ionizace za atmosférického tlaku (APCI), fotoionizace za atmosférického tlaku (APPI) a ionizace laserem za účasti matrice (MALDI). Ve spojení s plynovou chromatografií (GC) se používá zejména elektronová ionizace (EI), která patří mezi tvrdé ionizační techniky. V důsledku elektrického pole dochází k uvolnění nebo přijmutí valenčního elektronu, jehož důsledkem dochází ke vzniku radikál kationtu $M^{+\bullet}$ nebo radikál aniontu $M^{-\bullet}$. Pro EI jako jedinou ionizační techniku existují knihovny spekter. Mezi nejšetrnější techniky patří ESI a MALDI, která je využívána zejména pro analýzu velkých molekul jako jsou proteiny a biomolekuly [27][29].



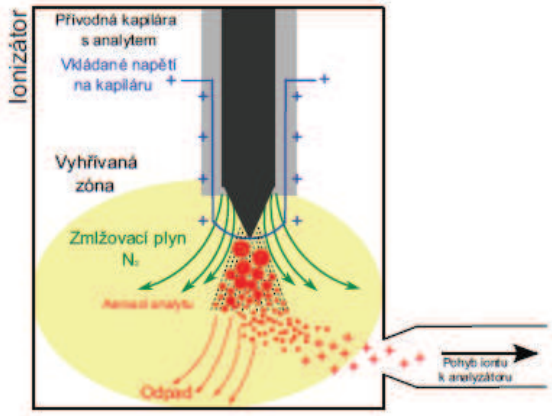
Obr. 13: Aplikace různých ionizačních technik v závislosti na molekulové hmotnosti a polaritě analytu. Zatímco elektronová ionizace (EI), chemická ionizace (CI) a ionizace polem (FI) jsou používány převážně pro aplikace GC-MS, s LC-MS technikou jsou spojeny ionizace elektrosprejem (ESI), chemická ionizace za atmosférického tlaku (APCI) a fotoionizace za atmosférického tlaku (APPI). Z diagramu je patrné, že elektrosprej je nejuniverzálnější ionizační technikou [30].

IONIZACE ELEKTROSPREJEM

ESI je využívána především pro analýzu velkých biomolekul, jako jsou proteiny, peptidy a nukleotidy, ale také k analýze středně a silně polárních malých molekul. Jedná se o nejčastěji používanou ionizační techniku ve spojení s LC.

Eluát vycházející z LC je pomocí vyhřívané kovové kapiláry přiváděn do iontového zdroje. V přítomnosti silného elektrického pole na kapiláře dochází k disociaci molekul analytu a vzniku nabitých kapiček. Pomocí sušícího plynu dochází k vypařování rozpouštědla z kapiček a tím k zakoncentrování náboje na jejich povrchu. Jakmile hustota povrchového náboje dosáhne kritické hodnoty, dojde k tzv. coulombické explozi. Jedná se o rozpad nabité kapičky na mnoho ještě menších kapiček nesoucích náboj, Obr. 14. Tento proces se opakuje až do vzniku dostatečně malé kapičky, aby z jejího povrchu mohla být uvolněná deprotonovaná nebo protonovaná molekula. Vzniklá nabitá molekula je pomocí iontové optiky fokusována do hmotnostního analyzátoru. Stupeň fragmentace lze ovlivnit hodnotou napětí vloženého na vypuzovací elektrodu. Zvýšením tohoto napětí se podpoří kolize neutrálních molekul a

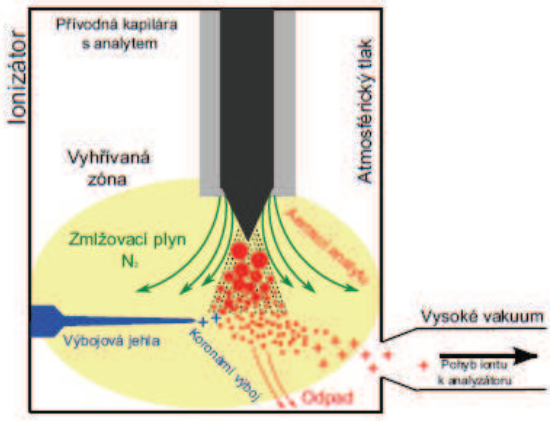
molekulárních iontů ve zdroji a dojde k jejich fragmentaci. Tento proces se nazývá kolizně indukovaná disociace (CID) v iontovém zdroji (in source CID) [27][28].



Obr. 14: Schéma ionizace elektrosprejem. Převzato ze zdroje [32] .

CHEMICKÁ IONIZACE ZA ATMOSFÉRICKÉHO TLAKU

Tato ionizační technika se používá ve spojení s kapalinovou chromatografií k analýze malých nepolárních a polárních sloučenin. Není vhodná pro analýzu málo těkavých analytů, protože před vlastní ionizací musí být molekuly analytu převedeny do plynné fáze, proto se nehodí např. pro biopolymery.



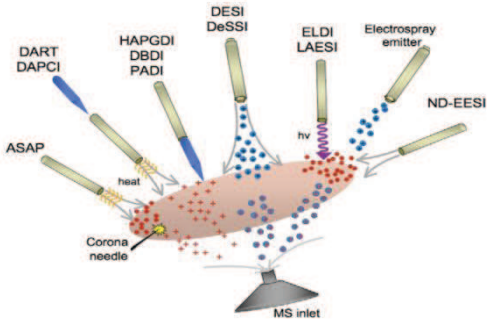
Obr. 15: Schéma chemické ionizace za atmosférického tlaku. Převzato ze zdroje [32].

Princip je stejný jako u chemické ionizace, ale je uskutečněna za atmosferického tlaku. Do iontového zdroje je přiváděn eluát z LC. Na výbojovou elektrodu umístěnou v iontovém zdroji je vloženo napětí 3-4 kV, čímž vzniká tzv. koronární výboj. Pomocí tohoto výboje jsou ionizovány nejprve molekuly mobilní fáze a následně pomocí ion-molekulárních interakcí dochází k ionizaci analytu. Vzniklé ionty jsou urychleny elektrodami k analyzátoru. Protiproud sušícího plynu (dusík) slouží k odpaření, vysušení a rozbití případných nekovalentních klastrů a asociátů [27][28].

Všechny výše uvedené ionizační techniky se řadí mezi konvenční a před jejich použitím je nutná úprava vzorku, která je v mnohých případech složitá a časově velmi náročná. Trend ionizačních

technik se vyvíjí směrem přímé analýzy reálného vzorku bez použití jakékoli jeho úpravy. Byly zavedeny tzv. ambientní desorpční ionizační techniky, které umožňují přímou analýzu objektu v otevřené atmosféře laboratoře nebo přímo v místě jeho výskytu. Jako první byly zavedeny techniky: desorpční ionizace elektrosprejem (DESI) v roce 2004 a přímá analýza v reálném čase (DART) v roce 2005. Kombinací různých desorpčních kroků a ionizačních technik, jako ESI, APCI a APPI došlo v posledních letech ke vzniku mnoha dalších technik založených na přímé analýze reálných vzorků. Seznam jednotlivých technik je vyobrazen na Obr. 16 [31].

Technika	zkratka
Desorption Electrospray Ionization	DESI
Surface Sampling Probe	SSP
Direct Analysis in Real Time	DART
Ambient Solid Analysis Probe	ASAP
Electrospray Laser Desorption/ Ionization	ELDI
Fused Droplet Electrospray Ionization	FD-ESI
Desorption Atmospheric Pressure Chemical Ionization	DAPCI
MALDI Assisted Electrospray Ionization	MALDESI
Extractive Electrospray Ionization	EESI
Desorption Sonic Spray Ionization	DeSSI
Plasma-Assisted Desorption/ Ionization	PADI
Dielectric Discharge Barrier Ionization	DBDI
Helium Atmospheric Pressure Glow Discharge Ionization	HAPGDI
Neutral Desorption Extractive Electrospray Ionization	ND-EESI
Laser Ablation Electrospray Ionization	LAESI
Atmospheric Pressure Thermal Desorption Ionization	APTDI
Desorption Atmospheric Pressure Photo Ionization	DAPPI



Obr. 16: Seznam jednotlivých technik přímé analýzy reálného vzorku. Převzato ze zdroje [31].

3.3.1.2.2. ANALYZÁTORY

Hmotnostní analyzátor je srdcem hmotnostního spektrometru. Je umístěn za iontovým zdrojem a před detektorem, Obr. 12. Analyzátor slouží k rozdělení jednotlivých iontů podle m/z a k urychlení a fokusaci do detektoru.

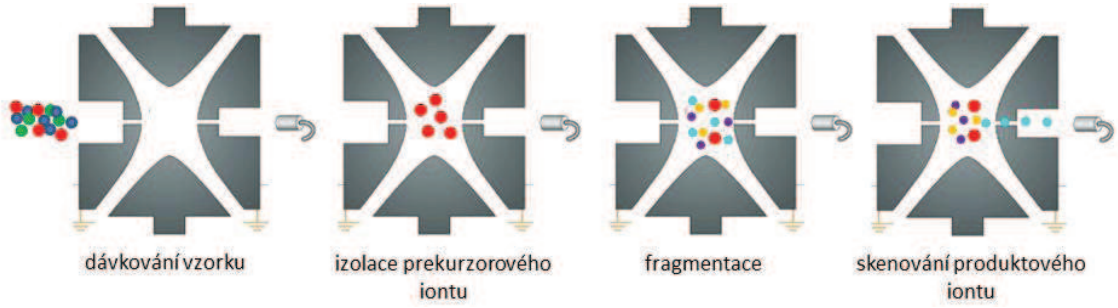
Separace jednotlivých iontů podle m/z lze dosáhnout na základě různých fyzikálních principů: (a) zakřivení dráhy letu iontů v elektrickém a magnetickém poli, např. magnetický analyzátor (B), (b) různá stabilita oscilací iontů v dvoj- nebo trojrozměrné kombinaci stejnosměrného a vysokofrekvenčního střídavého proudu, např. kvadrupól (Q), trojitý kvadrupól (QqQ) a iontová past (IT), (c) různá doba rychlosti letu iontů, např. analyzátor doby letu (TOF), (d) různá absorpce energie při cykloidálním pohybu iontů v kombinovaném magnetickém a elektrickém poli, iontová cyklotronová rezonance (ICR). Hmotnostní analyzátory jsou hodnoceny na základě následujících parametrů: hmotnostní rozsah, rozlišení, účinnost, správnost hmoty, lineární dynamický rozsah, rychlost a citlivost.

V současné době se trendy v oblasti hmotnostních analyzátorů vyvíjí dvěma hlavními směry, zvýšení rychlosti a zvýšení citlivosti. Došlo k rozvoji zejména v oblasti tandemové hmotnostní spektrometrie, analyzátorů s vysokým rozlišením (HRMS) a hybridních analyzátorů.

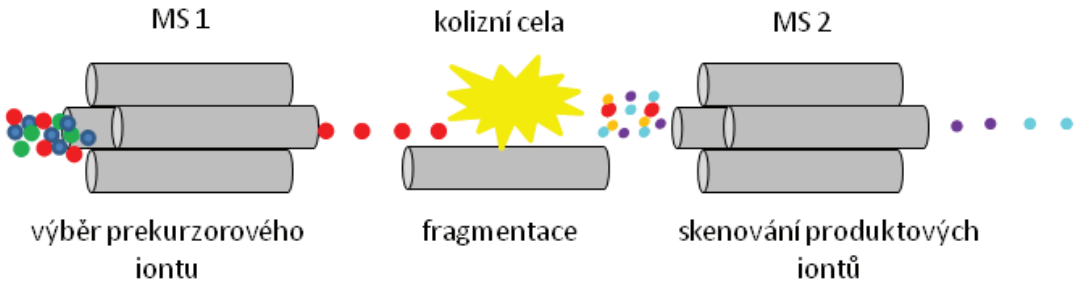
S ohledem na rozlišovací schopnost jednotlivých analyzátorů je lze rozdělit na analyzátory s nízkým rozlišením (Q, IT a lineární iontová past (LIT) a analyzátory s vysokým rozlišením a vysokou správností m/z (B, TOF, orbitální past, ICR). Zatímco na počátku vývoje HRMS byly využívány tyto analyzátory zejména k identifikaci neznámých látek a k určování správné hmoty,

v současnosti se stále více uplatňují i na poli kvantitativní analýzy. Jejich hlavní výhodou je možnost stanovení analytů, které jsou identifikovány na základě správné hmoty a zároveň kvantifikovány. Nevýhodou HRMS je jejich relativně nízká skenovací rychlost, omezený lineární dynamický rozsah a nižší limity detekce v porovnání s QqQ, který je zatím nejpoužívanější analyzátorem na poli kvantitativní bio-analýzy. V oblasti bio-analýzy se vytvořily dvě skupiny vědců. První, která preferuje kvantitativní analýzu pomocí trojitého kvadrupólu a druhá preferující HRMS. V této fázi vývoje hmotnostních analyzátorů má QqQ stále ještě nezastupitelnou roli zejména v oblasti analýzy cílených analytů, zatímco na poli necílené analýzy HRMS svými výhodami zcela předčila možnosti QqQ [33].

Při měkké ionizační technice nedochází k dostatečné fragmentaci analytů, a proto není možné získat dostatečné informace pro identifikaci a kvantifikaci analytů složitých směsí látek. Pro objasnění fragmentačních mechanismů, spolehlivou identifikaci a kvantifikaci při použití měkké ionizační techniky je nutné provést MS/MS experiment. Jednou z možností je tandemová hmotnostní spektrometrie. Jedná se o spojení dvou stejných hmotnostních analyzátorů v čase (IT) nebo v prostoru (QqQ). Při MS/MS experimentu v čase se jednotlivé kroky provádějí v různých časových intervalech, ale na stejném místě, Obr. 17. Zatímco při tandemové hmotnostní spektrometrii v prostoru se jednotlivé kroky provádějí ve třech odlišných částech hmotnostního analyzátoru. V prvním hmotnostním analyzátoru se provede výběr prekurzorového iontu, který je podroben kolizně indukované disociaci a dochází k jeho fragmentaci v druhém hmotnostním analyzátoru a ve třetím dojde k analýze produktových iontů, Obr. 18 [28]. Tandemová hmotnostní analýza se provádí nejčastěji jako MS^2, nicméně jsou možné i MS^n experimenty, nejčastěji pomocí iontové pasti.



Obr. 17: Znázornění tandemově hmotnostní spektrometrie v čase.

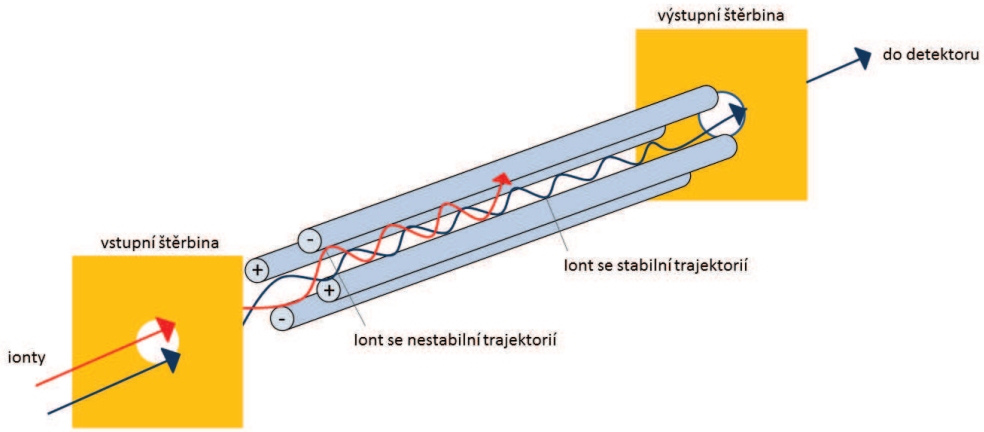


Obr. 18: Znázornění tandemově hmotnostní spektrometrie v prostoru.

Dalším důležitým trendem v oblasti hmotnostních analyzátorů jsou tzv. hybridní analyzátory. Jedná se o spojení dvou různých typů analyzátorů, kdy se kombinují jejich nejlepší vlastnosti. Nejpopulárnějším hybridním analyzátorem je spojení kvadrupólu a analyzátoru doby letu (Q-TOF) a spojení lineární iontové pasti s orbitální pastí (LIT-Orbitální past) [27].

QUADRUPÓLOVÝ ANALYZÁTOR

Skládá se ze čtyř kovových tyčí kruhového průřezu. Na dvě protilehlé je vloženo kladné stejnosměrné napětí a na zbývající dvě záporné stejnosměrné napětí. Na všechny je vloženo střídavé vysokofrekvenční napětí. V průběhu času je plynule měněno napětí střídavého elektrického pole, které způsobí oscilaci iontů přivedených do středu osy kvadrupólu. Každý iont s danou hodnotou m/z je stabilní při určité hodnotě napětí a tedy projde kvadrupólem do detektoru. Všechny ostatní ionty jsou zachyceny na tyčích kvadrupólu. Plynulou změnou napětí jsou postupně propuštěny všechny ionty do detektoru, Obr. 19.
Jedná se o jednoduchý, relativně levný hmotnostní analyzátor, který se uplatnil zejména ve spojení s kapalinovou chromatografií. Další analyzátory velmi často používané ve spojení s LC jsou například lineární a sférická iontová past.



Obr. 19: Schéma kvadrupólového analyzátoru. Převzato ze zdroje [34].

Své uplatnění na poli bio-analýzy našlo hlavně jeho tandemové uspořádání, tedy trojitý kvadrupól (QqQ). Skládá se ze tří kvadrupólových analyzátorů za sebou. Prostřední slouží jako kolizní cela. Zavedením kolizního plynu do hexapólu či oktapólu je provedena aktivace iontů vybraných prvním kvadrupólem a jejich následná fragmentace. V trojitém kvadrupólu na rozdíl od iontové pasti může docházet k opakovaným kolizím a tím ke vzniku více fragmentových iontů. Neposkytuje však tak čistá spektra jako iontová past.

IONTOVÁ PAST

Jedná se v podstatě o trojrozměrný kvadrupól, který se skládá ze dvou koncových elektrod a jedné kruhové elektrody. Na kruhovou elektrodu je vloženo kombinované stejnosměrné a střídavé napětí. Ionty přiváděné do detektoru jsou v iontové pasti nejprve zachyceny na základě vhodného poměru stejnosměrného a střídavého napětí a poté jsou plynulou změnou napětí vypuzovány na detektor podle jejich m/z.

Hlavními výhodami iontové pasti jsou možnost strukturní analýzy bez jakéhokoli rozšíření přístroje a možnost provádění fragmentací nejen do druhého (MS/MS), ale i více stupňů (MS^n) V poslední době došlo k přechodu od klasické sférické iontové pasti k pasti lineární (LIT). LIT je tvořena kvadrupólem, který je na obou stranách uzavřen kruhovými elektrodami. Vyznačuje se účinnější akumulací iontů, vyšší iontovou kapacitou a tím i vyšším dynamickým rozsahem [36].

SKENY V HMOTNOSTNÍ SPEKTROMETRII:

- **Základní sken (MS sken)** – měření hmotnostních spekter v plném rozsahu m/z.
- **Selektivní záznam jednoho nebo více iontů (SIM) (MS sken)** – měříme pouze závislost vybraného iontu na čase (nebo více iontů).
- **Sken produktových iontů (MS/MS sken)** – změříme hmotnostní spektrum produktů vzniklých fragmentací selektivně vybraného prekurzoru. Spektrum se využívá zejména k objasnění fragmentačních cest a tím k identifikaci sloučeniny.
- **Sken prekurzorových iontů (MS/MS sken)** – záznam poskytující spektrum všech prekurzorových iontů, jejichž fragmentace mohla vést ke vzniku daného fragmentu. Používá se k identifikaci strukturně podobných látek.
- **Sken neutrálních ztrát (MS/MS sken)** – slouží ke zjištění prekurzorového a produktového iontu, který může vést k charakteristické ztrátě, například m/z = 18 pro ztrátu vody. Používá se také k identifikaci strukturně podobných látek.
- **Selektivní záznam jedné nebo více reakcí – SRM (MS/MS sken)** – prvním analyzátorem se vybere ion prekurzoru, který je následně v kolizní cele fragmentován a sledován pouze jeden vybraný fragment pro daný prekurzor. Je možné však sledovat i více reakcí najednou. Tento záznam se využívá pro kvantitativní analýzu složitých směsí látek [35].

Schéma nastavení jednotlivých analyzátorů při sledování různých typů skenů je znázorněno na Obr. 20.

	MS 1	CID	MS 2	TYP SKENU
MS sken	skenuje	-	-	kvalitativní
SIM	fixní m/z	-	-	kvantitativní
sken produktových iontů	fixní m/z	+	skenuje	kvalitativní
sken prekurzorových iontů	skenuje	+	fixní m/z	kvalitativní
sken neutrálních ztrát	skenuje	+	skenuje	kvalitatiní (kvantitativní)
SRM	fixní m/z	+	fixní m/z	kvantitativní

Obr. 20: Typy skenů v hmotnostní spektrometrii a nastavení jednotlivých analyzátorů.

3.3.1.3. SPOJENÍ KAPALINOVÉ CHROMATOGRAFIE S HMOTNOSTNÍ DETEKCÍ

Spojení separačních technik s MS umožňuje analýzu reálných vzorků, jako jsou environmentální a biologické matrice. Jedná se o velmi složité a komplexní vzorky obsahující široké spektrum kontaminant. Proto je velmi důležitá separace kontaminant a jednotlivých analytů a zároveň selektivní a citlivá detekce. Separační techniky jako GC, HPLC, CE mohou být kombinovány s hmotnostně spektrometrickou detekcí, což umožňuje nejen kvantifikaci ale i identifikaci jednotlivých sloučenin ve vzorku. LC-MS je nejpoužívanějším spojením.

Protože se mobilní fáze účastní přímo ionizačního procesu, její výběr je značně omezen. Není vhodné použít například bezvodé mobilní fáze bez proton-donorového rozpouštědla nebo čistý hexan. Přednost mají těkavá aditiva v co nejnižší koncentraci, kyselina mravenčí, octová, amoniak či octan amonný nebo mravenčan amonný. Dále je nutné nahrazení konvenčních netěkavých pufrů těkavějšími analogy. Průtoky používané pro ionizační techniky za atmosférického tlaku se pohybují od 1 µl/min do 2 ml/min podle typu ionizace.

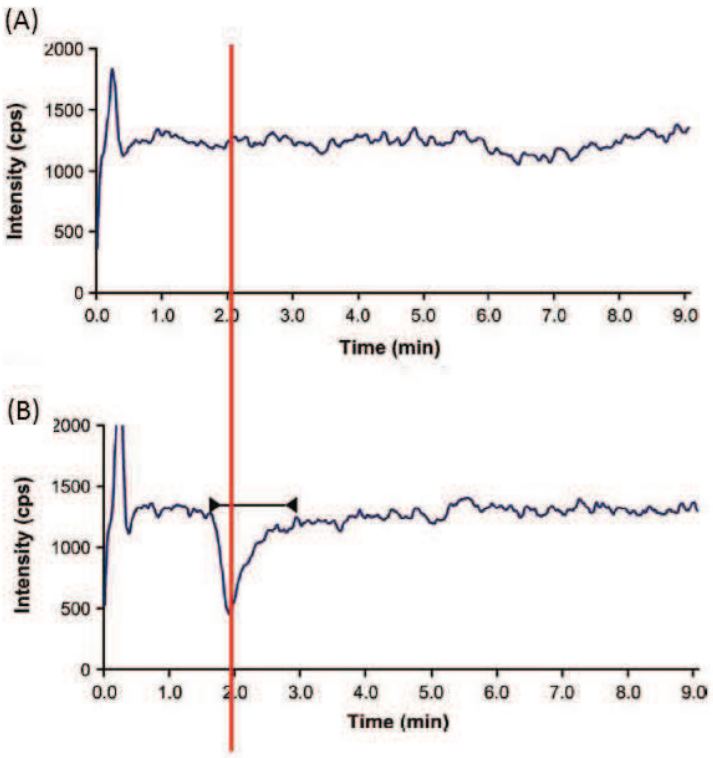
Mezi hlavní výhody spojení LC-MS patří: možnost analýzy složitých směsí, možnost odlišit koelující píky, získání informace o struktuře, citlivost MS detekce a nižší spotřeba vzorku než při off-line provedení [27].

Spojení kapalinové chromatografie s hmotnostní detekcí se vyznačuje vysokou citlivostí, a selektivitou. Kvantifikace pomocí LC-MS se provádí pomocí skenů SIM a SRM a velmi důležité je také použití vnitřního standardu. Přestože v kapalinové chromatografii je často používána i metoda vnějšího standardu, pro hmotnostní detekci je tento přístup nevhodný, protože není schopen kompenzovat vliv matrice. Nejvhodnější je vnitřní standard, který kompenzuje nejen kolísající opakovatelnost odezvy hmotnostního analyzátoru, ale zároveň eliminuje ztráty při úpravě vzorků. Mohou být využity tři typy vnitřních standardů: (a) vnitřní standard s podobnými fyzikálně-chemickými vlastnostmi, (b) strukturně homologické standardy a (c) pro LC-MS nejvhodnější stabilní izotopicky značený vnitřní standard (SIL-IS), který má s analytem téměř shodné vlastnosti. Nejlepší volbou jsou standardy s posunem o 4-5 hmotnostních jednotek. Metoda izotopicky značeného standardu je v současné době již nutností pro splnění validačních kritérií pro bio-analytické aplikace LC-MS analýzy.

Velmi problematický je vliv tzv. matricových efektů, který způsobují jednotlivé složky matrice a vedou ke vzniku pozitivních nebo negativních efektů, které jsou způsobené vlivem zesílení či potlačení odezvy hmotnostního spektrometru [27].

Pro zjištění přítomnosti matricových efektů existují **tři přístupy**:

1) Prvním a nejrychlejším způsobem ověření přítomnosti matricových efektů je **POSTKOLONOVÝ PŘÍDAVEK**. Do toku mobilní fáze je pomocí přímého vstupu do MS kontinuálně aplikován roztok standardu. Do tohoto průtoku je nadávkován nejprve roztok mobilní fáze a poté blanková plasma (nebo jiná testovaná tělní tekutina), která byla připravena příslušnou technikou přípravy vzorku. Matricový efekt se projeví vznikem výrazných pozitivních nebo negativních píků na záznamu blankové matrice v porovnání s čistou mobilní fází a vodou upravenou stejným postupem, Obr. 21 [37][38].



Obr. 21: Příklad hodnocení matricových efektů pomocí postkolonové infúze pro ESI-LC-MS analýzu. Obrázek (A) znázorňuje záznam mobilní fáze a záznam (B) v blanku reálné matrice. Je zde patrná přítomnost negativního efektu matrice. Převzato ze zdroje [39].

2) Druhým způsobem umožňujícím **kvantifikaci matricových efektů** je porovnání koncentrací analytů ve dvou roztocích lišících se přítomností matrice. Koncentrace roztoku blankové matrice upravené extrakční technikou a následně obohacené („spikované“) sledovaným analytem (c_x) je porovnávána s koncentrací odpovídajícího standardního roztoku (c_s) [37][38].

   Hodnota matricových efektů se vypočítá podle následující rovnice:

$$\%ME = \frac{c_x}{c_s} \times 100$$

   V případě, že ME = 100, nejsou matricové efekty přítomny. Je-li ME < 100 jsou přítomny negativní matricové efekty a v případě ME > 100 pozitivní matricové efekty.

3) Nejméně používaným způsobem je **porovnání směrnic kalibračních křivek** získaných měřením standardních roztoků analytů a matricových kalibračních křivek získaných metodou standardního přídavku. V případě, že nejsou přítomny žádné matricové efekty, koeficient „k“ v rovnici lineární závislosti jsou v obou případech shodné [40].

$$y = kx + q$$

Matricové efekty mohou být sníženy využitím SRM experimentu, izotopicky značeného vnitřního standardu, APCI ionizace a také preferencí negativního záznamu iontů, zlepšením chromatografické separace, zdokonalením techniky úpravy vzorků, využitím matricové kalibrační křivky či zředěním vzorku [38][40].
Izotopicky značené standardy se jeví jako nejlepší technika kompenzace matricových efektů. V některých případech může dojít k částečné separaci analytu a vnitřního standardu, což může být značnou komplikací. V případě výskytu matricového efektu blízko retenčního času vnitřního standardu, může dojít ke zkreslení výsledků a ovlivnění přesnosti a správnosti metody. Proto vhodnějšími izotopicky značenými standardy jsou ^{15}N, ^{13}C značené analogy, které se svými vlastnostmi podstatně méně liší než izotopy vodíku [37].

3.3.1.4. METODY PŘÍPRAVY VZORKŮ K ANALÝZE

Úprava vzorku tvoří nedílnou součást analytické metody pro stanovení látek pomocí chromatografických technik v reálných vzorcích. Přestože plynová a kapalinová chromatografie slouží k analýze složitých směsí, vysoce komplexní vzorky obsahující velké množství proteinů a jiných balastních látek nejsou vhodné pro přímou analýzu. Jednotlivé komponenty složitých matric mohou interferovat se sledovaným analytem. Úprava vzorku se používá k izolaci cílových analytů a k jejich zakoncentrování a to nejen u biologických vzorků, ale i rostlinného materiálu, potravin, odpadních vod a mnoha dalších. Jedná se o nejkritičtější a časově nejnáročnější krok analýzy reálných vzorků, který velmi vysokou měrou ovlivňuje výsledky celého stanovení.
Jednotlivé metody je možné rozdělit na dvě skupiny, konvenční a moderní techniky. Konvenční způsoby jsou běžně používány v mnoha analytických, ale i rutinních klinických laboratořích, protože se vyznačují bezproblémovostí, jednoduchostí a dobrou reprodukovatelností. Mají však také nevýhody, které vedou k vývoji novějších přístupů. Vznikla tedy druhá skupina zahrnující moderní metody úpravy vzorků. Tyto metody nejsou zatím běžně používány v rutinních analytických laboratořích a jsou zatím omezeny pouze na výzkumné laboratoře, kde jsou nadále zdokonalovány a rozšiřovány na další aplikace. Vývoj v oblasti metod přípravy vzorků je zaměřen na automatizaci, miniaturizaci, zejména objemu vzorku a jednotlivých rozpouštědel, a urychlení procesu při zachování dostatečné výtěžnosti a vysoké účinnosti extrakčního procesu. V současné době existuje mnoho způsobů přípravy vzorků, nicméně ještě nebyla nalezena univerzální technika splňující všechna požadovaná kritéria. Moderní přístupy lze dále dělit podle typu konvenční extrakce, která svým principem dala základ moderním metodám [22][41].

3.3.1.4.1. KONVENČNÍ TECHNIKY

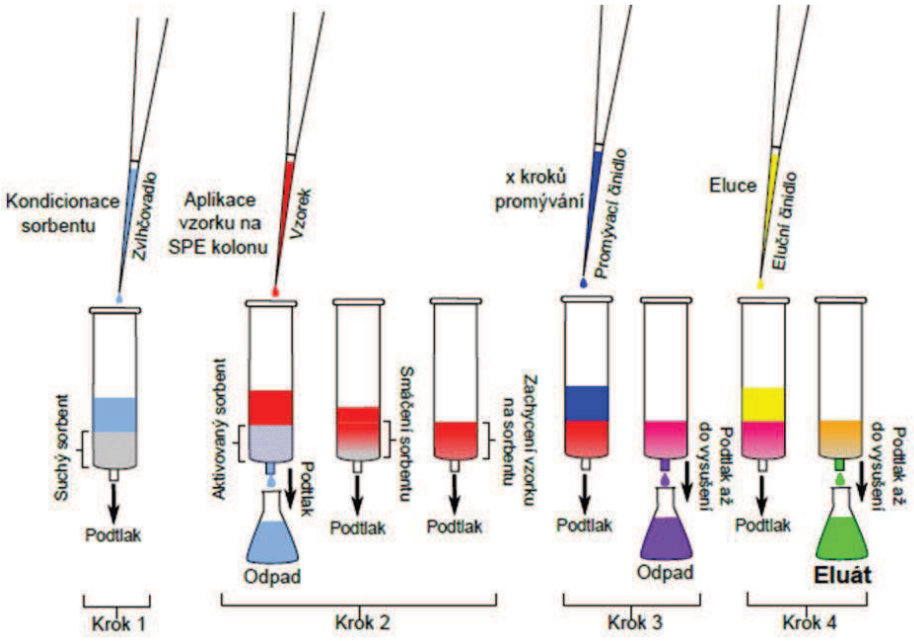
Konvenční přístupy úpravy vzorků se vyznačují časovou náročností a více krokovým postupem s relativně vysokou spotřebou rozpouštědel a vzorku. Zejména kvůli jejich časové náročnosti jsou v rozporu s trendy v oblasti kapalinové chromatografie a vývojem směru jako je rychlá chromatografie. Protože se však jedná o jednoduché, levné a instrumentálně nenáročné metody jsou stále velmi rozšířené v mnoha analytických laboratořích.
Klasickými konvenčními metodami přípravy vzorků před chromatografickou analýzou užívané v klinické praxi jsou (a) srážení proteinů (protein precipitation, PP), (b) extrakce z kapaliny do kapaliny (liquid-liquid extraction, LLE), (c) extrakce na tuhou fázi (solid phase extraction, SPE).

a) **Srážení proteinů** je tradiční jednoduchá technika úpravy biologických vzorků. Jako srážecí činidlo bývá nejčastěji používáno organické rozpouštědlo, např. acetonitril nebo methanol. Slouží zejména k odstranění proteinů ze vzorku, ovšem efektivita odstranění ostatních kontaminant je velmi nízká. Dochází ke vzniku nečistého supernatantu, což je společně s nízkou selektivitou hlavní nevýhodou. Přestože čistota supernatantu a selektivita je zcela zásadní, PP je pro její jednoduchou a rychlou optimalizaci stále velmi často používanou technikou [41].

b) **Extrakce z kapaliny do kapaliny** je jedna z nejstarších technik úpravy vzorků a je nadále využívána v praxi. Je založená na extrakci cílového analytu z vodného roztoku vzorku do organického rozpouštědla s vodou nemísitelného, vycházející z rozdělovacího koeficientu oktanol/ voda. Tato technika nese spoustu nevýhod, jako je velká spotřeba toxických organických rozpouštědel, produkce velkého objemu odpadu, tvorba emulze, nedostatečné oddělení jednotlivých vrstev rozpouštědel a často musí být na závěr rozpouštědlo odpařeno a analyt znovu rozpuštěn v mobilní fázi. Jedná se o drahou, časově náročnou techniku, která je nevhodná i z hlediska ekologie. Ovšem stále je často používanou metodu. Hlavním důvodem je zejména finanční a instrumentální nenáročnost, snadná manipulace a minimální požadavky na zručnost operátora. Některé nevýhody, zejména časová náročnost byly odstraněny vyvinutím automatického přístupu LLE [41].

c) **Extrakce na tuhou fázi** je nejrozšířenější technikou úpravy vzorků a to nejen pro biologický materiál, ale i pro mnoho dalších matric. Hlavní výhody představuje vysoká výtěžnost, schopnost zakoncentrování vzorku, nižší spotřeba rozpouštědel ve srovnání s ostatními konvenčními technikami a také snadná automatizace. SPE extrakce je založena na principu rozdělování mezi tuhou fází, ve formě extrakční kolonky, a kapalnou fází, kterou představuje vzorek s analytem. Během extrakčního procesu může být využito polárních, nepolárních či iontově výměnných interakcí. Hlavním předpokladem účinné SPE extrakce je vyšší afinita analytu k pevné fázi než ke kapalné fázi, tudíž matrici daného vzorku. Z tohoto předpokladu vyplývá, že zcela zásadním krokem při optimalizaci SPE extrakce je výběr vhodné extrakční kolonky. Mechanismus retence analytů na SPE sorbent je stejný jako v kapalinové chromatografii a proto i používané sorbenty jsou vlastně podobné. Postup je znázorněn na Obr. 22.

Přestože je nejvíce používanou technikou úpravy vzorků v analytických laboratořích, má mnoho nevýhod, jako vcelku vysokou spotřebu rozpouštědel a vzorku, mnohakrokový proces, jednorázové použití kolonek a časovou náročnost, která je často spojena s nutností odpaření vzorku a následným rozpuštěním analytu. Některé z těchto nevýhod byly odstraněny při modernizaci postupu SPE extrakce, a proto dala základ vzniku mnoha novým extrakčním postupům.



Obr. 22: Schéma postupu extrakce na tuhou fázi. V prvním kroku je provedena aktivace sorbentu. Následně v druhém kroku je dávkován vzorek na kolonku. Při průchodu analytu kolonkou, dochází k záchytu analytů na SPE sorbent. 3. krok je promytí sorbentu, což slouží k odstranění kontaminant matrice a ve 4. kroku je provedena cílená eluce analytů. Převzato ze zdroje [42].

V současné době existuje široká škála SPE sorbentů lišících se zejména jejich selektivitou a kapacitou. Lze je rozdělit na tři základní skupiny: sorbenty na reverzní fázi (C18, C8), na normální fázi, iontově výměnné (SAX, SCX). Sorbenty mohou být jednak na bázi silikagelu, ale také z polymerních materiálu, které umožňují práci v širokém rozmezí pH. V současné době jsou trendem v oblasti SPE extrakce tzv. „mixed-mode“ sorbenty, které obsahují jak nepolární skupinu, tak iontově-výměnnou, dále to jsou imunosorbenty, molekulárně vtištěné polymery (MIPs) a materiály s omezeným přístupem (RAM). Extrakce pomocí SPE může být prováděna jednak automaticky nebo manuálně. Existují již on-line přístupy umožňující přímé spojení s kapalinovým chromatografem. Sorbenty jsou dostupné jednak ve formě SPE kolonek, disků, či špiček naplněných sorbentem a existují i platíčka s 96 pozicemi pro automatické SPE [41].

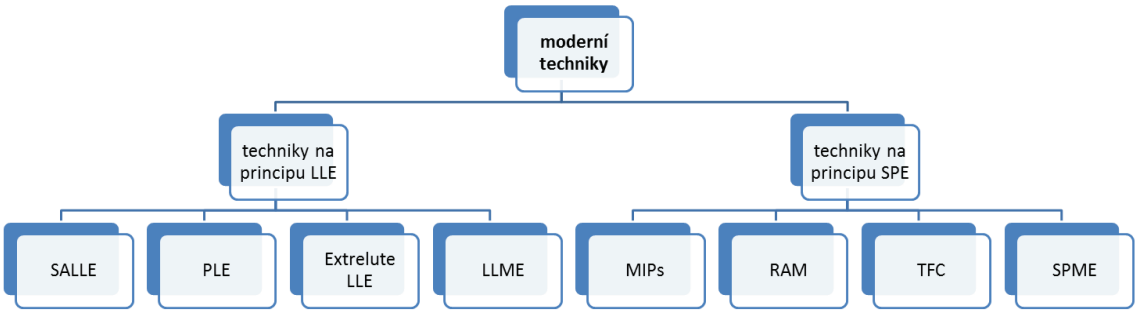
3.3.1.4.2. MODERNÍ TECHNIKY

Moderní metody úpravy vzorků odstraňují některé nevýhody dosud rutinně používaných metod a měly by zjednodušovat tuto komplikovanou a časově náročnou část analýzy. Hlavními požadavky na extrakční techniky jsou: miniaturizace (snížení spotřeby rozpouštědel a množství vzorku), zkrácení doby přípravy vzorků, možnost automatizace, zvýšení účinnosti a selektivity, on-line spojení s chromatografickými technikami a snížení ceny extrakčního procesu [43].

Na základě principu lze rozdělit moderní techniky pro úpravu vzorků do dvou základních skupin, Obr. 23:

- moderní přístupy na bázi extrakce z kapaliny do kapaliny
- moderní přístupy na bázi extrakce na tuhou fázi

Hlavním trendem obou základních skupin je miniaturizace, automatizace a možnost on-line propojení s chromatografickým systémem. Proto obě hlavní skupiny zahrnují mikroextrakční techniky, které užívají minimální množství vzorku a rozpouštědel.



Obr. 23: Schématické znázornění rozdělení technik úpravy vzorků.

1) MODERNÍ PŘÍSTUPY EXTRAKCE Z KAPALINY DO KAPALINY

Tato oblast zahrnuje moderní techniky úpravy vzorků, mikroextrakce z kapaliny do kapaliny (liquid liquid microextraction, LLME), Extrelut LLE, vysokotlaká extrakce kapalinou (pressurized liquid extraction, PLE) a extrakce z kapaliny do kapaliny pomocí vysolování (salting out liquid liquid extraction, SALLE), Obr. 22.

Extrelut LLE představuje metodu, která na rozdíl od klasické LLE využívá Extrelut kolonky naplněné inertní matricí s velkými póry. Vzorek s upraveným pH je aplikován na kolonku, kde dojde k vytvoření kapalného filmu vzorku na povrchu porézní matrice. Jako činidlo je použito nemísitelné organické rozpouštědlo, které při průchodu kolonkou vymývá cílené analyty. Tato alternativa LLE přináší některé výhody v porovnání s klasickou LLE. Jedná se zejména o prevenci tvorby emulze a možnost automatizace. Technika používá velké objemy toxických organických rozpouštědel a je časově náročná, a proto není v rutinních laboratořích rozšířena [22].

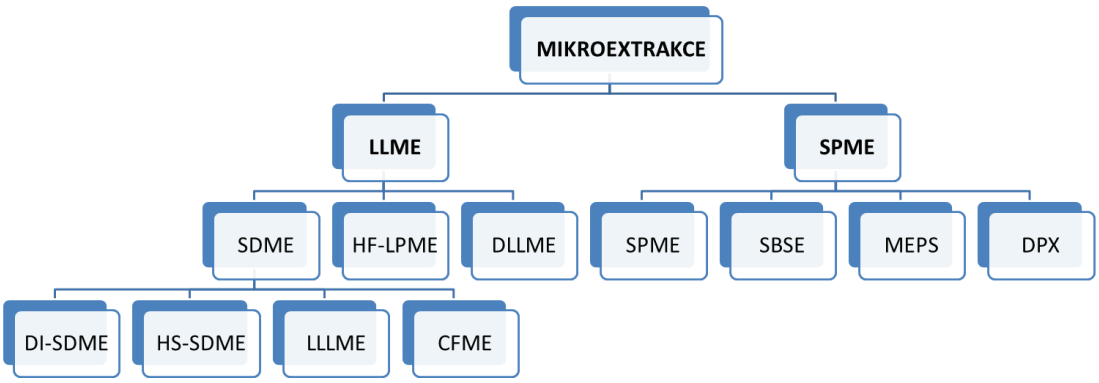
Vysokotlaká extrakce kapalinou je využívána k extrakci z tuhé matrice. Je provedena pomocí organického rozpouštědla, které se za vysokého tlaku a teploty vyskytuje v kapalném stavu. Zvýšený tlak udržuje rozpouštědlo jednak v kapalném stavu, ale také usnadňuje kontakt rozpouštědla s analytem. Zvýšená teplota urychluje transportní procesy a potlačuje interakce analyzované látky s matricí. Projevuje se to nižší spotřebou rozpouštědla, rychlejší, šetrnější a účinnější extrakcí. Tato metoda je v oblasti bio-analýzy používaná při extrakci tkání nikoli tělních tekutin [22].

Extrakce z kapaliny do kapaliny pomocí vysolování je založena na rozdělování mezi acetonitrilem a vodným roztokem vzorku, který byl obohacen organickou nebo anorganickou solí. Přináší určité výhody jako je snadná automatizace a v porovnání s klasickou LLE aplikovatelnost na širokou skupinu analytů [22][41].

Mikroextrakce z kapaliny do kapaliny

Vyznačuje se použitím velmi malého množství rozpouštědel a minimalizací počtu jednotlivých extrakčních kroků. Jsou používány nejen ve farmaceutické, ale i v potravinářské a environmentální analýze. Jejich rozšíření do klinických laboratoří brání časová náročnost,

v některých případech také obtížná manipulace a složitost výběru vhodného rozpouštědla. V současné době existuje několik přístupů LLME: mikroextrakce jednou kapkou (SDME), mikroextrakce do kapalné fáze s využitím dutého vlákna (HF-LPME) a disperzní mikroextrakce z kapaliny do kapaliny (DLLME). Přehled mikroextrakčních technik zařazených do mikroextrakce z kapaliny do kapaliny je znázorněn na Obr. 24.



Obr. 24: Zařazení jednotlivých mikroextrakčních technik do skupiny mikroextrakce na tuhé fázi a mikroextrakce z kapaliny do kapaliny.

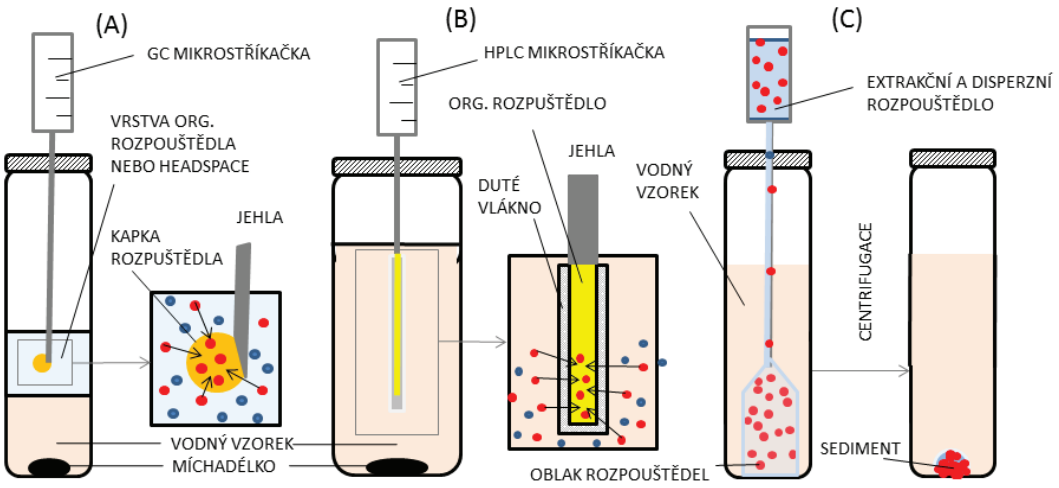
- **<u>Mikroextrakce jedinou kapkou (SDME, Single-drop microextraction)</u>**

  SDME byla vyvinuta roku 1996 jako prekoncentrační technika založená na extrakci analytu z vodného roztoku vzorku do mikrokapky (1-10 µl) organického rozpouštědla vytvořené na konci jehly. Po extrakci je kapka vtažena do jehly a může být přímo nastřikována do chromatografického systému, což eliminuje kroky odpaření a následného rozpuštění analytu. Množství použitého rozpouštědla je sníženo o 99% v porovnání s LLE. Velmi problematická je špatná reprodukovatelnost extrakce způsobená nestabilitou kapky a výběr vhodného rozpouštědla, který je limitován mnoha kritérii. Jedno z nich představuje netěkavost rozpouštědla. Nejčastěji používanými rozpouštědly jsou n-oktyl acetát, isoamylalkohol, oktan či ethylenglykol, Obr. 25 [22][43].

  Pro různé aplikace byly vyvinuty různé módy SDME [43]:

  - mikroextrakce jedinou kapkou pomocí přímého ponoření (DI-SDME, direct immersion SDME)
  - mikroextrakce jedinou kapkou pomocí head-space (HS-SDME, head-space SDME)
  - mikroextrakce z kapaliny do kapaliny a zpět (LLLME, liquid-liquid-liquid microextraction)
  - mikroextrakce kontinuálního toku (CFME, continuous-flow microextraction)

  SDME vyžaduje pracnou a pečlivou optimalizaci jednotlivých podmínek z důvodu nestability kapky, která je ovlivněna zejména vysokou teplotou, rychlejším mícháním či analýzou více kontaminovaných vzorků. Nestabilita kapky vede ke značnému prodloužení doby úpravy vzorku, a proto není vhodnou technikou pro rutinní aplikace a on-line zakoncentrování vzorku.



Obr. 25: Schématické znázornění jednotlivých mikroextrakčních technik z kapaliny do kapaliny, (A) SDME, (B) HF-LPME, (C) DLLME. Převzato a upraveno z reference [43].

- **<u>Mikroextrakce do kapalné fáze s využitím dutého vlákna (HF-LPME, Hollow-fibre liquid phase microextraction)</u>**

  Tato extrakční technika je založena na extrakci pomocí dutého vlákna, které má charakter membrány vyrobené z polypropylenu nebo jiného hydrofobního materiálu. Hydrofobní membrána je smočena organickým rozpouštědlem, které vnikne do pórů a vytvoří tzv. ochranný film. Lumen jehly je naplněn akceptorovou tekutinou a podle toho, zda je zastoupena hydrofilní nebo hydrofobní tekutinou, je možné rozdělit tento typ extrakce na dvoufázovou a třífázovou HF-LPME. V případě třífázové HF-LPME je akceptorová tekutina hydrofilní a dochází nejprve k extrakci analytu do organického rozpouštědla tvořícího ochranný film a následně do vodné akceptorové fáze. Ostatní módy jsou založeny na dvoufázovém systému, kdy je akceptorová tekutina tvořena také organickým rozpouštědlem. Hlavní výhody zahrnují velmi malou spotřebu rozpouštědel, pozoruhodnou účinnost čistícího procesu, která je způsobena neschopností velkých molekul přecházet membránou, vysokým koncentračním faktorem pro organické i anorganické sloučeniny v široké oblasti polarity a možností on-line spojení s chromatografií nebo dalšími instrumentálními technikami [22][43], Obr. 25.

  HF-LPME je nejčastěji používanou mikroextrakční technikou na bázi LLE. Představuje velmi užitečnou techniku kvůli využitelnosti pro široký rozsah analytů bez ohledu na jejich polaritu, nízké spotřebě rozpouštědel, vysoké účinnosti a snadné automatizaci. Problém může přinést kontaminace vlákna popřípadě jeho křehkost. Tato technika se jeví jako vhodná pro rutinní použití, nicméně v současné době neexistuje komerčně dostupná sestava pro HF-LPME [22][43].

- **<u>Disperzní mikroextrakce z kapaliny do kapaliny (DLLME, Dispersive LLME)</u>**

  DLLME technika využívá systém trojice rozpouštědel. Odpovídající směs extrakčního činidla a disperzního rozpouštědla je rychle vstříknuta do roztoku vzorku, kde za jemného míchání vzniká oblak rozpouštědel. Po centrifugaci dojde k usazení jemných částic na dně zkumavky. Následně je sediment odsát pomocí mikro-stříkačky a nastříknut přímo do GC systému. Hlavní podmínkou pro správné provedení extrakce je, že extrakční činidlo musí být

s vodou nemísitelné a zároveň mít vyšší hustotu než voda, zatímco disperzní roztok musí být mísitelný jak s extrakčním rozpouštědlem, tak s vodným roztokem vzorku, Obr. 25 [22][43].

2) MODERNÍ PŘÍSTUPY EXTRAKCE NA TUHOU FÁZI

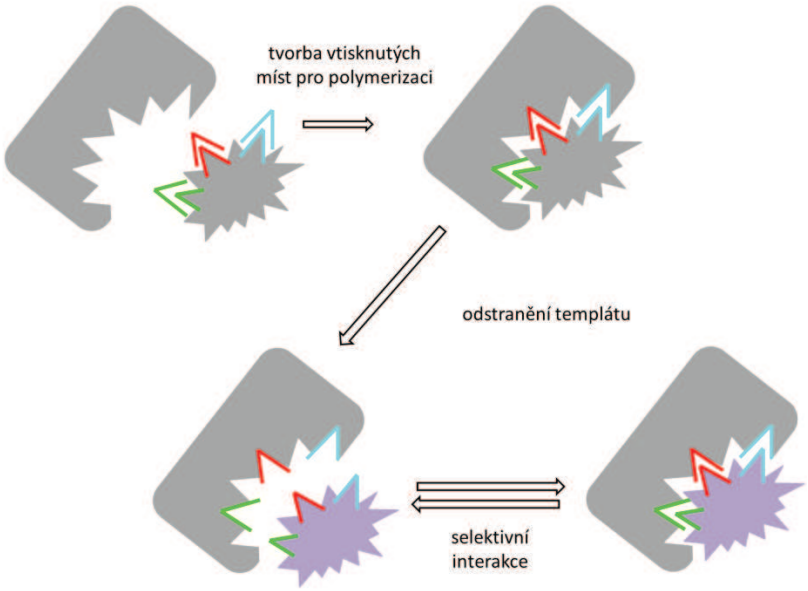
Jedná se o další rozsáhlou skupinu metod, které jsou založena na SPE. Mezi tyto techniky jsou zahrnuty: turbulentní průtoková chromatografie (TFC, turbulent flow chromatography), SPE pomocí sorbentu s omezeným přístupem (RAM, restricted access materiál) a pomocí vysoce selektivního materiálu (MIPs, molecular imprinted polymer) a velká skupina mikroextrakčních technik na tuhou fázi [41][43].

- **Turbulentní průtoková chromatografie (TFC, turbulent flow chromatography)**
  Tato technika umožňuje on-line přečištění vzorku. Je využívána pro přímý nástřik biologického vzorku do kolony o malém vnitřním průměru (0,18 µm nebo 1 mm) a naplněné velkými částicemi (20 – 60 µm). TFC využívá výhod turbulentního proudění způsobeného vysokým průtokem mobilní fáze (1,5 – 5 ml/min). Průtok mobilní fáze přes TFC částice způsobí zhruba 100x vyšší lineární průtokovou rychlost než v klasické HPLC koloně. V TFC koloně jsou vytvořeny velké mezičásticové prostory, které umožní tvorbu turbulencí. Principem TFC je separace malých molekul od makromolekulární složky. Malé molekuly vzorku se dostanou snadněji do póru a dochází k jejich vazbě na částice, což způsobí, že velké molekuly a proteiny jsou z kolony vypuzeny mnohem dříve. Následnou změnou mobilní fáze dojde k eluci malých molekul z kolony a jsou unášeny do detektoru nebo do druhé analytické kolony určené k separaci [22].

- **Materiály s omezeným přístupem (RAM, restricted access materiál)**
  Tato technika umožňuje také přímý nástřik biologického materiálu do chromatografického systému. Je určena především pro analýzu malých molekul (léčiva, xenobiotika) ve složité matrici obsahující vysokomolekulární sloučeniny, nejčastěji proteiny. RAM HPLC kolony jsou v mnoha případech využívány jako předkolony, které spolu s analytickou kolonou a systémem přepínáním kolon umožní odstranění makromolekulárních látek ještě před chromatografickou separací. K jejich odstranění dochází na základě omezeného vstupu těchto sloučenin do RAM sorbentu. A protože vznikají pouze povrchové interakce, jsou z kolony brzo eluovány. Jinak tomu je u malých molekul, které do RAM částic vstupují, a proto jsou zadržovány na koloně a eluovány později. Omezení vstupu makromolekul do RAM sorbentu může být způsobeno jak na základě chemické, tak fyzikální bariéry. Tyto sorbenty jsou prakticky hodně využívány zejména pro analýzu léčiv v biologickém materiálu [22].

- **Molekulárně vtištěné polymery (MIPs, molecularly imprinted polymers)**
  Molekulárně vtištěné polymery jsou selektivní materiály používané pro extrakci na tuhou fázi. Hlavní výhodou tohoto přístupu je možnost přípravy sorbentu specifického pro cílovýc analyt nebo skupinu strukturních analogů, což umožňuje vysoce selektivní izolaci daného analytu od reálné matrice a zvýšení selektivity a citlivosti následné chromatografické separace.
  Vznik molekulárně vtištěného polymeru je složitý mnohakrokový proces, který spočívá ve třech základních mechanismech vtištění analytu: nekovalentním, kovalentním a kombinací

obou. Může dojít ke vtisknutí jednoho nebo více monomerů. Následně dochází k polymerizaci a odstranění templátu. Vzniklý MIP obsahuje stérickou (velikost a tvar) a chemickou paměť pro templát, Obr. 26 [48][49]. První MIP byl aplikován v roce 2004 na stanovení pentaimidu v moči [50].



Obr. 26: Znázornění přípravy molekulárně vtištěného polymeru.

SPE pomocí MIP může být provedena jednak off-line ale i on-line. Pro biologické aplikace je využíváno zejména off-line provedení. V současné době je dostupná řada molekulárně vtištěných polymerů, např. pro izolaci cefalosporinu, fluorochinolonů, β-blokátorů a mnoha dalších látek.

Přestože tato technika přináší velké množství výhod, které představuje zejména vysoká selektivita, stabilita a možnost opakovaného použití, má tato technika také některé nevýhody. Velká část polymerů je připravována nekovalentním vtištěním, které je zatíženo nízkou výtěžností a tudíž vznikem malého počtu specifických vazebných míst. Další výzkum je proto zaměřen na zvýšení kapacity a selektivity polymerů [22][41].

- **Imunoafinitní SPE**

  Princip extrakce je shodný jako SPE. SPE kolonka je naplněná sorbentem s navázanými protilátkami specifickými pro cílený analyt. Při průchodu vzorku sorbentem vznikají vysoce specifické vazebné interakce mezi analytem a protilátkami navázanými na sorbentu. Vlivem těchto specifických interakcí se jedná o vysoce selektivní a specifickou metodu, která je však použitelná pro užší skupinu látek.

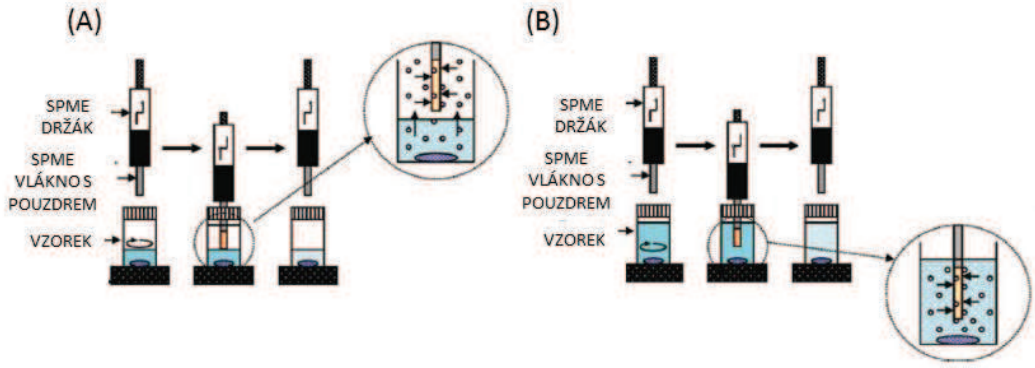
MIKROEXTRAKCE NA TUHOU FÁZI

Mezi mikroextrakční techniky na tuhou fázi řadíme sorpční extrakce míchátkem (SBSE – stir bar sorptive extraction), mikroextrakce na tuhou fázi (SPME, solid phase microextraction), mikroextrakce tuhým sorbentem (MEPS, microextraction by packed sorbent) a extrakce pomocí naplněných špiček pipet (DPX, disposable pipette tip extraction).

- **Mikroextrakce na tuhou fázi (SPME, solid phase microextraction)**

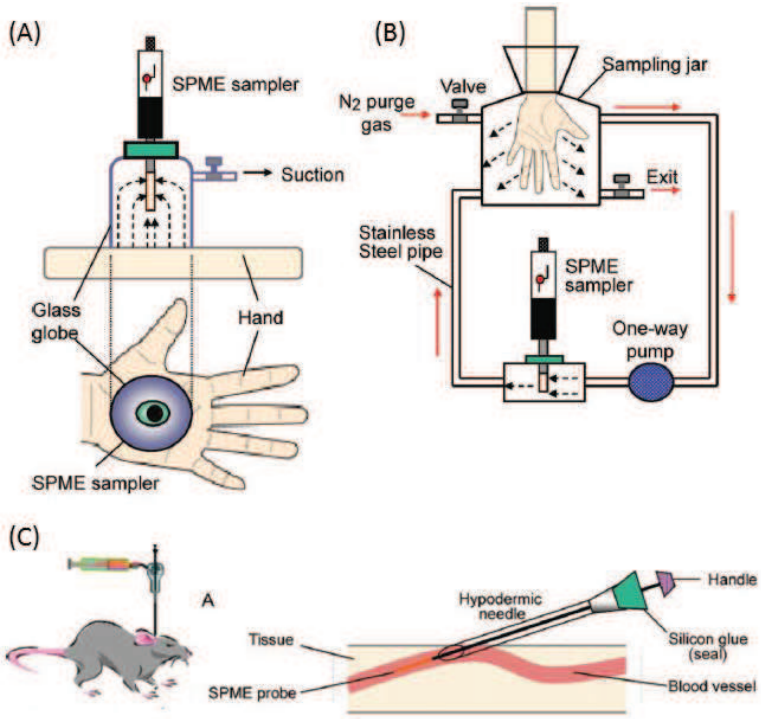
  Byla vyvinuta v roce 1990 jako jednoduchá a efektivní technika adsorpce a desorpce, která eliminuje spotřebu rozpouštědla. Lze ji rozdělit na SPME pomocí vlákna (fiber SPME) a SPME v kapiláře (in tube SPME), která je řazena mezi on-line techniky.

  SPME pomocí vlákna (fiber SPME) je prováděna pomocí křemíkového vlákna, které je na povrchu pokryto organickým polymerem (obvykle polydimethylsiloxanem). Vlákno je umístěno v nerezovém pouzdře a může být zasouváno a vysouváno. Vlastní extrakce probíhá ve vysunuté poloze. Podle toho zda se jedná o head space SPME (HS-SPME) nebo SPME s přímým ponořením (DI-SPME) je vlákno umístěno buď přímo do roztoku vzorku anebo nad jeho hladinu. Při propichování septa při vstupu do GC systému je vlákno zastrčeno do kovového pouzdra a po vysunutí vlákna dojde k desorpci přímo do GC systému, Obr. 27. Hlavní výhodou toho přístupu je provedení extrakce, zakoncentrování a derivatizace v jednom kroku, což umožňuje snadnou automatizaci. Tato technika přináší další výhody jako vysokou citlivost a snadnou automatizaci. Dříve však přinášela nevýhody jako nízkou výtěžnost, časovou náročnost, nízkou kapacitu vlákna a možnost užití pouze pro některé bio-analýzy z důvodu problematického stanovení netěkavých analytů ve složité matrici [43].



Obr. 27: Schématické znázornění mikroextrakce na tuhou fázi, (A) HS-SPME, (B) DI-SPME. Převzato a upraveno ze zdroje [45].

V posledních letech došlo v oblasti SPME k velkému vývoji. Vzniklo mnoho nových vláken s různými stacionárními fázemi (např. PA – polyakrylát), které umožňují analýzu jak polárních, tak nepolárních analytů. Také byly vyvinuty směsné fáze s porézními pevnými částicemi, například PDMS/DVB (polydivinylbenzen), čímž se výrazně zvýšila retenční kapacita vlákna. Vývoj směřuje také k in vivo SPME, která umožňuje studii dynamických chemických procesů přímo v živém systému. Může se vyskytovat také v uspořádání HS- a DI-SDME. Dalším možným provedením je aplikace SDME sondy pomocí jehly přímo do žíly nebo tkáně zvířete. Uspořádání in vivo SDME je zobrazeno na Obr. 28.

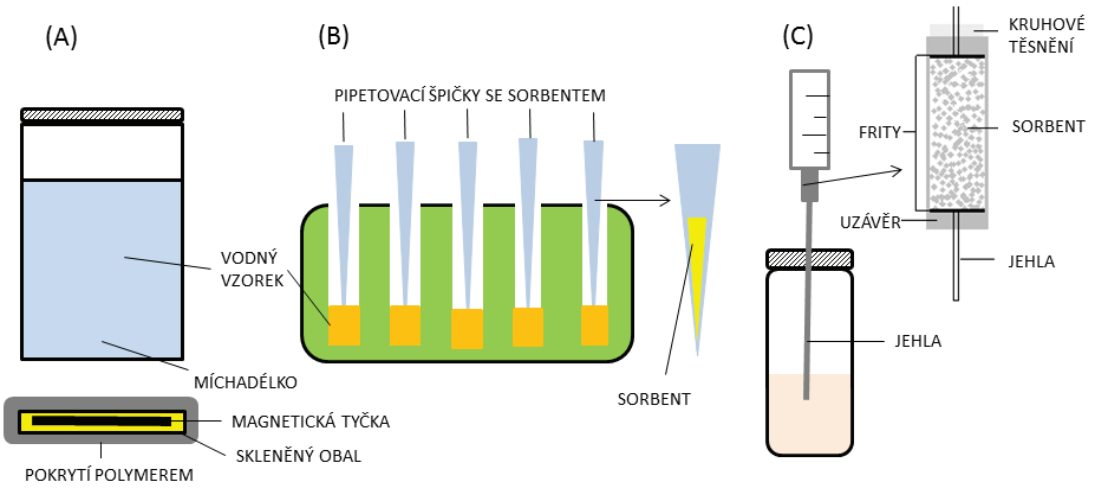


Obr. 28: Schematická zobrazení in vivo SPME, (A) DI- in vivo SPME, (B) HS- in vivo SPME, (C) aplikace sondy přímo do žíly. Převzato ze zdroje [45].

SPME v kapiláře (in-tube SPME) používá otevřenou křemíkovou kapiláru místo vlákna a tím usnadňuje spojení s LC. Tyto kapilární kolony jsou umístěné v dávkovači místo dávkovací smyčky. Tloušťka, délka a potažení kapiláry jsou individuální a měnitelné podle potřeb aplikace. Roztok vzorku je několikrát nasáván a vypuzován z kapiláry, čímž dochází k adsorpci analytu. Desorpce se potom provede už vlastní mobilní fází, která je transportována do LC systému. Dříve byly pro extrakci používány GC kolony, ale v současné době je dostupných mnoho nových komerčně dostupných kapilár (kapiláry s MIP částicemi, imunoafinitní a monolitní in-tube SPME kapiláry), což umožňuje analýzu široké skupiny analytů. Tato technika představuje jednu z nejlepších technik pro bio-aplikace, přináší mnoho výhod jako je velmi snadná automatizace, rychlost extrakce, vyšší přesnost a selektivita než všechny off-line techniky a nízká cena. Velmi pozitivní je také minimální styk pracovníků s biologickým vzorkem a organickými rozpouštědly [41][43].

- **Sorpční extrakce míchátkem (SBSE, Stir bar sorptive extraction)**

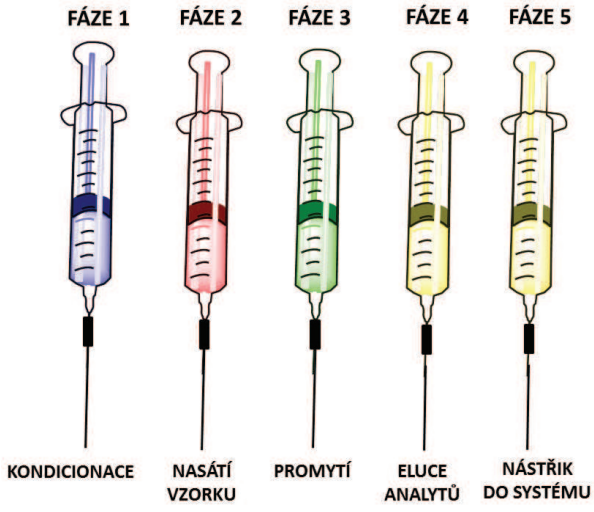
  Tato technika je založena na stejném principu jako SPME, liší se pouze v tom, že extrakční fáze je nanesena ne na vlákně, ale na magnetickém míchadle. V porovnání s SPME se SBSE vyznačuje vyšší extrakční účinností, způsobenou 50x-250x větším množstvím fáze pokrývající míchadélko. Desorpce analytu může být prováděna pomocí termální desorpce nebo malým množstvím rozpouštědla, pak se jedná o kapalnou desorpci (LD). Protože míchadlo je pokryto nepolární fází, tato extrakce je vhodná zejména pro slabě polární sloučeniny. V případě analýzy polárních či dokonce silně polárních analytů, musí být provedena in-situ derivatizace. Tento proces sice poskytuje vysokou citlivost, nicméně jeho hlavní nevýhodou je doba extrakce, která může dosáhnout až 240 minut, což je velmi nepraktické pro rutinní laboratoře, Obr. 29.



Obr. 29: Schematické znázornění mikroextrakčních technik na tuhou fázi. (1) SBSE, (2) DPX, (3) MEPS. Převzato a překresleno ze zdroje [49].

- **<u>Mikroextrakce tuhým sorbentem (MEPS, microextraction by packed sorbent)</u>**

  MEPS byla popsána v roce 2004 ve švédské laboratoři AstraZeneca. Jedná se o techniku, která redukovala množství sorbentu a umožnila použití širokého rozsahu objemu vzorku (10 µl – 1000 µl). Extrakce je prováděná pomocí stříkačky o velikosti 100 – 500 µl. 1 mg sorbentu je vložen do válečku, který je upevněn na konci jehly a vstupuje do stříkačky. V současné době je už poměrně široký výběr extrakčních fází zahrnující materiály na bázi silikagelu (C2, C8, C18 a SCX), ale RAM a MIP sorbenty zatím nejsou komerčně dostupné.
  MEPS je založena na stejném principu jako SPE extrakce i jednotlivé kroky těchto extrakcí jsou shodné, Obr. 30.



Obr. 30: Schématický postup MEPS extrakce.

Vzorek je nasáván pomocí stříkačky. Při průchodu vzorku sorbentem dochází k adsorpci analytu na pevnou fázi. Následně jsou kontaminanty ze sorbentu odstraněny pomocí vhodných promývacích činidel. Analyt je vymyt organickým rozpouštědlem nebo mobilní fází. Získaný eluát může být přímo dávkován do LC systému. Proces nasávání a vypouštění vzorku může být několikrát opakován, což umožňuje zakoncentrování vzorku.

MEPS existuje v manuálním, poloautomatickém nebo automatickém provedení. Manuální přístup s sebou přináší některé nevýhody. Z hlediska výtěžnosti je problematická tvorba vzduchových bublin a závislost na plynulém pohybu pístem. Tento přístup je také omezen z hlediska použitého množství vzorku. Pro získání reprodukovatelných a správných výsledků, není vhodné nasávání menšího objemu vzorku než 50 µl. Špatná reprodukovatelnost nasávání malých objemů a tvorba bublin může být odstraněna použitím automatické pipety. Nejedná se však o plně automatický přístup, který MEPS také umožňuje. V současné době pro některé LC systémy existuje i on-line provedení.

Na poli bio-analytických stanovení hrají velmi významnou roli zejména výhody jako je: snížení množství rozpouštědel a reálného vzorku, rychlost provedení extrakce a také možnost opakovaného použití sorbentu bez významných přenosů mezi jednotlivými vzorky (až 100x pro plazmu a moč). Tato technika může být použita pro komplexní složité matrice jako je například plazma nebo moč. Nevýhody této techniky jsou spojeny zejména s jejím manuálním přístupem, nicméně i ten představuje dostatečně citlivou a selektivní techniku úpravy vzorků, vhodnou pro biologické aplikace, Obr. 29 [22][41][44].

Obr. 31: Poloautomatické a automatické provedení MEPS. Převzato ze zdroje [46][47] .

- **<u>Extrakce pomocí naplněných špiček pipet (DPX, disposable pipette tip extraction)</u>**

  DPX představuje jednoduchou, rychlou, cenově výhodnou a přesnou variantu SPE. Sorbent naplněný ve špičce je z obou stran ohraničen fritami. Na rozdíl od klasické extrakce na tuhou fázi, při které je analyt ve styku se sorbentem pouze jednou, při DPX dochází k několikanásobnému kontaktu, a proto se vyznačuje vysokou účinností. Jednotlivé extrakční kroky, jako kondicionace, nanesení vzorku, promytí a eluce, jsou shodné se SPE. Nevýhodou této techniky je komerční dostupnost pouze jedné velikosti špiček a omezeného počtu druhů sorbentu. Obr. 29 [41].

Technika	Doba extrakce [min]	Selektivita	Mnohakrokový proces	Možnost automatizace	Spotřeba rozpouštědel
KONVENČNÍ TECHNIKY ÚPRAVY VZORKU					
LLE	15 – 25	střední	ano	+	velmi vysoká
SPE	15 – 25	střední	ano	+	relat. vysoká
PP	< 10	nízká	ne (centrifugace)	+	vysoká
MODERNÍ MIKROEXTRAKČNÍ TECHNIKY ÚPRAVY VZORKU ZALOŽENÉ NA LLE					
SPME	10 – 60	střední	adsorpce/desorpce	+	NE
SBSE	30 – 240	střední	adsorpce/desorpce	-	NE
MEPS	1 – 5	střední	ano	+	velmi nízká
DPX	15 – 20	střední	ano	+	velmi nízká
MODERNÍ MIKROEXTRAKČNÍ TECHNIKY ÚPRAVY VZORKU ZALOŽENÉ NA LLE					
LLME	5 – 60	střední	adsorpce/desorpce	-	velmi nízká

Tab. 5: Porovnání základních vlastností konvenčních a moderních mikroextrakčních technik.

3.3.2. VALIDACE ANALYTICKÉ METODY

Účelem validace je prokázání vhodnosti vyvinuté metody pro zamýšlený účel. Každá nově zaváděná analytická metoda musí být validovaná v plném rozsahu validačních parametrů. V případě, že dojde ke změnám v analytickém postupu, musí být metoda revalidována. Validace analytické metody aplikované na biologickou matrici se řídí platnými předpisy, zejména ICH směrnicí (quidelines) Q2R1 (Text on Validation of Analytical Procedures) [51], která je zaměřena obecně na validaci analytických metod. V roce 2010 vydala společnost EMEA (European Medicines Agency), výbor zabývající se zdravotními produkty pro lidské použití, směrnici na validaci bio-analytických metod. EMEA směrnice je na rozdíl od ICH předpisů zaměřena speciálně na metody určené k monitorování lékových hladin v biologických vzorcích (plazmě, moči, slinách nebo tkáních) [52]. Hlavními parametry, které jsou posuzovány v rámci validace podle EMEA směrnice jsou: selektivita, limit kvantifikace, odezva kalibrační křivky, přesnost, správnost, matricové efekty a stabilita všech analytů včetně vnitřního standartu v biologické matrici, zásobním a pracovním roztoku. Jedná se o shodné parametry jako u obecně platných směrnic pro analytické metody s výjimkou matricových efektů a parametru zohledňujícího přenos analytů mezi jednotlivými nástřiky vzorků. Tato kritéria nejsou ostatními směrnicemi zatím požadována. Dalším rozdílem mezi EMEA a ICH směrnicí je v počtu stanovení nutných pro hodnocení jednotlivých parametrů a v jejich limitních hodnotách. Zatímco ICH směrnice neurčila jednotlivé limity, EMEA přesně specifikovala limitní hodnoty jednotlivých parametrů, Tab. 6.

	ICH	EMEA	
Parametr	počet vz.	počet vz.	limitní hodnota
selektivita	+	6	< 20% LOQ
přenos vzorku (carry-over)	+	+	bez vlivu na správnost a přesnost
limit kvantifikace (LOQ)	+	+	
kalibrační křivka			
lineární rozsah	5 x 1	6 x 3	správnost ± 15%, pro LOQ 20%
sklon kalibrační křivky	-	6 x 3	
správnost	3 x 3	4 x 5	± 15%, pro LOQ ± 20%
Přesnost	3 x 3	4 x 5	± 15%, pro LOQ ± 20%
vliv ředění vzorku	-	5	správnost i přesnost ± 15%
matricové efekty	-	6	< 15%

+ směrnice parametr zahrnuje, - směrnice parametr nezahrnuje

Tab. 6: Porovnání ICH a EMEA směrnice.

Hodnota přesnosti, správnosti a matricových efektů bio-analytické metody je omezena odchylkou 15%, což s ohledem na složitost biologické matrice přináší značné problémy. Biologické vzorky jsou velmi komplexní matrice obsahující širokou škálu biogenních látek, které mohou mít často velmi podobné vlastnosti jako analyt, a proto jsou velmi těžko odstranitelné pomocí technik úpravy vzorků nebo rozdělitelné pomocí chromatografické separace. Účinnější, ale nikoli všemocná, je technika vnitřního standardu, za použití stabilního izotopicky značeného standardu. Zde ovšem může nastat problém, že nejen z ekonomických důvodů, ale i kvůli špatné dostupnosti jednotlivých standardů, není často možné použít standard individuálně pro všechny analyty včetně metabolitů. Protože ICH směrnice neurčovala přesný limit, bylo zavedení tohoto limitu 15 % velmi přísné a těžko splnitelné.

Další změny jsou také v počtu koncentračních hladin a počtu vzorků na jednotlivých hladinách, na kterých jsou parametry stanovovány. Také je zde zcela zásadní podmínka, že v případě užití jedné analytické metody na více matric je nutné validaci provádět nejen pro každou matrici individuálně, ale zároveň každá matrice musí být získána minimálně ze šesti různých zdrojů.

Vydáním EMEA směrnice došlo ke specifikaci limitů jednotlivých parametrů přímo pro biologický materiál, což přineslo velké komplikace ve validaci jednotlivých bio-analytických metod nejen pro klinické, ale i výzkumné laboratoře. Značně se také prodloužil čas potřebný na vývoj a validaci jednotlivých metod, což ovlivňuje ekonomiku zejména ve výzkumném odvětví.

3.3.3. METODY STANOVENÍ STATINŮ

Pro vývoj léčiv je nutné mít vždy dostupnou metodu jejich stanovení v biologických vzorcích. Nejpoužívanější technikou v oblasti monitorování lékových hladin v biologickém materiálu je kapalinová chromatografie ve spojení s hmotnostní spektrometrií. UHPLC-MS metody použité pro stanovení statinů jsou shrnuty v Tab. 7. Každý statin je v tabulce zastoupen třemi reprezentativními metodami.

Z Tab. 7 je patrné, že v oblasti stanovení statinů je nejpoužívanějším analyzátorem trojitý kvadrupól a ionizace elektrosprejem. Převážná většina statinů ionizuje v pozitivním i negativním módu. Existují i výjimky, které umožnují stanovení pouze v negativním záznamu iontů.

UHPLC-MS/MS metody byly aplikovány na lidskou plasmu nikoli na ostatní matrice jako je moč, tkáň či plazma pocházející z jiného živočišného druhu. S ohledem na složitost biologické matrice je překvapivé, že většina LC metod užívá izokratickou eluci, která není zcela vhodná pro analýzu komplexních matric. Dochází k pozdní eluci jednotlivých složek matrice a tím k prodloužení času analýzy a vyšším matricovým efektům. Při využití gradientové eluce jsou jednotlivé složky matrice snadněji vymyty z kolony a tudíž je vliv matrice nižší. Pro analýzu statinů byl používaný především HPLC systém a kolony s částicemi o velikosti 3 a 5 µm. Pouze velmi úzká skupina metod aplikuje tzv. rychlou chromatografii pomocí UHPLC [58].

Zcela zásadní při analýze statinů je udržení pH v rozmezí 4 až 5, proto použití octanu amonného a mravenčanu amonného o daném rozsahu pH je vhodnou volbou. Zcela zásadní je použití těchto pufrů při uchování a úpravě vzorků, zatímco jako součást mobilní fáze. Existuje mnoho metod využívajících kyselinu mravenčí nebo octovou [55][56][57][65][68][72].

Z tabulky je také patrné, že v případě bio-analytických metod stále ještě převažují konvenční techniky přípravy vzorků, jako je srážení proteinů a extrakce z kapaliny do kapaliny. Minimum jich využívalo některý z moderních přístupů úpravy vzorků [74] .

Velmi důležitá je také metoda vnitřního standardu při stanovování analytů v biologickém materiálu. Nejvhodnějším druhem IS jsou izotopicky značené standardy, které mohou

potlačovat vliv matrice. Více než 50 % metod neužívá SIL-IS, ale uplatňují tzv. strukturně homologické standardy, Tab. 7. V ideálním případě by měl být izotopicky značený standard pro každý analyt, což přispívá ke zvýšení přesnosti, správnosti a také k odstranění matricových efektů. V roce 2011 byla publikována práce zabývající se kvantifikací všech aktivních metabolitů, atorvastatinu a atorvastatinu laktonu, která využila deuteriem značených standardů pro každý analyt, což je v poslední době poměrně výjimečné [53].

Statiny mohou být v játrech přeměňovány na aktivní metabolity, jako například atorvastatin, který poskytuje dva aktivní hydroxy metabolity, nebo rosuvastatin, který je metabolizován na N-desmethyl rosuvastatin. Během analýzy statiny mohou podléhat interkonverzi hydroxy kyseliny statinu na laktonovou formu a zpět. Vyskytuje se v mnoha fázích přípravy vzorku: v biologickém vzorku, ještě před jeho odběrem, v průběhu extrakce, odpařování, rozpouštění a také v roztoku elučního činidla určeném k analýze. V případě použití MS detekce byla interkonverze prokázána i v iontovém zdroji. Její eliminace je možná pomocí vhodně nastavených podmínek a zvolených činidel. Stěžejní je zejména pH vzorku, mobilní fáze a extrakčních činidel, které by mělo být v rozmezí 4 až 5. Intekronverzi ve zdroji je možné odhalit dostatečnou separací analytů, v tomto případě laktonové a hydroxy formy statinů. Pro vývoj spolehlivé a přesné metody jejich stanovení je velmi důležité současné monitorování obou forem (hydroxy kyseliny a laktonu) a všech aktivních metabolitů [53].

analyt	vnitřní standard	objem vzorku	technika úpravy vzorku	kapalinová chromatografie a hmotnostně spektrometrická detekce			matrice	citace rok
				analytická kolona	mobilní faze, průtok a čas analýzy	ionizační mód, analyzátor		
AT 2-OH AT 4-OH AT ATL 2-OH ATL 4-OH ATL	AT-d_5 2-OH AT-d_5 4-OH AT-d_5 ATL-d5 2-OH ATL-d_5 4-OH ATL-d_5	0,05 ml	PP	Zorbax-SB phenyl (100 × 2,1 mm, 3,5 µm)	0,1% AcAc v 10% MeOH: 40% MeOH v ACN 0,35 ml/min gradientová eluce, 7 min	ESI, pozitivní QqQ	lidská plazma	**[54] 2011**
AT	merivapin	0,5 ml	LLE	Zorbax-SB C18 (50 x 4,6 mm, 5 mm)	0,1% FAc: ACN (15: 85) 1,0 ml/min izokratická eluce, 2,5min	ESI, pozitivní QqQ	lidská plazma	**[55] 2010**
AT 2-OH AT	diklofenak	0,025 ml	LLE On-line SPE	Sunfire C18 (100 × 2,1 mm, 3,5 µm)	100% MeOH 0,5 ml/min isokratická eluce, 4 min	ESI, pozitivní QqQ	lidská plazma	**[56] 2009**
LV	SV	0,5 ml	LLE	Kromasil C18 (150 x 4,6 mm, 5 µm)	voda: MeOH (13: 87) 1 ml/min izokratická eluce, 5 min	ESI, pozitivní QqQ	lidská plazma	**[57] 2008**
LV	SV	0,5 ml	LLE	Acquity™ UPLC BEH C18 (50 x 2,1 mm, 1,7 µm)	ACN: 5 mmol/l AcAc, (85:15) 0,35 ml/min izokratická eluce, 2 min	ESI, pozitivní QqQ	lidská plazma	**[58] 2008**
LV	nevirapin	0,5 ml	LLE	Alltima HP C_{18} (50 x 4,6 mm, 5 µm)	ACN: 5 mM AmAc (80:20) 1 ml/min izokratická eluce, 3 min	ESI, pozitivní QqQ	lidská plazma	**[59] 2011**

AT – atorvastatin, 2-OH AT – 2-hydroxy atorvastatin, 4-OH AT – 4-hydroxy atorvastatin, ATL – atorvastatin lakton, 2-OH ATL – 2-hydroxy atorvastatin lakton, 4-OH ATL – 4-hydroxy atorvastatin lakton, LV – lovastatin, SV - simvastatin, PP – srážení proteinů, LLE – extrakce z kapaliny do kapaliny, SPE – extrakce na tuhou fázi, AcAc – octová kyselina, FAc – mravenčí kyselina, AmAc – octan amonný, MeOH – methanol, ACN – acetonitril, ESI – ionizace elektrosprejem, QqQ – trojitý kvadrupól

Tab. 7: Příklady LC-MS metod pro stanovení atorvastatinu a lovastatinu.

analyt	vnitřní standard	objem vzorku	technika úpravy vzorku	kapalinová chromatografie a hmotnostně spektrometrická detekce			Matrice	citace rok
				analytická kolona	mobilní faze, průtok a čas analýzy	ionizační mód, analyzátor		
SV SVA	SV-d_3 SVA-d_3	0,1 ml	SALLE	Synergy MAX-RP (50 x 2,0 mm, 2,5 µm)	ACN: voda: 0,1 M methyl-AmAc (300:145:5), 0,45 ml/min izokratická eluce, 1,5 min	ESI, pozitivní, negativní QqQ	lidská plazma	**[60] 2010**
SV	propanolol hydrochlorid	0,2 ml	PP	Gemini-C18 (50 x 2,0 mm, 3 µm)	ACN:0,5% FAc pH 3,5 (90:10) 0,2 ml/min izokratická eluce, 5 min	ESI, pozitivní QqQ	lidská plazma	**[61] 2009**
SV SVA	LV LVA	0,3 ml	PP + LLE	YMC ODS-A C18 (50 x 4,0 mm, 5 µm)	ACN: 5 mM AmAc pH 4,5 (82:18) 0,7 ml/min izokratická eluce, 2 min	ESI, pozitivní, negatiní QqQ	lidská plazma	**[59] 2008**
FV	RV	0,2 ml	SPE	Chromolith speed ROD RP-18e (50 x 4,6 mm)	MeOH: 10 mM AmAc (98:2) 1,5 ml/min izokratická eluce, 1,5 min	ESI , negativní QqQ	Lidská plazma	**[63] 2006**
FV	warfarin	0,5 ml	LLE	Chiralcel OD-R (250 x 4,6 mm, 10 µm)	ACN: MeOH: 0,1%FAc (24:36:40)	ESI, pozitivní QqQ	lidská plazma	**[64] 2006**

SV – simvastatin, SVA – simvastatin kyselina, FV – fluvastatin, LV – lovastatin, LVA – lovastatin kyselina, PP – srážení proteinů, LLE – extrakce z kapaliny do kapaliny, SPE – extrakce na tuhou fázi, SALLE – extrakce z kapaliny do kapaliny pomocí vysolování, AmAc – octan amonný, FAc – mravenčí kyselina, MeOH – methanol, ACN – acetonitril, ESI – ionizace elektrosprejem, QqQ – trojitý kvadrupól

Tab. 8: Příklady LC-MS metod pro stanovení simvastatinu a fluvastatinu.

analyt	vnitřní standard	objem vzorku	technika úpravy vzorku	kapalinová chromatografie a hmotnostně spektrometrická detekce			matrice	citace rok
				analytická kolona	mobilní faze, průtok a čas analýzy	ionizační mód, analyzátor		
RV	estron	1 ml	LLE	Luna C_{18} (150 x 4,6 mm, 5 µm)	ACN:2% FAc: (80:20) 0,25 ml/min izokratická eluce, 5 min	ESI, pozitivní QqQ	lidská plazma	**[65] 2007**
RV	dichlorthiazid	0,2 ml	LLE	Zorbax XDB-C18 (150 x 4,6 mm, 5 µm)	ACN: roztok amoniaku pH 6, (75: 25), 0,5 ml/min izokratická eluce, 3 min	ESI, negativní QqQ	lidská plazma	**[66] 2007**
RV	RV-d6	1,7 ml	automatická SPE	Luna C_{18} (150 x 4,6 mm, 5 µm)	MeOH:0,2% FAc (70:30) 1 ml/min, izokratická eluce, 5,5 min	ESI, pozitivní QqQ	lidská plazma	**[67] 2002**
PV	hydrochlorthiazid	0.5 ml	SPE	Betabasic C8 (100 x 4,6 mm, 5 µm)	0,1 % amoniak: ACN, (20:80) 0,5 ml/ min izokratická eluce, 3 min	ESI, negativní QqQ	lidská plazma	**[68] 2007**
PV 3α- isoPV 6α –epiPV	PV- d_3	0,02 ml	PP (ACN)	Atlantis dC18 (150 x 2,1 mm, 3 µm)	ACN: 0,1% FAc ve vodě (29: 71) 0,5 ml/min izokratická eluce, 10min	ESI, negativní QqQ	myší plazma, tkáňový homogenát	**[69] 2010**
PV PVL 3-OH PV	triamcinolone	1,4 ml	automatická SPE	Synergi Max-RP (150 × 2,0 mm, 4 µm)	ACN: MeOH: 5mM AmAc pH 4,5 (30: 30: 40), 0,2 ml/min izokratická eluce, 8 min	ESI, pozitivní QqQ	lidská plazma	**[70] 2008**

PV – pravastatin, PVL – pravastatin lackton, RV – rosuvastatin, PP – srážení proteinů, LLE – extrakce z kapaliny do kapaliny, SPE – extrakce na tuhou fázi, AmAc – octan amonný, FAc – mravenčí kyselina, MeOH – methanol, ACN – acetonitril, ESI – ionizace elektrosprejem, QqQ – trojitý kvadrupól

Tab. 9: Příklady LC-MS metod pro stanovení rosuvastatinu a pravastatinu.

analyt	vnitřní standard	objem vzorku	technika úpravy vzorku	kapalinová chromatografie a hmotnostně spektrometrická detekce			Matrice	Citace rok
				analytická kolona	mobilní faze, průtok a čas analýzy	ionizační mód, analyzátor		
PTV PTVL	racemic i-prolakt	0,2 ml	LLE	BDS Hypersil C_8 (50 x 2,1 mm, 3 µm)	MeOH: 0,2% AcAc (70:30) 0,2 ml/min Izokratická eluce, 3 min	ESI, pozitivní QqQ	lidská plazma, moč	**[71] 2008**
PTV	RV	0,5 ml	SPE	Shim-pack VP-ODS (150 × 4,6 mm, 5 µm)	MeOH: voda: FAc (75:25:0,05) 1 ml/min Izokratická eluce, 6 min	ESI , pozitivní QqQ	lidská plazma, moč	**[72] 2008**
PTV	FV	0,3 ml	SPE	Luna C_{18} (50 × 2,0 mm, 3 µm)	ACN: 0,1% FAc (90:10) Izokratická eluce, 2 min	ESI, negativní QqQ	lidská plazma	**[73] 2008**

PTV – pitavastatin, PTVL – pitavastatin lakton, LLE – extrakce z kapaliny do kapaliny, SPE – extrakce na tuhou fázi, AcAc – octová kyselina, FAc – mravenčí kyselina, MeOH – methanol, ACN – acetonitril, ESI – ionizace elektrosprejem, QQQ – trojitý kvadrupól

Tab. 10: Příklady UHPLC-MS metod pro stanovení pitavastatinu.

4. VÝSLEDKY A DISKUSE

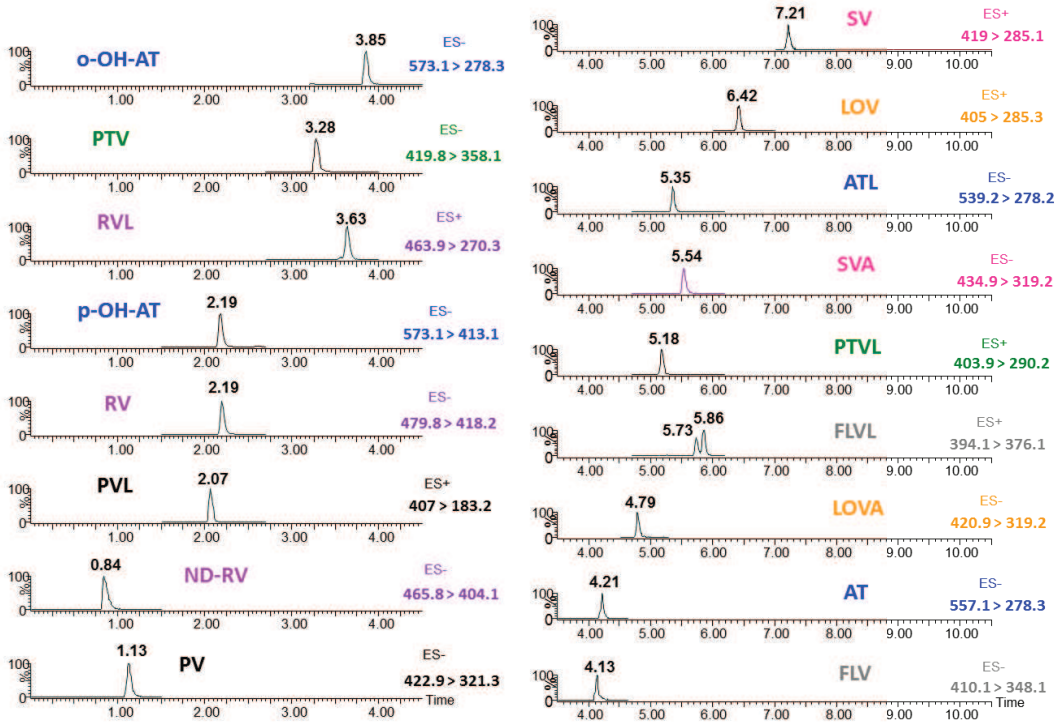
Výsledky prezentované v této disertační práci byly naměřeny na katedře analytické chemie Farmaceutické fakultě v Hradci Králové a ve výzkumné laboratoři Gerontologické a metabolické kliniky ve Fakultní nemocnici v Hradci Králové.
Výsledky lze rozdělit podle použité techniky úpravy vzorků do dvou celků: využití konvenční techniky extrakce na tuhou fázi v bio-analytických stanoveních statinů a využití moderní techniky mikroextrakce tuhým sorbentem pro stanovení statinů v biologických vzorcích.

4.1. VYUŽITÍ KONVENČNÍ TECHNIKY EXTRAKCE NA TUHOU FÁZI PRO BIO-ANALYTICKÉ STANOVENÍ STATINŮ

Cílem práce zařazené do této části byl vývoj a validace UHPLC-MS/MS metod s využitím extrakce na tuhou fázi jako techniky úpravy vzorků před chromatografickou separací.
Jednalo se zejména o vyvinutí rychlé, citlivé a selektivní metody pro **stanovení atorvastatinu, simvastatinu, jejich metabolitů a interkonverzních forem s použitím SPE-UHPLC-MS/MS**. Pro separaci byla využita kolona Acquity BEH C18 s částicemi menšími než 2 µm, což umožňuje vyšší účinnost a zkrácení doby analýzy. Optimalizace složení mobilní fáze musela být prováděna s ohledem nejen na hmotnostní detekci, ale i stabilitu analytů. Byly testovány tři typy aditiv při hodnotách pH 4,0 a 4,5 a různých koncentracích, ale i další aditiva jako kyselina mravenčí, octová a amoniak. 0,5 mM octan amonný pH 4,0 ve směsi s ACN byl zvolen jako mobilní fáze. Byl použit průtok 0,25 ml/min a gradientová eluce. Kvantifikace jednotlivých analytů byla provedena v ESI^+ módu. 2 SRM přechody pro každý analyt a deuteriem značené vnitřní standardy přispěly ke zvýšení selektivity a identifikační hodnoty metody. Protože metoda měla být využita pro stanovení cílových analytů v biologickém materiálu, musela být vyvinutá také metoda úpravy vzorku před chromatografickou analýzou. S ohledem na vysokou výtěžnost a čistotu eluátu byla zvolena technika extrakce na tuhou fázi. Byly vyzkoušeny tři typy extrakčních kolonek, z nichž nejvhodnější byla Discovery DSC-18 (100mg, 1ml). Vymytí analytů z kolonky bylo provedeno pomocí směsi acetonitrilu a 0,1 M octanu amonného o pH 4,5 v poměru 95:5.
Vyvinutá metoda byla validována a následně aplikována na reálné vzorky. Validace byla provedena v souladu ICH směrnicí a splnila veškeré její požadavky. Byl proveden test způsobilosti systému (SST). Opakovatelnost retenčního času byla < 1 % a opakovatelnost plochy píku < 7 % pro všechny analyty. Linearita byla ověřována nejen pomocí standardní kalibrační křivky v rozsahu 0,1 – 100 nmol/l, ale také na základě matricové kalibrační křivky ve stejném koncentračním rozsahu s korelačními koeficienty > 0,999 pro všechny analyty s výjimkou simvastatin kyseliny (0,998). Přesnost a správnost metody byla ověřena na třech koncentračních hladinách s mezidenní přesností < 15 % a výtěžností v rozsahu 75 – 100% pro všechny analyty. Dostatečná citlivost s LOQ 0,08 – 5,46 nmol/l umožnila využití této techniky k monitorování hladin statinů v biologických tekutinách. V porovnání s některými dříve vyvinutými metodami používá deuteriem značené standardy jako vnitřní standard a stanovuje také jednotlivé laktonové formy a metabolity [2][55][56]. Z finančních důvodů se ovšem nezabývá stanovením metabolitů laktonových forem atorvastatinu, jak je tomu v metodě publikované v roce 2011 [54]. Výsledky této práce byly publikovány v odborném mezinárodním časopise Journal of Chromatography B (Příloha I).

Na tuto práci navazuje studie provedená ve spolupráci s Fakultní nemocnicí v Hradci Králové. **Vyvinutá a validovaná metoda stanovení atorvastatinu a simvastatinu a jejich metabolitů byla využita pro reálné vzorky séra a lipoproteinových frakcí HDL, LDL a VLDL pacientů s familiární hypercholesterolémiía léčených vysokými dávkami statinů.** Jedná se dosud o jedinou metodu, která se zabývá stanovením statinů v lipoproteinových frakcích. V případě homozygotní formy FH je léčba statiny nedostatečná a pro tyto pacienty je nezbytná kombinace užívání velmi vysokých dávek statinů s extrakorporální eliminační procedurou (EEP) odstraňující LDL cholesterol. Protože vysoké dávky cholesterolu zvyšují výskyt nežádoucích účinků, je zcela stěžejní monitorovat hladiny statinů v séru těchto pacientů. Kvůli složitosti a finanční náročnosti nebylo toto stanovení zavedeno do rutinní praxe klinických laboratoří. Hlavním cílem této studie bylo ověřit a případně kvantifikovat možné ztráty statinů při EEP. Dále byla snaha objasnit mechanismus ztráty statinů během EEP a upravit dávkovací schéma tak, aby byly ztráty minimalizovány.
V průběhu experimentu byla zjištěna snížená koncentrace statinů v séru pacientů po EEP, čímž se ověřila domněnka vlivu EEP na hladinu statinů a tudíž jejich účinek. Přítomnost statinů byla také prokázána v proplachových tekutinách a filtrech používaných během EEP. Tyto informace vedly k objasnění mechanismu způsobených ztrát a byly nápomocné k úpravě dávkovacího schématu tak, aby časový odstup mezi podáním léčiva a provedením EEP procedury byl vyšší než je vlastní biologický poločas léčiva, tudíž 6 hodin. Výsledky studie byly publikovány v odborném mezinárodním časopise (Příloha II).

Pro velký zájem externích pracovišť o analýzu jednotlivých druhů statinů, byl započat **vývoj kompletní metody pro stanovení všech klinicky významných statinů a jejich metabolitů v lidském séru.** Jedná se o analýzu celkem 17 analytů, pro které byla vyvinuta UHPLC-MS/MS metoda. Byla použita kolona Acquity BEH C18 (50 x 2,1 mm, 1,7 µm), mobilní fáze směs acetonitrilu a 0,5 mM octanu ammoného pH 4,0 a průtok 0,3 ml/min. Ionizace elektrosprejem v systému přepínání polarit a SRM mód byly uplatněny pro jejich kvantifikaci. Byla ověřena linearita, opakovatelnost a citlivost metody. Korelační koeficient byl pro všechny analyty > 0,999 s výjimkou lovastatin kyseliny a limit kvantifikace nižší než 5 10^{-9} ng/ml. Pouze pro tři analyty byl stanoven vyšší limit kvantifikace. Záznam jednotlivých SRM přechodů separace analytů je na Obr. 32.
V současné době byl započat vývoj SPE metodiky pro úpravu lidského séra a bude sloužit k monitorování hladin jednotlivých statinů. V budoucnu by mohla být rozšířena nejen na analýzu dalších biologických materiálů, ale také na odpadní vody. Dosud neexistuje metoda stanovující všechny klinicky významné statiny včetně jejich laktonových forem a metabolitů v jedné analýze.



Obr. 32: Chromatogramy jednotlivých statinů, jejich laktonových forem a metabolitů.

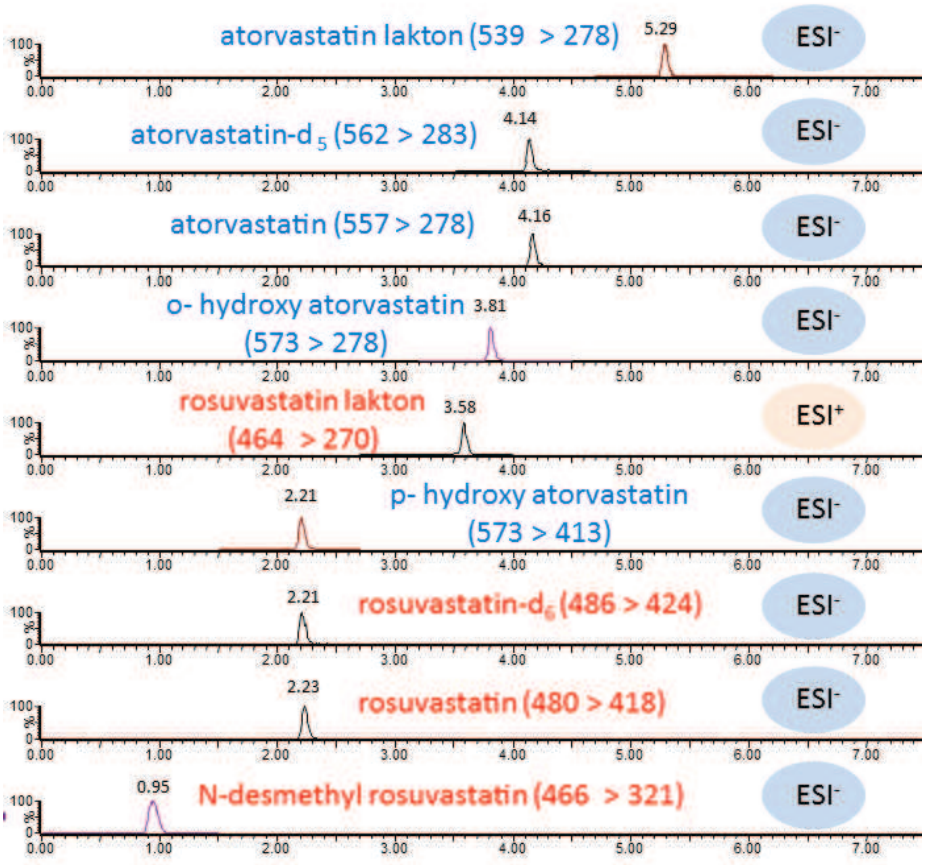
4.2. VYUŽITÍ MODERNÍ TECHNIKY MIKROEXTRAKCE TUHÝM SORBENTEM PRO STANOVENÍ STATINŮ V BIOLOGICKÝCH VZORCÍCH

K získání ucelených informací o jednotlivých trendech nejen na poli chromatografických technik, ale zejména úpravy vzorků byl sepsán souhrnný článek (Příloha III). Chromatografické techniky jsou nejrozšířenější separační technikou v bio-analytických laboratořích. Hlavním trendem v této oblasti je tzv. rychlá chromatografie, která zahrnuje několik směrů: monolitní technologii, použití kolon s částicemi s pevným jádrem, kapalinovou chromatografii za vysoké teploty a ultra vysokoúčinnou kapalinovou chromatografii. UHPLC technologie umožňuje použití částic menších než 2 µm, což přináší vyšší separační účinnost a rozlišení. UHPLC je již rozšířená i v rutinních analytických laboratořích požadujících rychlé a citlivé stanovení.
Přestože na poli HPLC došlo ke značnému pokroku a urychlení analýzy, konvenční techniky úpravy vzorků využívané v klinické praxi představují složitý a časově náročný proces v porovnání s přístupy rychlé LC. Z těchto důvodů je snaha mnoha laboratoří vyvíjet nové extrakční techniky. Hlavními směry, kterými tento vývoj směřuje je miniaturizace, zvýšení selektivity, zkrácení doby extrakce, minimalizace spotřeby rozpouštědel a potřebného objemu vzorku. V současné době existuje už několik nových přístupů přípravy vzorků, jako jsou RAM, MIP, SPME, LLME a mnoho dalších. Ovšem stále ještě nebyla vyvinuta metoda, která by v klinické praxi nahradila konvenční techniky jako PP, LLE, SPE. Zatímco většina nových extrakčních přístupů přináší značné nevýhody, MEPS je velmi perspektivní a vhodnou techniku pro úpravu biologických vzorků a díky své jednoduchosti, rychlosti a automatizaci umožňuje aplikaci i do klinické praxe.

Na základě těchto poznatků, práce v oblasti **stanovení atorvastatinu směřovala k využití MEPS techniky pro úpravu biologických vzorků před UHPLC-MS analýzou**. Byla vyvinuta rychlá a jednoduchá metoda přípravy vzorků používající malé objemy vzorku a rozpouštědel, což v klinické praxi představuje nižší zátěž pro pacienta a ekonomičtější provoz. Optimalizace metody vycházela z již dříve vyvinuté SPE extrakce (Příloha I). Optimalizovány byly zejména objemy vzorku a jednotlivých promývacích a elučních kroků. Přestože SPE technika je dostupná v automatickém i on-line provedení, v průběhu tohoto experimentu bylo využito manuálního přístupu. Bylo ověřeno, že na rozdíl od automatického provedení se manuální použití potýká s některými omezeními a nevýhodami. Dávkování objemů vzorku menších než 50 µl není vhodné pro off-line přístup, na rozdíl od on-line spojení, které může využít objemy menší jako např. 10 µl. Protože účinnost MEPS extrakce je značně závislá na rychlosti a plynulosti pohybu pístu stříkačky je on-line spojení vhodnější pro rutinní analýzu velkého počtu vzorků.
Nicméně v případě přesné a pečlivé manipulace se stříkačkou, použití větších objemů vzorku než 50 µl a udržení plynulého pohybu pístu se MEPS extrakce vyznačuje vysokou výtěžností, opakovatelností a zkrácením času extrakce. Další výhodou, která snižuje cenu analýzy, je možnost opakovaného použití sorbentu a to až 100x pro moč a sérum.
Metoda byla aplikována na reálné vzorky pacientů s FH léčených statiny v kombinaci s EEP. MEPS metoda tedy nahradila dříve vyvinout SPE metodu používanou pro stanovení atorvastatinu. Dosud byly publikovány pouze práce využívající konvenční techniky úpravy vzorků (PP, LLE, SPE) pro analýzu statinů, např. [55][57]-[66][71]-[73]. Tyto techniky představují

časově náročný proces s výjimkou on-line SPE, která byla v některých případech také využita [56][67][70]. Aplikací MEPS techniky došlo ke zkrácení času extrakce zhruba z 30 na 7 minut, což přináší značné výhody zejména v klinické praxi. Výsledky této práce byly publikovány v odborném mezinárodním časopise (Příloha IV).

Protože v klinické praxi je simvastatin v poslední době nahrazován rosuvastatinem, dalším cílem bylo **vyvinutí vhodné metody pro stanovení atorvastatinu a rosuvastatinu v lidském séru s využitím MEPS jako techniky úpravy vzorků.** Byly nalezeny vhodné podmínky UHPLC-MS/MS metody pro kvantifikaci atorvastatinu, rosuvastatinu a jejich metabolitů s využitím vnitřních standardů a SRM módu. MEPS extrakce byla prováděna pomocí C8 sorbentu. Jednotlivé analyty byly eluovány pomocí směsi acetonitrilu a octanu ammoného o pH 4,5. Při volbě vhodného promývacího činidla se musel brát ohled zejména na výtěžnost N-desmethyl rosuvastatinu, který je v porovnání s ostatními analyty výrazně hydrofilní. Maximální možný obsah acetonitrilu v promývacím činidle je 5%. Na MEPS sorbent je dávkováno 50 µl vzorku a eluce je provedena pomocí 100 µl elučního činidla.



Obr. 33: Chromatogramy standardu atorvastatinu, rosuvastatinu a jejich metabolitů.

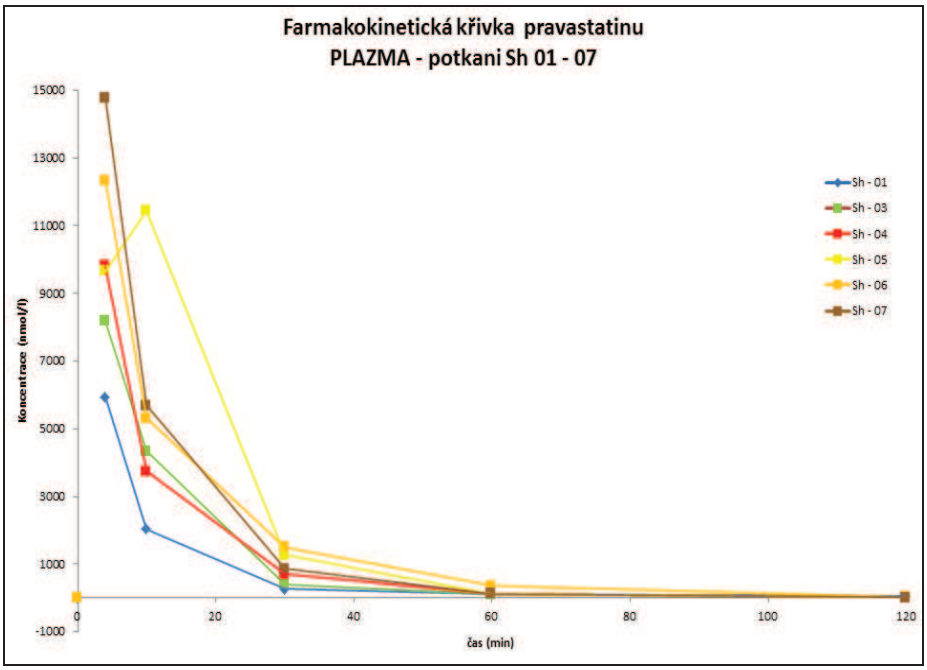
Další **MEPS-UHPLC-MS/MS metodou je stanovení pravastatinu a pravastatinu laktonu**. Na rozdíl od všech dříve popisovaných metod byla **aplikována na potkaní plasmu a moč**. Chromatografická separace byla provedena na koloně Acquity BEH C18 (50 x 2,1 µm, 1,7 µm) s mobilní fází ACN: 1 mM AmAc pH 4,0 (05:95) a průtokem 0,2 ml/min. V průběhu hmotnostní detekce bylo využito systému přepínání polarit, umožňující ionizaci pravastatinu v negativním

módu a pravastatinu laktonu v pozitivním módu. Kvantita jednotlivých analytů byla provedena pomocí SRM skenů pro každý analyt, což zvyšuje selektivitu dané metody.

PP a MEPS extrakce byly použity jako techniky úpravy plazmy, zatímco pro úpravu moči byla dostačující mikroextrakce tuhým sorbentem. Srážení proteinů umožňuje snadné a rychlé odstranění proteinů, což zvyšuje životnost MEPS sorbentů. Samotné PP není však vlivem nedostatečné účinnosti čištění vhodné. MEPS byla zvolena kvůli mnoha výhodám, jako je například miniaturizace, která umožní analýzu potkaních vzorků, které jsou dostupné ve velmi malém objemu. Extrakce byla provedena pomocí eVol poloautomatické MEPS pipety, která umožňuje snadnější a přesnější manipulaci se stříkačkou a odstraňuje negativní vliv operátora.

V průběhu vývoje metody se vyskytlo několik problémů. Jedním z nich je interkonverze hydroxy formy pravastatinu na jeho laktonovou formu. K potlačení interkonverze je zcela klíčové udržení pH v rozmezí 4 a 5, což značně omezuje výběr vhodného srážecího, promývacího a elučního činidla. Dalším problémem byl výskyt matricových efektů, které se nepodařilo odstranit pomocí extrakčních technik. V tomto ohledu bylo využito vyšší separační účinnosti UHPLC systému, který společně s deuteriem značenými vnitřními standardy. (PV-d_3, PVL-d_3) pomohl k částečnému potlačení vlivu matrice.

Nově vyvinutá MEPS-UHPLC-MS/MS metoda pro stanovení pravastatinu a pravastatinu laktonu byla aplikována na vzorky plazmy a moči potkanů, kterým byl podán pravastatin. Od každého potkana byla odebrána sekvence vzorků, která umožnila sestrojení farmakokinetických křivek a zároveň napomohla objasnit způsob exkrece pravastatinu, Obr. 34. Výsledky studie byly shrnuty v publikaci a odeslána k publikaci do odborného mezinárodního časopisu (Příloha V).



Obr. 34: Farmakokinetické křivky potkanů léčených pravastatinem.

5. ZÁVĚR

Disertační práce „Stanovení statinů v biologickém materiálu“ se zabývá využitím trendů v oblasti kapalinové chromatografie a úpravy vzorků zaměřených na bio-analytické aplikace. Trendy v oblasti chromatografických metod směřují ke zrychlení analýzy a zvýšení separační účinnosti. Vývoj v oblasti úpravy vzorků je zaměřen především na miniaturizaci, automatizaci, selektivitu, snížení času extrakce a objemu vzorku a rozpouštědel.

Bylo vyvinuto několik citlivých UHPLC-MS/MS metod pro stanovení statinů v biologickém materiálu. Jako techniky úpravy vzorků bylo využito jednak konvenční SPE extrakce, ale také mikroextrakční metody MEPS, která přinesla mnoho výhod. Jedná se zejména o zkrácení doby extrakce, snížení objemu vzorků a použitých rozpouštědel. Využití malého objemu vzorku umožnilo aplikaci dané metody na potkaní plazmu, která je dostupná v omezeném množství.

Protože statiny mohou během analýzy podléhat interkonverzi z hydroxy kyseliny statinu na laktonovou formu a zpět, všechny vyvinuté metody stanovovaly nejen hydroxy kyselinu, ale i laktonovou formu. V průběhu vývoje metod byla snaha o minimalizaci interkonverze a to zejména úpravou pH vzorku a jednotlivých roztoků použitých v průběhu extrakce. V případě použití MS detekce může k interkonverzi docházet také v iontovém zdroji. Tento děj je možné odhalit na základě dostatečné separace hydroxy kyseliny a laktonu. Statiny mohou být také v játrech metabolizovány na aktivní metabolity, a proto bylo důležité monitorovat i hladiny metabolitů statinů.

Na základě výše zmíněných vlastností statinů je pro klinické účely zcela zásadní současně s jednotlivými statiny stanovovat také jejich metabolity a laktonové formy, což všechny metody zahrnuté v této práci splňují. Byly vyvinuty metody pro stanovení atorvastatinu, simvastatinu, pravastatinu a rosuvastatinu, jejich metabolitů a laktonových forem.

Vyvinuté metody byly publikovány v mezinárodních odborných časopisech s impakt faktorem a jejich kopie tvoří nejrozsáhlejší část disertační práce.

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7. PŘÍLOHY

7.1. PŘEHLED PRACÍ ZAHRNUTÝCH DO DISERTAČNÍ PRÁCE

PŘÍLOHA I

NOVÁKOVÁ Lucie, VLČKOVÁ Hana, ŠATÍNSKÝ Dalibor, SADÍLEK Petr, SOLICHOVÁ Dagmar, BLÁHA Milan, BLÁHA Vladimír, SOLICH Petr

Ultra high performance liquid chromatography tandem mass spectrometric detection in clinical analysis of simvastatin and atorvastatin

Journal of Chromatography B, 2009, vol. 877, p. 2093–2103.

(IF 2,971)

Počet citací: 8

PŘÍLOHA II

BLÁHA Milan, VLČKOVÁ Hana, NOVÁKOVÁ Lucie, SOLICHOVÁ Dagmar, SOLICH Petr, LÁNSKÁ Miriam, MALÝ Jaroslav, BLÁHA Vladimír

The Importance of ultra high performance liquid chromatography tandem mass spectrometry in clinical monitoring of simvastatin, atorvastatin and its metabolites after extracorporeal LDL-cholesterol elimination

Journal of Biomedicine and Biotechnology, 2011, vol. 2011, článek ID 912471, 9 stran.

(IF 1,23)

Počet citací: 0

PŘÍLOHA III

NOVÁKOVÁ Lucie, VLČKOVÁ Hana

Current trends and advances in modern bio-analytical methods: chromatography and sample preparation- a review

Analytica Chimica Acta, 2009, vol. 656, p. 8-35.

(IF 4,311)

Počet citací: 45

PŘÍLOHA IV

VLČKOVÁ Hana, SOLICHOVÁ Dagmar, BLÁHA Milan, SOLICH Petr, NOVÁKOVÁ Lucie

Microextraction by packed sorbent as sample preparation step for atorvastatin and its metabolites in biological samples - critical evaluation

Journal of Pharmaceutical and Biomedical Analysis, 2011, vol. 55, p. 301–308.

(IF 2,733)

Počet citací: 1

PŘÍLOHA V

VLČKOVÁ Hana, RABATINOVÁ Martina, KOLOUCHOVÁ Gabriela, MIČUDA Stanislav, SOLICH Petr, NOVÁKOVÁ Lucie

Determination of pravastatin and pravastatin lactone in rat plasma and urine using UHPLC- MS/MS and microextraction by packed sorbent as the sample preparation step

podáno k publikaci.

Počet citací:

7.2. PŘEHLED PRACÍ NEZAHRNUTÝCH DO DISERTAČNÍ PRÁCE

1. GREGUŠ Petr, VLČKOVÁ Hana, BUCHTA Vladimír, KESTŘANEK Jan, KŘIVČÍKOVÁ Lucie, NOVÁKOVÁ Lucie **UHPLC-MS/MS in qualitative and quantitative analysis of quorum sensing molecules of candida albicans**
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   Počet citací:

7.3. PŘEDNÁŠKY A POSTERY PREZENTOVANÉ NA KONFERENCÍCH

PŘEDNÁŠKY

1. VLČKOVÁ Hana
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6th Nordic separation science society conference, Riga, Lotyšsko, 24. – 27.10.2011

7.4. JEDNOTLIVÉ PŘÍLOHY

PŘÍLOHA I

NOVÁKOVÁ Lucie, VLČKOVÁ Hana, ŠATÍNSKÝ Dalibor, SADÍLEK Petr, SOLICHOVÁ Dagmar, BLÁHA Milan, BLÁHA Vladimír, SOLICH Petr

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Journal of Chromatography B, 2009, vol. 877, p. 2093–2103.

Journal of Chromatography B, 877 (2009) 2093–2103

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Ultra high performance liquid chromatography tandem mass spectrometric detection in clinical analysis of simvastatin and atorvastatin

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ARTICLE INFO

Article history:
Received 25 January 2009
Accepted 26 May 2009
Available online 17 June 2009

Keywords:
Atorvastatin
Simvastatin
Hemodialysis
UPLC
Tandem mass spectrometry
Bio-analytical method

ABSTRACT

Simvastatin and atorvastatin belong to the group of hypolipidemic drugs, more exactly to the second generation of inhibitors of microsomal 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. They induce a significant reduction in total cholesterol, low-density lipoprotein cholesterol and plasma triglycerides, therefore they are widely used in the treatment of hypercholesterolemia even of its severe form-familiar hypercholesterolemia. Simvastatin and atorvastatin as the most widely used statins in clinical treatment and their hydroxy-acid/lactone forms were determined by means of UPLC in connection with triple quadrupole mass spectrometer. Deuterium labeled reference standard compounds were used as internal standards for the quantitation. Separation was performed on Acquity BEH C18 (100 mm × 2.1 mm, 1.7 μm) using gradient elution by mobile phase containing acetonitrile and ammonium acetate pH 4.0, which is convenient in order to prevent interconversion of analytes. ESI in positive mode was used for the ionization of all compounds. Two SRM (selected reaction monitoring) transitions were carefully optimized for each analyte in order to get high sensitivity and selectivity. SPE on Discovery DSC-18 was used as a sample preparation step. Intra-day precision was generally within 10% RSD, while inter-day precision within 15% RSD. Method accuracy expressed as recovery ranged from 75 to 100%. The method was validated with the sensitivity reaching LOQ 0.08–5.46 nmol/l and LOD 0.01–1.80 nmol/l in biological samples. Atorvastatin, simvastatin, its metabolites and hydroxy-acid/lactone forms were monitored in human serum and in lipoprotein fractions (LDL, HDL and VLDL) at patients with end stage renal diseases.

1. Introduction

Statins are drugs widely used for the treatment of severe forms of hypercholesterolemia, such as familiar hypercholesterolemia. They have potent cholesterol-lowering effect and they could significantly reduce morbidity and mortality associated with coronary heart disease as it was proven by many clinical trials [1–4]. They possess high effectiveness in reducing total cholesterol and low-density lipoprotein (LDL) cholesterol levels in human body. HMG-CoA reductase is the key enzyme that catalyzes the conversion of HMG-CoA to mevalonate, which is an early rate-limiting step in the cholesterol biosynthetic pathway. Statins are effective HMG-CoA inhibitors, however some of statins exhibit a number of adverse effects, such as myopathy or rhabdomyolysis [1]. Therapeutic range of statins is typically 10–80 mg/day. Maximum plasma concentration (c_{max}) has been reported to be 27–66 ng/ml for atorvastatin and 10–34 ng/ml for simvastatin [5,6]. High doses could be used with caution in the elderly, in patients with renal or hepatic insufficiency, hypothyroidism or diabetes. Therapeutic drug monitoring is not routinely done in patients treated by statins. They are only advised to report to their doctors if muscle aches, pains or weakness develop. Therefore it would be highly convenient and helpful to monitor the levels of statins in biological materials in order to establish and control appropriate dosage scheme, which would minimize adverse effects and keep the cholesterol lowering effect. Moreover, the method is useful when some extracorporeal elimination procedure (e.g. hemodialysis) is used in order to determine if loses of statins do not occur during the procedure.

Patients with chronic renal disease often suffer from a secondary form of complex dyslipidaemia [7]. The most important abnormalities in the lipid profile are an increase in triglyceride levels, the presence of small, dense LDL particles and low high-density

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1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.05.052

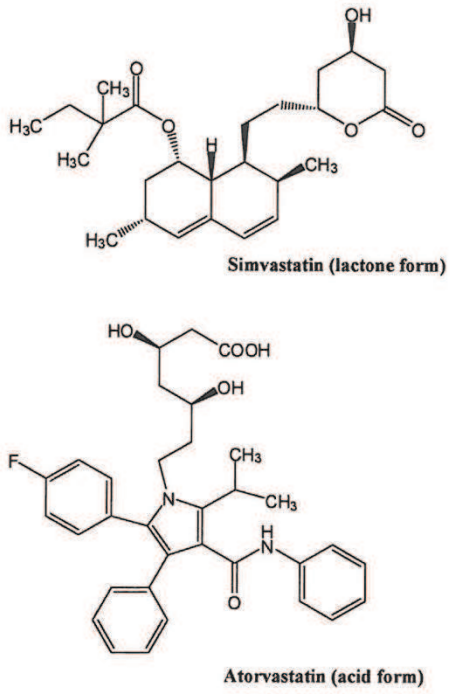

Fig. 1. Chemical structures of simvastatin and atorvastatin.

lipoprotein (HDL) cholesterol levels. The increase in triglyceride levels is due to elevated levels of very-low-density lipoprotein (VLDL) remnants and intermediate-density lipoprotein (IDL). Each of these parameters has been associated with increased risk of cardiovascular disease [7].

Atorvastatin and simvastatin are two drugs worldwide the most commonly occurred in commercially available pharmaceutical formulations used in the clinical treatment of hypercholesterolemia. Structures could be seen in Fig. 1. Because of the complex and difficult-to-treat dyslipidaemia in dialysis patients, higher doses of statin might be of value in the treatment of hypercholesterolaemic patients on hemodialysis. Hemodialysis is not expected to enhance significantly the clearance of statin, since the drug is extensively bound to plasma proteins (atorvastatin 80–90%, simvastatin 94–98%). However, it is known that renal dysfunction may hamper the hepatic metabolism of drugs [8,9], which could lead to accumulation of statin and/or its long-lived metabolites, in turn increasing the risk of clinically important adverse events such as rhabdomyolysis. Moreover, as both atorvastatin and simvastatin are lipophilic agents, it can be assumed that not only the changes in liver lipoprotein metabolism, but also the distribution of statin in already abnormally modified lipoprotein fractions can be clinically important. This warrants the performance of supplemental studies on the plasma statin levels and its distribution in lipoprotein fractions, and this is the reason why the study of the presence of atorvastatin in different lipoprotein fractions would be of high significance.

Statin molecules exist in two forms, lactone and open-ring hydroxy acid form [10,11]. In vivo, the hydroxy acid forms are the active drugs to lower plasma cholesterol while the lactone forms are inactive (prodrug). Lactone form of statin can be absorbed from the gastrointestinal tract and transformed to the active drugs in liver and non-hepatic tissues [11]. Simvastatin is a prodrug, which is administered as an inactive lactone form. The lactone is absorbed from gastrointestinal tract and hydrolyzed to the active β-hydroxy acid form in the liver [12,13].

Atorvastatin is administered in the open-ring hydroxy acid form—the active form. It is absorbed from the gastrointestinal tract and it undergoes extensive first-pass metabolism in the liver. Liver metabolism produces two active hydroxy metabolites being ortho-hydroxyatorvastatin and para-hydroxyatorvastatin and three inactive corresponding lactone forms. More than 90% is bound to plasma proteins. About 70% of the total plasma HMG-CoA activity is attributed to active metabolites of atorvastatin, even if their concentrations are very low [12–14]. As it figures out from the information above, the levels of statins in biological fluids are very low, probably because only about 5% of dosed statin reaches the systemic circulation. Typical plasma concentrations are in ng/ml levels. The active metabolites of atorvastatin are present at plasma concentration corresponding to pg/ml levels [13], typical concentration range being between 0.1 and 20 ng/ml.

Statins are a typical example of drugs, where the interconversion between lactone and open-ring hydroxy acid occurs [10,11]. When the development of a method for the quantitation of two analytes that can undergo interconversion is performed—the first step is to select the conditions that will eliminate or minimize the interconversion. The second step is to judiciously select the composition of the QC samples and the composition of calibration standards, which should cover the spectrum of the composition of real samples. For the samples of hydroxy acid chemical structure and the corresponding lactone forms it is important to maintain pH between 4 and 5 in order to minimize interconversion. Increasing the pH above 6 facilitates the conversion of the lactone to the acid (in the ionized form), contrariwise, lowering pH facilitates the conversion of the acid to lactone form or the lactone to the acid (in the non-ionized form). The most of assays utilizes pH around 4.5 [10–15].

As it figures out from the different structures of simvastatin and atorvastatin, analytical methods for their quantitative determination were developed individually. Because of the structure properties, there are not many analytical methods which determine these two compounds together in one analytical run or even in combination with other statin molecules. This is also probably due to the fact, that statins are not used with other statins simultaneously during the treatment of hyperlipidemic patients. The methods for the determination of simvastatin and atorvastatin were recently reviewed by our group [15]. In clinical applications HPLC-MS/MS was unequivocally the method of choice in analysis of both simvastatin [16–21] and atorvastatin together with its metabolites [22–25] using typically ESI (electrospray ionization) in positive ion mode.

The aim of this work was to develop fast, reliable, sensitive and selective analytical method for the determination of simvastatin and atorvastatin together with metabolites and lactone/hydroxy-acid interconversion forms using UPLC-MS/MS method. In spite of the fact, that statins are not use simultaneously during the treatment of hypercholesterolemia, such a procedure is useful in daily routine sample handling, when many samples from patients taking either atorvastatin or simvastatin are analyzed in one laboratory. Thus the laboratory does not need to distinguish among samples to be analyzed, to perform two different procedures for individual statins, which increases the sample throughput of the laboratory.

2. Experimental

2.1. Chemicals and reagents

Working standards of simvastatin were obtained from Sigma–Aldrich (Prague, Czech Republic). Working standards of simvastatin acid, atorvastatin lactone and atorvastatin, p-hydroxyatorvastatin, o-hydroxyatorvastatin, simvastatin deuterium labeled (D6-methyl

Table 1
Optimization of specific transitions for all analytes.

	Compound	Precursor	Precursor type	Fragment	Dwell time	Cone voltage	Collision energy	t_R
1	p-Hydroxyatorvastatin	575.0	$[M+H]^+$	440.1 466.2	0.05	30 30	20.0 15.0	2.49
1	o-Hydroxyatorvastatin	575.0	$[M+H]^+$	440.1 466.2	0.05	30 30	20.0 15.0	2.89
2	Atorvastatin	559.0	$[M+H]^+$	440.1 466.1	0.05	30 30	20.0 15.0	2.89
3	Atorvastatin-deuterium labeled	564.0	$[M+H]^+$	445.1 471.0	0.05	30 30	20.0 15.0	2.89
4	Atorvastatin lactone	541.0	$[M+H]^+$	448.0 422.1	0.05	30 30	15.0 20.0	3.19
5	Simvastatin acid	437.0	$[M+H]^+$	303.00 285.3	0.05	15 15	10.0 15.0	3.48
6	Simvastatin	419.0	$[M+H]^+$	199.2 285.3	0.05	20 20	10.0 10.0	4.39
7	Simvastatin-deuterium labeled	425.1	$[M+H]^+$	199.2 285.3	0.05	20 20	10.0 10.0	4.40

groups) and atorvastatin deuterium labeled (D5-phenyl ring) were purchased from Toronto Research Chemicals (Ontario, Canada).

The acetic acid, reagent grade, the ammonium, reagent grade, the formic acid, reagent grade and the acetonitrile, LC-MS grade, were purchased from Sigma–Aldrich. HPLC grade water was prepared by Milli-Q reverse osmosis Millipore (Bedford, MA, USA) and it meets European Pharmacopoeia requirements.

2.2. Chromatography

UPLC System Acquity (Waters, Prague, Czech Republic) was used for the purposes of this study. It consists of ACQ-binary solvent manager, ACQ-sample manager and ACQ-tunable UV detector. All UPLC analyses were performed on BEH C_{18} analytical column (100 mm × 2.1 mm, 1.7 μm, Waters, Prague, Czech Republic) based on Bridged Ethyl Hybrid (BEH) particles. Mobile phase was composed of acetonitrile and 0.5 mM ammonium acetate buffer pH 4.0 using gradient elution, initial mobile phase composition being acetonitrile, ammonium acetate buffer (30:70). Thereafter the concentration was changed within 1.5 min to 30% of ammonium acetate buffer and subsequently to 5% of the buffer within 5.25 min. Flow rate was 0.25 ml/min. The analytical column was kept at 35 °C by column oven. The solutions were stored in the autosampler at 4 °C. The full loop injection mode was set up to inject 5 μl using 5 μl injection loop. Acetonitrile was used as a strong wash and 20% acetonitrile in water was used as a weak wash solvent.

2.3. Mass spectrometry

The MS/MS triple quadrupole system was used for the purposes of this study. Quattro Micro (Micromass, Manchester, GB) was equipped with a Multi-Mode Ionization Source (ESCI), which combines high-speed switching between electrospray ionization (ESI) and Atmospheric Pressure Chemical Ionization (APCI) within one ion source.

Ion source set-up was carefully tuned as follows: capillary voltage: 3500 V, ion source temperature: 130 °C, extractor: 3.0 V, RF lens: 1.0 V. The desolvation gas was nitrogen at flow 500 l/h and at the temperature 375 °C. Cone voltage was set up individually for each analyte (Table 1). Nitrogen was used also as a cone gas (120 l/h) to prevent the contamination of sample cone. Quantitation of all analytes was performed in ESI positive ion mode using SRM (selected reaction monitoring) experiment. Two specific transitions were optimized for each molecule to increase selectivity of the method. Argon was used as collision gas and collision energy was optimized for each analyte individually (Table 1).

The MassLynx 4.1 Data System was used for data MS control and data gathering. QuanLynx software was used for data processing and quantitation–regression analysis of standard curves and calculation of concentrations.

2.4. Preparation of standard solutions

The stock solutions of standards were prepared by dissolving of the amount corresponding to 1.0 mmol/l concentration of appropriate working standard into 1.0 ml of solution media according to the solubility properties, because the molecules differ significantly in solubility. The stock solutions of simvastatin, simvastatin D6 and atorvastatin lactone were prepared in pure acetonitrile. The stock solutions of atorvastatin, atorvastatin D5, atorvastatin hydroxy-metabolites and simvastatin acid were prepared in mobile phase used at initial step of gradient elution—acetonitrile, ammonium acetate buffer 0.5 mM, pH 4.0 (30:70).

Stock solutions were further diluted by mobile phase (from stability reason to keep pH of solution between 4.0 and 5.0 to prevent interconversion) to achieve a concentration 10 nmol/l for SST (System suitability test) measurements, and to get individual points of calibration curve in the range 0.1–100 nmol/l, using seven calibration points (100, 50, 10, 5.0, 1.0, 0.5 and 0.1 nmol/l).

2.5. Sample preparation

Serum and lipoprotein fraction samples were prepared using SPE (solid phase extraction) procedure. These following sorbents were tested: ZORBAX SPE C-18 (100 mg, 1 ml) (Agilent Technologies), Oasis HLB (hydrophilic–lipophilic balance) SPE (60 mg, 3 ml) (Waters), and Discovery DSC-18 (100 mg, 1 ml) (Supelco). SPE columns Discovery DSC-18 were chosen as optimal for final validation of the method.

I.S. (100 μl) was added to 900 μl of the serum samples containing the analytes. This sample was diluted with 1 ml of ammonium acetate buffer and mixed. The mixture was loaded on Discovery DSC-18 sorbent previously activated with 1 ml of acetonitrile and conditioned with 1 ml of 0.1 M ammonium acetate buffer pH 4.5.

The SPE cartridge with loaded sample was washed with 1 ml of mixture acetonitrile:0.01 M ammonium acetate buffer pH 4.5 (15:85, v/v), and subsequently the analytes were eluted with 1 ml of acetonitrile:0.1 M ammonium acetate buffer pH 4.5 (95:5, v/v). The eluate was filtered via 0.20 μm PTFE filter and sample was injected onto HPLC system.

2.6. System suitability test and validation

An important part of method validation is the SST, details of which are usually given in Pharmacopoeias [26,27]. The SST was performed under optimized chromatographic conditions. In mass spectrometric methods only repeatability of retention times and peak area is checked.

Calibration curves of all analytes in the concentration range 0.1–100 nmol/l were measured. Method precision and accuracy were established. For the precision, spiked blank serum at three different concentration levels were measured in three replicates to calculate RSD, which describes the closeness of agreement between series of measurements. Accuracy was determined as a method recovery using spiked blank serum, again at three different levels in three replicates to establish the closeness of agreement between the true and measured value as it corresponds to ICH (International Conference on Harmonization) requirements [28]. QC samples were prepared at the same concentrations as were the concentration levels prepared for precision and accuracy experiments. Lyophilized standard serum samples were used for the purposes of method validation.

Selectivity and matrix effects were also verified. For the determination of selectivity the injection of blank serum treated by the same sample preparation step was used. Matrix effects were established using direct inlet by Hamilton syringe, where standard mix solution was introduced to the mass spectrometer by direct infusion and the blank serum was injected by the autosampler to observe matrix suppressions or enhancements as positive or negative peaks influencing data plot of analytes.

Limit of detection and quantitation was established based on signal-to-noise (S/N) ratio approach. Limit of detection was expressed as S/N = 3, limit of quantitation was expressed as S/N = 10.

2.7. Patients

Plasma levels of statins have been already analyzed in healthy individuals [5]. However the presence of disease or concomitant therapy are important variables modifying the plasma statin levels [6,29]. Because the aim of this work was to study potential benefit from lipid-lowering treatment by statins in a group of high-risk patients on chronic hemodialysis, and because data on statin levels in hemodialysis patients are incomplete, following patients were randomly selected.

Ten end stage renal disease (ESRD) patients (8 females, 2 males, median age 68 years (range 55–83 years)) on chronic hemodialysis (median duration 30 months, range 4–63 months) were randomly selected. All the patients were recruited at the hemodialysis center in Hradec Králové, Czech Republic. Hemodialysis was performed for three times a week, using bicarbonate buffer and polysulfone dialysis membranes. Dialysis adequacy was estimated by Kt/V according to Daugirdas formula [30]. All patients were on a stable anticoagulation regimen using heparin. None of the patients showed clinical evidence of any acute disease, had malignancies, took corticosteroids, or immunosuppressive therapy at the beginning of the study. All of the patients were informed and Local Ethics Committee of our hospital approved the study.

The following concomitant drugs were not permitted during this study: (i) other lipid-lowering drugs or preparations (acipimox, niacin, fibrates, bile sequestrants, other statins, soluble fibre preparations like psyllium and Metamucil); (ii) other drugs known to modulate lipid parameters (corticosteroids, isotretinoin); (iii) antioxidant vitamins; (iv) immunosuppressive drugs; (v) drugs known to be associated with myopathy in combination with HMG-CoA reductase inhibitors, due to competition for metabolic pathways (cyclosporin, macrolide antibiotics, azole antifungals). Permitted medications, e.g. antihypertensive drugs and phosphate-binding drugs, were to be kept constant throughout the study, both in dosage and time of intake. The occasional use of antacids was permitted. Any concurrent medications were to be taken at least 30 min after the study medication. Patients were asked not to change their eating habits during the course of the study.

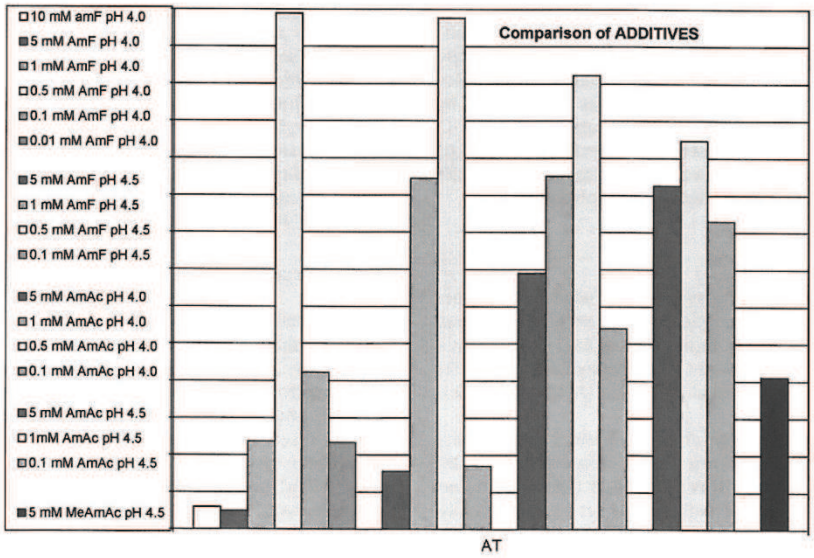

Fig. 2. Optimization of mobile phase additives—the influence of ammonium formate and ammonium acetate at various pH and concentrations.

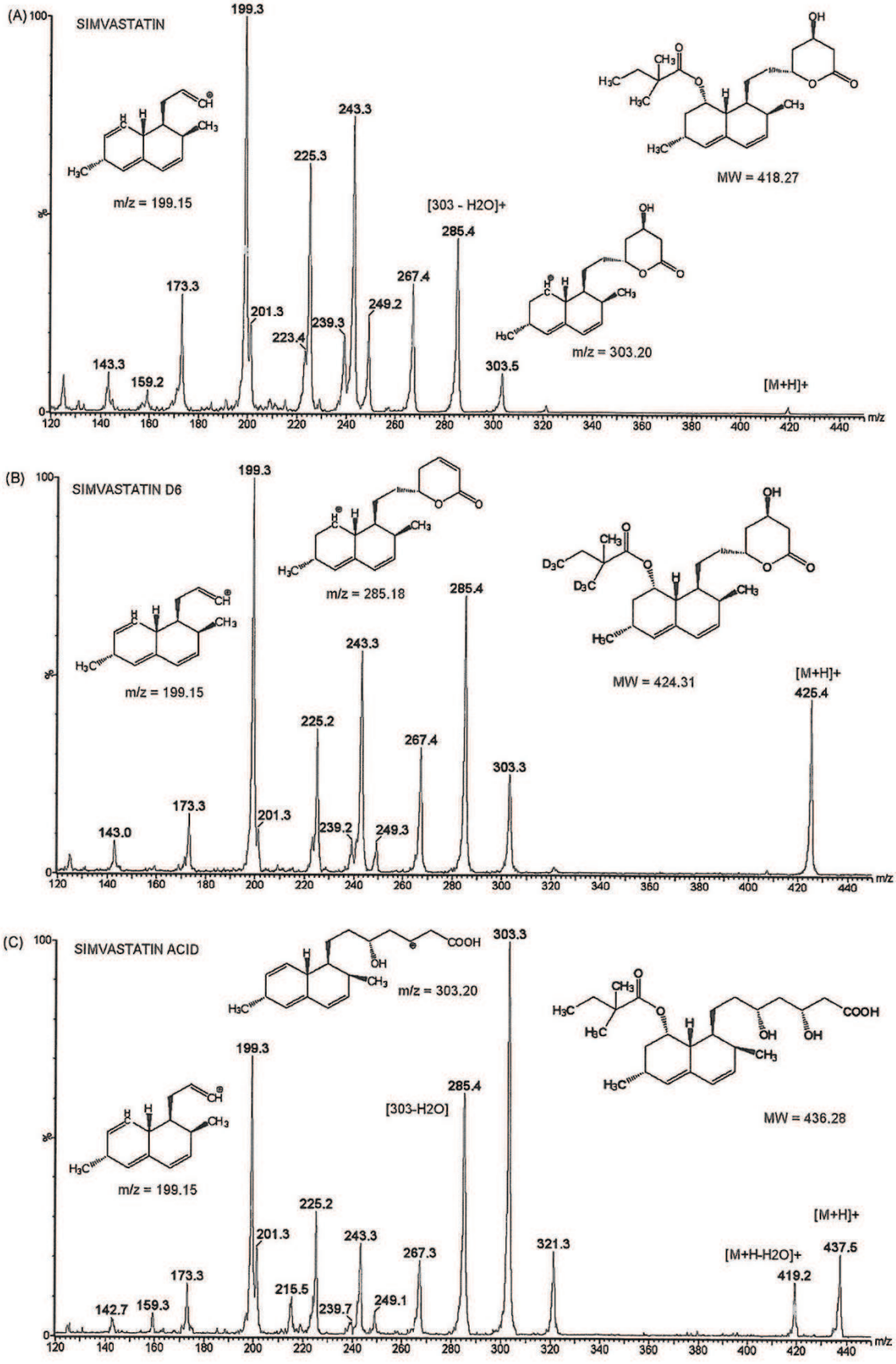

Fig. 3. Product ion spectra of simvastatin (A), simvastatin D6 (B) and simvastatin acid (C).

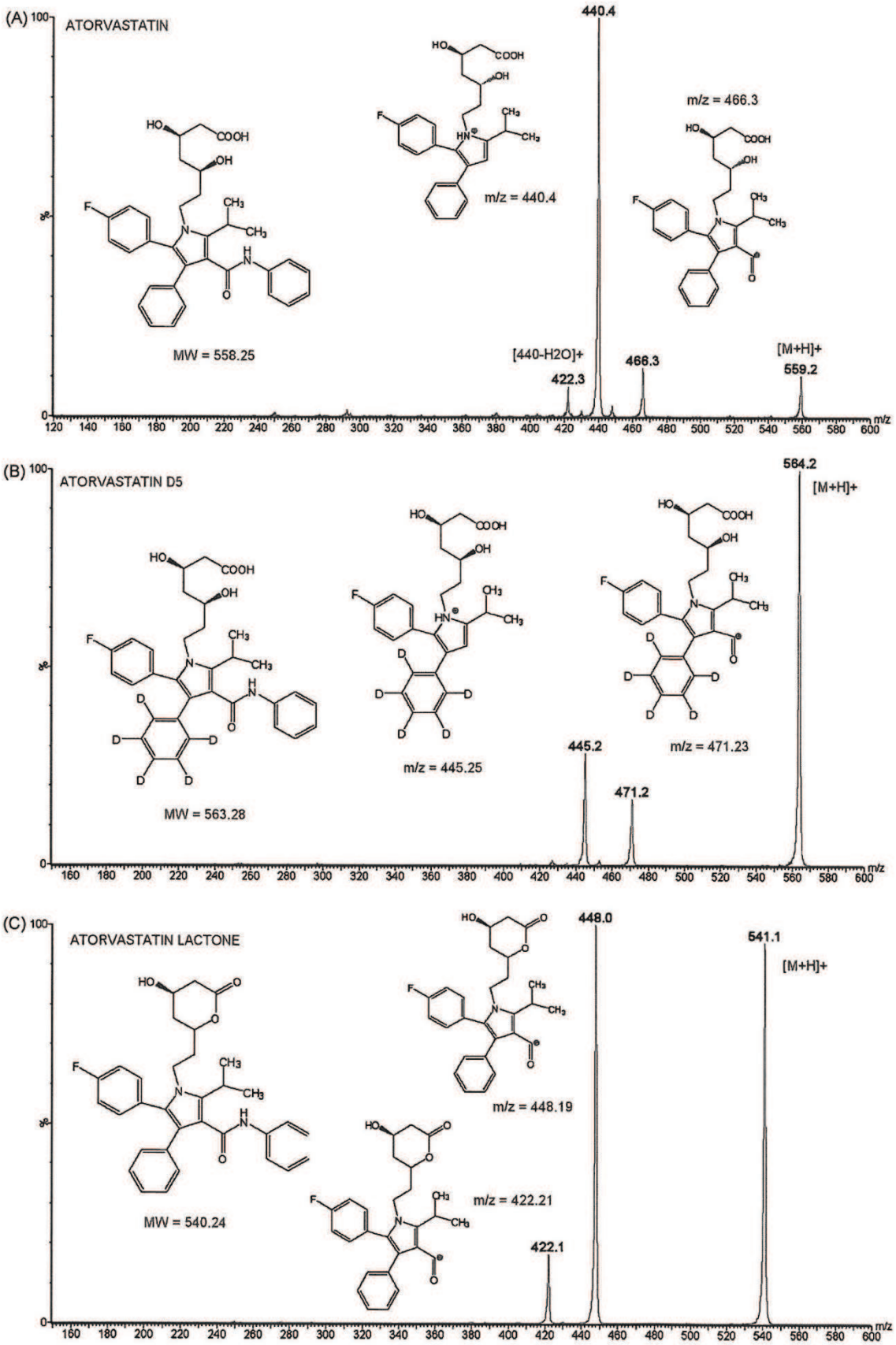

Fig. 4. Product ion spectra of atorvastatin (A), atorvastatin D5 (B) and atorvastatin lactone (C).

2.7.1. Protocol of drug administration and blood sampling

Participants were treated by 40 mg of atorvastatin or 20 mg of simvastatin daily. Drug intake had to be performed at 9.00 p.m. and started at least 4 weeks prior the study.

2.8. Biochemical analyses

For the evaluation of statin levels and its metabolites, two blood samples were taken: one just before the start of the dialysis session, the second just after dialysis. Blood samples were collected at the beginning of the study. The blood was drawn from needle inserted in vascular access for dialysis in fasting state before the start of hemodialysis at 7.00 a.m., and the second blood sample was drawn just after dialysis i.e. after 4 h of hemodialysis. After separation, serum aliquots were stored at −80 °C until analysis. The samples were assayed in random order. All samples were analyzed by personnel who had no knowledge of the subjects' clinical data. Serum lipoprotein fractions were prepared using NaCl density gradient ultracentrifugation (Beckman TL 100, Palo Alto, USA). The lipoprotein fractions were distinguished in the following density ranges: VLDL < 1.006 g/ml; LDL < 1.063 g/ml; HDL > 1.063 g/ml

3. Results and discussion

3.1. Ultra performance liquid chromatography and mass spectrometry

UPLC was used as separation method for the analysis of statins, their interconversion products and metabolites. In early experiments isocratic elution was applied. Minimally 70% of acetonitrile were necessary to elute statins in reasonable retention times. The separation was developed with the regard to the stability of analytes and mass spectrometric detection, which is quite limited in terms of solvents that could be used. Only few additives could enable

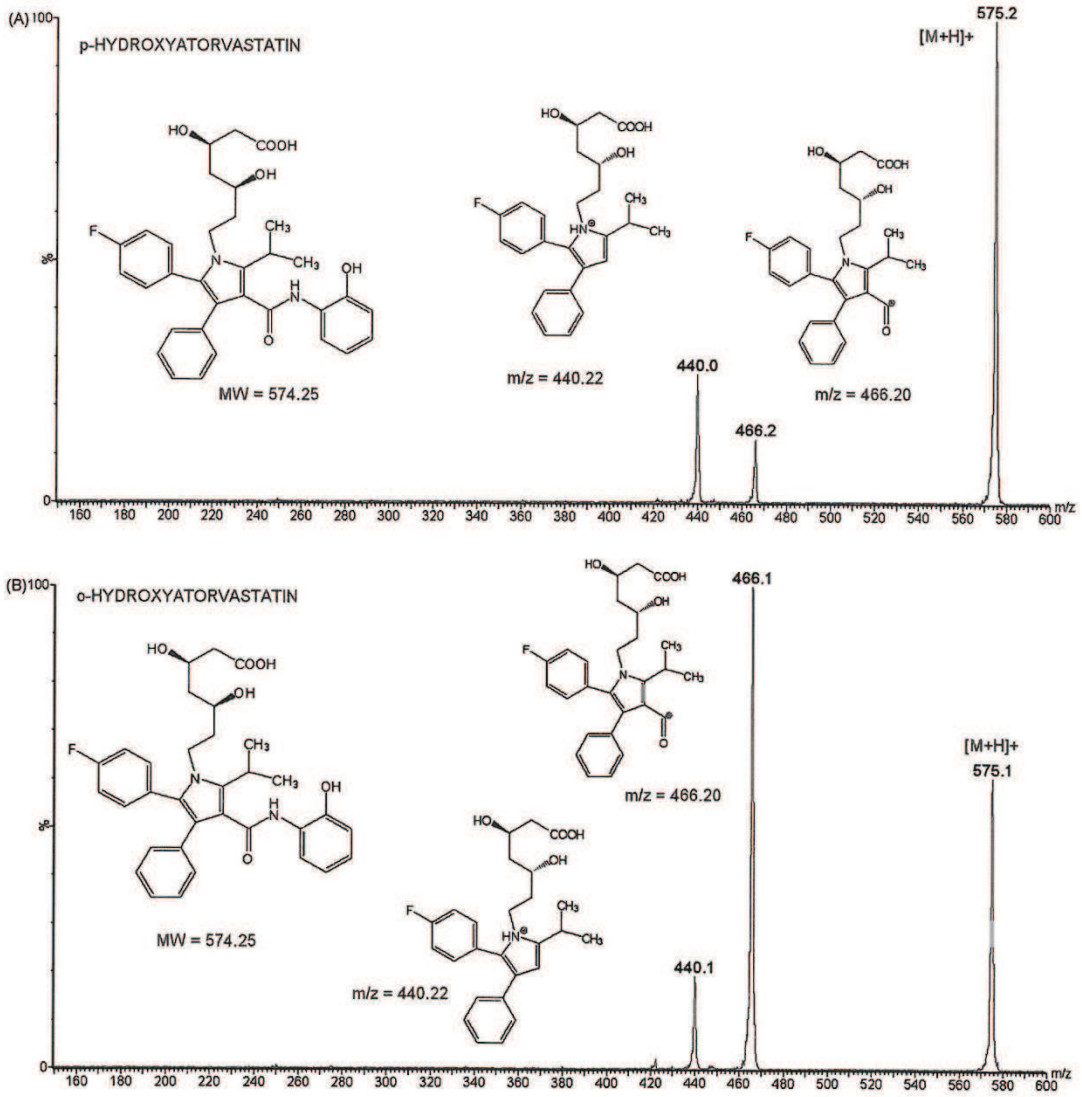

Fig. 5. Product ion spectra of p-hydroxyatorvastatin (A) and o-hydroxyatorvastatin (B).

Table 2
The results of SST, linearity and sensitivity test.

compound	t_R	Repeatability t_R [% RSD]	Repeatability A [% RSD]	Linearity [r^2]	LOQ [nmol/l]	LOD [nmol/l]
p-Hydroxyatorvastatin	2.49	0.27	6.29	0.9997	0.57	0.19
o-Hydroxyatorvastatin	2.89	0.18	1.49	0.9996	0.33	0.11
Atorvastatin	2.89	0.11	1.69	0.9999	0.15	0.05
Atorvastatin-deuterium labeled	2.89	0.18	2.06	0.9996	0.26	0.08
Atorvastatin lactone	3.19	0.21	1.44	0.9993	0.09	0.03
Simvastatin acid	3.48	0.15	4.67	0.9986	4.38	1.46
Simvastatin	4.39	0.12	1.76	0.9997	0.16	0.03
Simvastatin-deuterium labeled	4.40	0.16	1.11	0.9995	0.20	0.05

good stability at pH range 4–5 and volatility together with sensitive mass spectrometric response. In Fig. 2 there is an example of optimization given for atorvastatin, where the buffer pH and concentration in order to get the best S/N ratio of MS detector is performed. Other compounds gave similar response profile. Ammonium formate and ammonium acetate at pH 4.0 and 4.5 were tested at the concentration range 0.01–10 mmol/l. The best response was observed at 0.5 mmol/l buffers, which is in agreement with previously published works concerning the influence of additives—the concentrations higher than 5 mmol/l can significantly decrease the response of mass spectrometer [31]. On the other hand, the concentrations lower than 0.5 mmol/l were not sufficient to keep buffering capacity and they had negative influence to the response of mass spectrometer. Ammonium acetate was preferred before ammonium formate because of better peak shapes. Finally, the mobile phase composition was 70% of acetonitrile and 30% of ammonium acetate buffer 0.5 mmol/l pH 4.0.

In all cases protonated molecule $[M+H]^+$ was monitored in electrospray positive ionization mode. For atorvastatin and its metabolites it was the most intensive ion in mass spectra as published in many papers [22–25] before, however, concerning simvastatin there were strong discussion about the choice of precursor ion [15]. As proposed by Miao and Metcalfe [32], the addition of methylammonium acetate could enhance the formation of methylammonium adduct and that way highly enhance the sensitivity for quantitation using this adduct. In our experiment we did not observe any methylammonium adduct at all using this additive, thus protonated molecule was chosen for quantitation of simvastatin as well. Monitoring of adducts, such as $[M+Na]^+$ or $[M+CH_3CN+Na]^+$ is not correct in quantitative approach even if it was previously published [16–18].

Subsequently, all the parameters of mass spectrometer were finely tuned in order to get good sensitivity of precursor ion $[M+H]^+$ for all analytes—see Section 2.3. Cone voltage was set up individually for each analyte—the results could be seen in Table 1.

Quantitation of all analytes was performed in ESI positive ion mode using SRM mode. Two specific transitions were optimized for each molecule to increase selectivity and identification value of the method. Product ions were chosen according to the fragmentation pathways in Product ion scan mode—see Figs. 3–5. Argon was used as collision gas and collision energy was optimized for each analyte and for each of its two transitions individually in order to get high sensitivity—see Table 1.

3.2. Sample preparation

The sample preparation procedure was optimized using three different SPE sorbents—ZORBAX SPE C-18 (100 mg, 1 ml) (Agilent Technologies), Oasis HLB SPE (60 mg, 3 ml) (Waters), and Discovery DSC-18 (100 mg, 1 ml) (Supelco). Good results of recovery suitable for validation of the method were obtained with using Discovery DSC-18 SPE sorbents. Oasis HLB sorbent showed very different recovery values for atorvastatin (about 56%) and its metabolites (48–140%), thus it could not be used for their simultaneous determination. Sufficient and repeatable recoveries were observed for ZORBAX SPE C-18 but they were withdrawn from commercial market circulation. Serum sample preparation procedure was performed according to the procedure described in Section 2.5.

3.3. System suitability test and validation

The SST was performed by 10 subsequent injections of mixed solutions of standard mixture of statins at the concentration 10 nmol/l. Parameters such as the repeatability of reference standard solution injection were established (retentions times and peak areas were checked, the repeatability was expressed as RSD in %). SST results could be seen in Table 2.

3.3.1. Linearity–calibration range

Calibration curves of all analytes were measured in the concentration range 0.1–100 nmol/l, using seven calibration points (100,

Table 3
The results of validation—accuracy and precision.

Method validation		p-OH-AT	o-OH-AT	AT	ATL	SVA	SV
Accuracy [%] recovery	L1	84.9	65.3	86.0	93.2	78.1	75.6
	L2	89.3	74.4	84.6	91.8	90.5	98.8
	L3	78.8	86.0	86.4	78.5	100.0	89.6
Precision [% RSD] Intra-day	L1	1.5	1.2	1.0	0.8	0.6	9.8
	L2	3.8	3.3	1.6	2.8	4.5	8.6
	L3	8.4	4.7	6.7	11.8	1.8	9.1
Precision [% RSD] Inter-day	L1	2.5	3.9	10.6	6.6	3.5	6.9
	L2	5.5	11.2	10.4	3.2	15.3	6.6
	L3	9.2	4.3	13.7	8.9	11.9	9.6
Repeatability of calibration curve slope [% RSD]		6.0	16.9	14.4	8.4	7.1	9.9
Method selectivity		No interference	No interference	No interference	No interference	No interference	No interference

p-OH-AT = p-hydroxyatorvastatin, o-OH-AT = o-hydroxyatorvastatin, AT-atorvastatin, ATL = atorvastatin lactone, SVA = simvastatin acid, SV = simvastatin, OK = no matrix effect observed, L1, L2, L3 = concentration level 1, 2, 3 (10^{-7} to 10^{-9} mol/l).

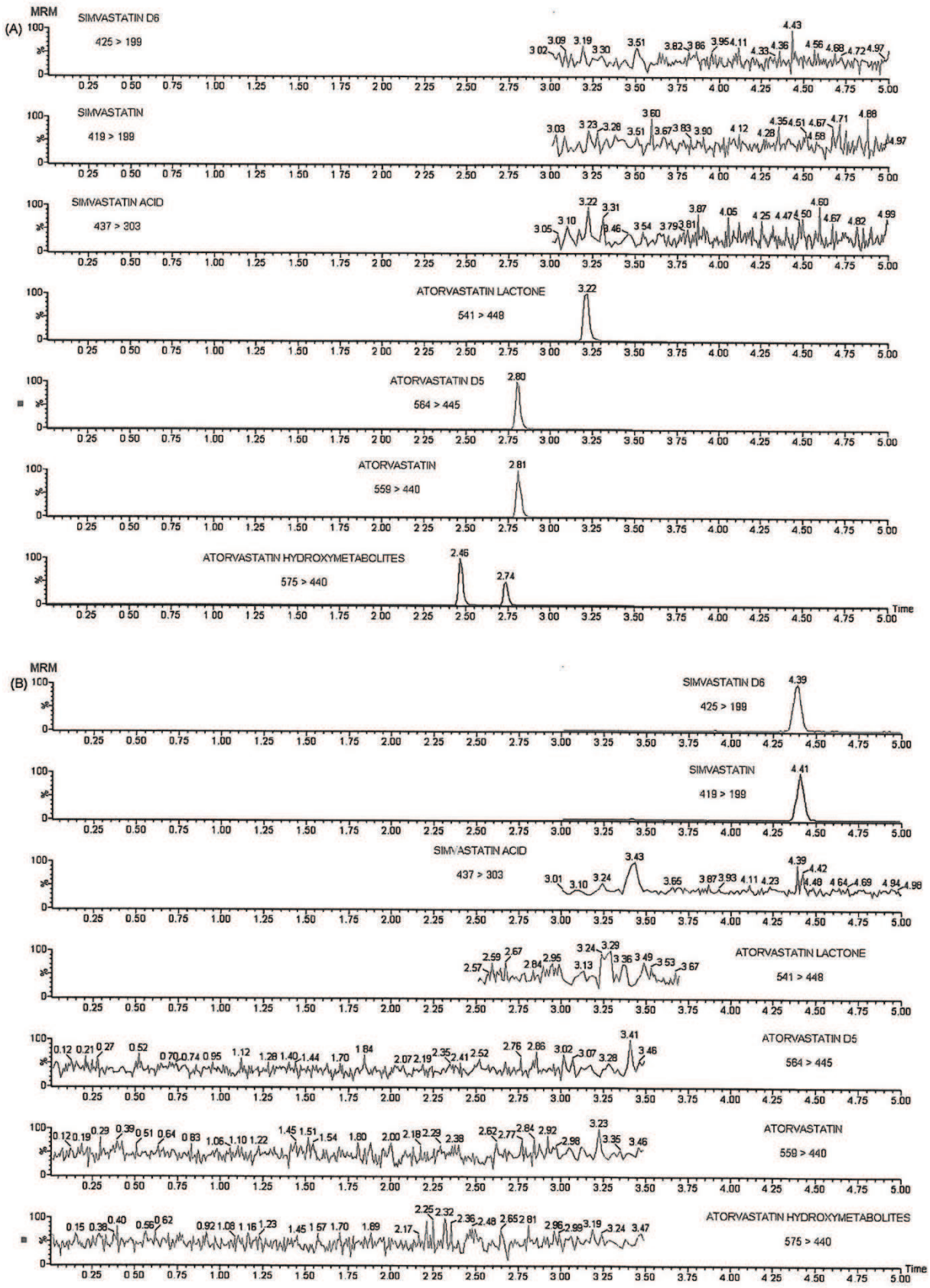

Fig. 6. Chromatogram of analysis of serum samples—patient on atorvastatin (A) and patient on simvastatin (B).

Table 4
Atorvastatin and metabolites—in serum and lipoprotein fractions (VLDL, LDL, HDL) before and after hemodialysis displayed for patients 1 and 2. VLDL = very-low-density lipoprotein, LDL = low-density lipoprotein, HDL = high-density lipoprotein.

Atorvastatin and metabolites in serum and lipoprotein fractions (40 mg dosed)							
compound [nmol/l]		p-OH-AT	o-OH-AT	AT	ATL	SV	SVA
Patient no. 1							
Before hemodialysis	serum	3.82	8.28	20.46	14.34	0	0
	VLDL	0.48	3.26	7.43	5.18	0	0
	LDL	0	2.04	3.4	3.63	0	0
	HDL	2.1	4.43	10.44	9.36	0	0
After hemodialysis	serum	0.49	2.56	3.03	5.64	0	0
	VLDL	0	1.27	0.13	1.01	0	0
	LDL	LOD	0.94	LOD	1.41	0	0
	HDL	LOD	1.69	1.63	4.49	0	0
Patient no. 2							
Before hemodialysis	serum	0.29	3.21	2.19	3.19	0	0
	VLDL	0.05	1.04	0.58	0.58	0	0
	LDL	0	0.74	0.19	0.24	0	0
	HDL	0	1.17	1.11	0.83	0	0
After hemodialysis	serum	0.24	1.95	1.38	0.92	0	0
	VLDL	0	0.8	1.07	0.27	0	0
	LDL	0	0.16	0.65	0.31	0	0
	HDL	0	1.18	1.83	0.89	0	0

50, 10, 5.0, 1.0, 0.5 and 0.1 nmol/l). Results concerning linearity can be seen in Table 2. Matrix calibration curves were prepared using the same calibration points by spiking blank serum samples with standard solutions and subsequent treatment by SPE preparation step. The linearity was found to be satisfactory for all compounds. Such calibration curves were used for quantitation purposes.

3.3.2. *Accuracy and precision*

Accuracy and precision were established by spiking blank serum samples at three levels of calibration curve—at high (10^{-7} mol/l), medium and low (10^{-9} mol/l) concentration using SPE step described in Section 3.2. Method precision was determined as intra-day and inter-day variability of three determinations at three different levels expressed as % RSD, see Table 3. Intra-day precision was generally within 10% RSD, while inter-day precision within 15% RSD. QC samples were prepared at the same concentration levels.

Method accuracy was determined as % of recovery using blank serum samples spiked with standard solutions treated by SPE extraction and blank serum samples treated by SPE extraction and subsequently spiked by the standard solution at three concentration levels—results could be seen in Table 3. Recovery typically ranged from 75 to 100%.

3.3.3. *Method selectivity–matrix effects*

For the determination of selectivity and measurement of matrix effects the injection of blank serum treated by the same sample preparation step was used. Matrix effects were established using direct inlet by Hamilton syringe, where standard mix solution was introduced to the mass spectrometer by direct infusion and the blank serum was injected by the autosampler to observe matrix suppressions or enhancements as positive or negative peaks influencing data plot of analytes. First, strong matrix effect was observed at retention time of atorvastatin and its metabolites. It was eliminated by the change of chromatographic conditions. Isocratic elution was changed to gradient profile, starting from 30% of acetonitrile, where statins are not eluted yet to allow interfering compounds from the matrix to be eluted. Thereafter the gradient was run up to 95% of acetonitrile to wash out all other possible interfering compounds—details in Section 2.2. Matrix effects were tested again using the same procedure. Neither negative nor positive peaks were observed at retention times of all analytes.

3.3.4. *Limits of detection and quantitation*

Limits of detection and quantitation were calculated based on S/N ratio. They were established first using standard solutions in mobile phase by the injection of the smallest amounts which provide S/N = 3. Subsequently this was confirmed by measurements in real matrix, which gave similar values. The results are displayed in Table 2. The method had excellent sensitivity to be able to perform the determination of statins in biological samples reaching LOQ 0.08–5.46 nmol/l and LOD 0.01–1.80 nmol/l.

3.4. *Application to real samples*

The samples of serum and lipoprotein fractions—HDL, LDL and VLDL were measured using developed UPLC-MS/MS method. A typical chromatogram could be seen in Fig. 6A and B, first transition, which was used for quantitation purpose is displayed. In patients using atorvastatin as a treatment both metabolites and also lactone form of atorvastatin were determined in all samples (Fig. 6A). In total, eight patients taking atorvastatin were included in our study. Atorvastatin levels typically found ranged from 1.33 to 20.46 nmol/l with 6.63 being a mean value. At patients taking simvastatin only simvastatin and simvastatin acid was determined in serum and lipoprotein fractions of patients (Fig. 6B). Only two patients taking simvastatin were included into the study. The levels of simvastatin were substantially lower, probably due to lower biological half-time, they were in the range 0.54–1.74 nmol/l.

The data from the first patient, who was treated by 40 mg of atorvastatin daily, are shown in detail in Table 4. High levels of atorvastatin and its metabolites were found in serum and lipoprotein fractions. The dose of 40 mg atorvastatin was administered at 9.00 p.m. The blood sampling was done the next morning before the start of hemodialysis at 7.00 a.m. The data from the second patient, who was treated by 40 mg of atorvastatin daily, are shown in Table 4 as well. Low levels of atorvastatin and its metabolites were found in serum and lipoprotein fractions. The dose of 40 mg atorvastatin was administered at 9.00 p.m. The blood sampling was done the next morning before the start of hemodialysis at 7.00 a.m.

A high inter-subject variability in pharmacokinetic parameters seen in this study is noteworthy. A high variability in statin kinetic parameters has also been observed in subjects without renal disease. Age, gender, food intake, and level of CYP3A4 expression and

activity all influence the body's handling of atorvastatin [33]. An important characteristic of CYP3A4 is the large inter-individual variability in activity (about 5-fold), which reflects genetic polymorphism combined with modulation by environmental factors [34]. Intake of known strong inhibitors or inducers of CYP3A4 did not occur in this study. However, in hemodialysis patients, who are polymedicated and have complex metabolic disturbances, uncharacterized interactions with concomitant drugs and endogenous substances may have contributed to the large variation in atorvastatin pharmacokinetic parameters.

4. Conclusions

Fast, sensitive and selective method for the determination of simvastatin, atorvastatin, its metabolites and interconversion products of both statins was developed. The method employed UPLC-MS/MS technique as a tool enabling high separation efficiency, speed of analysis and low solvent consumption. MS/MS detection utilized two SRM transitions for each compound to ensure high selectivity and reliability of the method. Deuterium labeled internal standards were used for the purposes of accurate and precise quantitation. Sample pretreatment of serum samples and lipoprotein fractions included stabilization by ammonium acetate buffer pH 4.0 during SPE sample preparation step. This was necessary to prevent the interconversion of analytes. Therefore ammonium acetate was also an inherent part of mobile phase during chromatographic separation. Its concentration was crucial in terms of the support of the ionization of statin molecules. While the concentration higher than 1 mmol decreased significantly ionization of statin molecules, the concentration lower than 0.5 mmol/l was not sufficient to ensure sufficient ionization, buffering capacity and the stability of analytes and therefore the response of mass spectrometer decreased.

The method was validated with good results for linearity (>0.9990, except of SVA), precision (RSD < 15% for all analytes), accuracy (recovery 75–100%) and selectivity showing no interferences with measured compounds. Analytes could be quantified at nmol/l concentrations with typical LOQ 0.09–0.57 nmol/l, except of SVA LOQ being 4.38 nmol/l. The method is applicable to analysis of serum samples and lipoprotein fractions containing atorvastatin or simvastatin. The advantage of the method was simultaneous determination of two clinically widely used statins—one chromatographic run and one sample preparation. It was not necessary to distinguish among the samples of patients and all samples could be analyzed using one procedure, which was very convenient for routine purposes.

This study revealed differences in the inter-individual processing of statins in hemodialysis patients with hyperlipidemia and is addressing the clinically relevant information with respect to achieve adequate levels of exposure to active compound in this group of patients.

Acknowledgement

The authors gratefully acknowledge the financial support of IGA MZ CR no. 1A/8689-4.

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PŘÍLOHA II

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The Importance of ultra high performance liquid chromatography tandem mass spectrometry in clinical monitoring of simvastatin, atorvastatin and its metabolites after extracorporeal LDL-cholesterol elimination

Journal of Biomedicine and Biotechnology, 2011, vol. 2011, článek ID 912471, 9 stran.

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2011, Article ID 912472, 9 pages
doi:10.1155/2011/912472

Research Article

Use of Ultra High Performance Liquid Chromatography-Tandem Mass Spectrometry to Demonstrate Decreased Serum Statin Levels after Extracorporeal LDL-Cholesterol Elimination

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Received 26 July 2010; Revised 19 September 2010; Accepted 13 October 2010

Academic Editor: Leonid Medved

Background. Using our statin analysis method, it was possible to uncover a significant drop in statin levels (atorvastatin, simvastatin, and metabolites) after extracorporeal LDL-cholesterol elimination (EE) in severe familial hypercholesterolemia (FH). The purpose of this work was to identify the mechanism underlying this drop and its clinical significance as well as to propose measures to optimize a pharmacotherapeutical regimen that can prevent the loss of statins. *Methods.* Ultra High Performance Liquid Chromatography (UHPLC) connected to the triple quadrupole MS/MS system was used. *Patients.* A group of long-term treated patients (3–12 years of treatment) with severe FH (12 patients) and treated regularly by LDL-apheresis (immunoadsorption) or haemorheopheresis (cascade filtration) were included in this study. *Results.* After EE, the level of statins and their metabolites decreased (atorvastatin before/after LDL-apheresis: 8.83/3.46 nmol/l; before/after haemorheopheresis: 37.02/18.94 nmol/l). A specific loss was found (concentration of atorvastatin for LDL-apheresis/haemorheopheresis: 0.28/3.04 nmol/l in washing fluids; 11.07 nmol/l in filters). To prevent substantial loss of statin concentrations, a pharmacotherapeutic regimen with a longer time interval between the dose of statins and EE is recommended (15 hours). *Conclusions.* A specific loss of statins was found in adsorbent columns and filters. The decrease can be prevented by the suggested dosage scheme.

1. Introduction

Familial hypercholesterolemia (FH) is a severe metabolic disorder that leads to accelerated atheromatosis [1]. A complete change in lifestyle, a strict diet, and intensive combined pharmacotherapy are necessary in patients with FH and provide very good results in most patients [2]. In spite of these measures to resolve FH, extracorporeal elimination (EE) of low-density lipoprotein cholesterol (LDL-cholesterol) is still necessary for patients who are homozygous for the disease-causing gene and a small number of heterozygous patients. EE is carried out using currently accepted methods, including heparin-induced extracorporeal LDL precipitation (HELP), direct adsorption of lipoprotein (DALI), LDL-apheresis (immunoadsorption), dextran-sulfate adsorption (lipopheresis), and hemorheopheresis (cascade filtration) [3–6]. Statins, combined with other cholesterol-lowering agents (ezetimibe, fibrates, or bile acid sequestrants), are the most efficient and the most widely used pharmacotherapeutics for FH treatment [7–9]. The therapeutic range of statins is relatively low (typically 10–80 mg/day) [10, 11], and patients with severe FH must take the maximally tolerable dose of statins. Such doses have a potent cholesterol-lowering effect and significantly reduce the morbidity and mortality associated with coronary heart disease, as shown by many clinical trials [9, 12–14]. However, some statins

exhibit a number of adverse effects, such as myopathy or rhabdomyolysis [1, 15–18]. Nevertheless, therapeutic drug monitoring is not routinely carried out for patients treated with statins. Patients are only advised to report the development of muscle aches, pains, or weakness, which increases the risk of clinically important adverse effects. The determination of statin levels in biological fluids is clinically important, but measurements are technically difficult and quite expensive; therefore, statin levels cannot be routinely determined in a common biochemical laboratory. Such determination requires specialized laboratory processing and analytical equipment; many important and interesting data were described elsewhere [19–22].

A sensitive and selective analytical method for statin analysis during EE was developed in our laboratories. After the initial analyses, we observed considerable decreases in statin levels after EE in some patients. This decrease could adversely influence our patients, although no data were found in the literature to allow us to evaluate the possible effects of this decrease. The aims of the present work were to evaluate the quantity of statin loss (as well as active metabolites of statins) during EE, to identify the mechanism of statin loss during EE (possibly captured also in absorbers or filters), and to propose measures to reduce losses, such as adjustments to the pharmacotherapeutical regimen.

2. Materials and Methods

2.1. Chemicals and Reagents. Working standards of simvastatin were obtained from Sigma Aldrich (Prague, Czech Republic). Working standards of simvastatin acid, atorvastatin lactone and atorvastatin, p-hydroxyatorvastatin, o-hydroxyatorvastatin, deuterium-labeled simvastatin (D6-methyl groups), and deuterium-labeled atorvastatin (D5-phenyl ring) were purchased from Toronto Research Chemicals (Ontario, Canada).

Acetic acid (reagent grade), ammonium (reagent grade), and acetonitrile (LC-MS grade) were purchased from Sigma Aldrich. High-performance liquid chromatography (HPLC) grade water was obtained with a Milli-Q reverse osmosis Millipore (Bedford, MA, USA) and met the requirements of the European Pharmacopoeia.

2.2. Therapeutic Procedures. All patients treated in the long term with EE in the Czech Republic were chosen for the purpose of this study. The following two EE approaches were used in twelve patients.

2.2.1. LDL-Apheresis. The patients were treated by LDL-apheresis based on the principle of immunoadsorption. The procedure consisted of the following two main steps: (1) separation of plasma using a Cobe-Spectra continual centrifugation separator (Cobe, Denver, USA) followed by (2) passage of plasma through a pair of Lipopak 400 adsorbers (Pocard, Moscow, Russia) with sheep antibodies against apolipoprotein B. The adsorbers are placed into an automatic adsorption-desorption device (Adasorb, Medicap, Ulrichstein, SRN). The pair of columns was alternated until the cholesterol level was significantly below the limit. The target LDL-cholesterol value used at the start of this research was less than 1 mmol/L, but the procedure has become much more effective, with a target value decreased to below 0.5 mmol/L (normal values typically range from 1.5 to 3.36 mmol/L).

2.2.2. Hemorheopheresis (Cascade Filtration). Cell-free plasma was obtained by high-speed centrifugation using a Cobe-Spectra separator, as described above. The plasma was then pumped through the second level (filters). Based on their properties, Evaflux 4A and 5A (Kuraray) filters were used in our modification of cascade filtration (named "hemorheopheresis" in our paper). The filters contained hollow fibers made from ethylene-vinyl alcohol material with a pore size of 0.03 or 0.04 μm. Anticoagulation was performed with ACD-A (Baxter, Munich, Germany) and an initial intravenous bolus injection of 4000 IU of heparin. The body plasma volume was calculated using the Cobe-Spectra separator computer, and we washed 1.5X of the body plasma volume.

2.2.3. Monitored Parameters. To monitor the safety, efficiency, and cost-effectiveness of the therapy, the patients were regularly tested for a set of basic hematological, biochemical, and immunological parameters (data not shown) [23–26].

This study was approved by the institutional ethics committee, and all examinations were in agreement with the principles of the currently valid version of the Helsinki declaration. All patients signed an informed consent form.

2.3. Chromatography and Mass Spectrometry. The previously developed and validated ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) technique was applied to measure the levels of simvastatin, atorvastatin, and their metabolites in serum and washing liquids [27]. A UHPLC system (Waters, Prague, Czech Republic) was used for the purposes of this study, and the system consisted of an ACQ-binary solvent manager and an ACQ-sample manager. An MS/MS triple quadrupole system was also used for the purposes of this study. A Quattro Micro apparatus (Micromass, Manchester, GB) was equipped with a multimode ionization source (ESCI). Following SRM (selected reaction monitoring) transitions were monitored: atorvastatin (559 > 440 and 559 > 466), and atorvastatin D5 (564 > 445), p-hydroxyatorvastatin (575 > 440 and 575 > 466), o-hydroxyatorvastatin (575 > 440 and 575 > 466), atorvastatin lactone (541 > 448 and 541 > 422).

2.4. Preparation of Standard Solutions and Samples. The standard stock solutions used to measure statin levels were prepared according to the procedure described in [27]. Stock solutions were further diluted by the mobile phase (for stability reasons, the pH of the solution was maintained between 4.0 and 5.0 to prevent interconversion). Serum samples were prepared using solid phase extraction (SPE) with a Discovery DSC-18 SPE support (SPE procedure I). An internal standard (100 μL) was added to 900 μL of the serum

samples containing the analytes. The mixture was loaded onto a Discovery DSC-18 sorbent previously activated with 1 mL of acetonitrile and conditioned with 1 mL of 0.1 M ammonium acetate buffer, pH 4.5. An SPE cartridge with a loaded sample was washed two times with 1 mL of 0.1 M ammonium acetate, pH 4.5, then once with 1 mL of a mixture of acetonitrile [0.01 M ammonium acetate buffer, pH 4.5 (15 : 85, v/v)]. Finally, the analytes were eluted with 1 mL of acetonitrile [0.1 M ammonium acetate buffer, pH 4.5 (95 : 5, v/v)]. The eluate was filtered through a 0.20 μm PTFE filter, and the sample was injected onto the UHPLC system [27].

The samples of washing liquids were prepared using a modified version of SPE procedure I. Four cycles were usually used during LDL-apheresis. First, the plasma of the patient was administered through the adsorbent columns to bind cholesterol. Subsequently, in the washing step, cholesterol was washed away by glycine acidification. In this phase, statins potentially retained in the adsorbent columns might be released as well. Therefore, the glycine washing solution was also analyzed by UHPLC-MS/MS. The sample was obtained during the first cycle, when the highest amount of cholesterol was present in the plasma. During the hemorheopheresis procedure, all washing liquid was collected in the waste bag and examined by UHPLC-MS/MS. A homogenization step was necessary to obtain representative samples of washing liquids. Therefore, waste bags were shaken for 30 minutes. Prior to the extraction of washing liquids, the samples were centrifuged at 3000 × g for 10 minutes. If necessary, the samples were also filtered through a 0.20 μm PTFE filter. For SPE, 5 mL of supernatant was used. Conditioning, washing, and elution steps were performed according to SPE procedure I.

Preparation of samples from filters was performed as follows. After the filtering procedure, all liquids were removed from the filters. The filters were then washed in acetonitrile to remove any remaining statins. All liquid was homogenized by shaking. Subsequently, the samples were centrifuged at 3000 × g for 10 minutes prior to extraction of the washed filters. If necessary, the samples were also filtered through 0.20 μm PTFE filters. For SPE, 5 mL of supernatant was used. Conditioning, washing, and elution steps were performed according to SPE procedure I. However, it was not possible to conduct this experiment quantitatively because some amount of liquid always remained in the filters, so the measurements were only an approximate value.

2.5. Patients. The patient pool included all long-term treated patients undergoing EE procedures in the Czech Republic. This set of 12 patients was gradually generated after 1996. The patients were treated for 3–12 years (average: 7.2 ± 2.96 years, range: 2–11.5, median: 7.25). The group of 12 patients consisted of 7 men and 5 women. The average age of the patients was 47 ± 16.6 years (range: 21–63, median: 52 years). The clinical phenotype of FH was characterized by an increased level of total plasmatic cholesterol and LDL-cholesterol as well as by the occurrence of xanthomas and premature symptoms of ischemic heart disease. The MedPed criteria [28], using thresholds for total cholesterol and LDL cholesterol levels above the 95th percentile specific to the Czech population [29], and the individual's age and family history were applied. Furthermore, DNA-based evidence of a mutation in the low-density lipoprotein receptor (LDLR) gene was used as the criterion for homozygous FH. None of the patients carried a mutation in the apolipoprotein B (APOB) gene.

All patients were treated with high-dose statins (40 mg simvastatin or 40–80 mg atorvastatin daily). One patient was treated with combined fenofibrate (200 mg daily) and a statin, two patients were treated with combined biliary acid-binding resins (6 g daily) and a statin, and 10 patients were treated with combined Ezetrol (ezetimibe, 10 mg daily) and a statin. Rosuvastatin was not available in the Czech Republic during the study period.

EE procedures were performed regularly, with three homozygous FH patients receiving EE every 10–14 days and other hypercholesterolemic patients receiving EE every 3-4 weeks. Five patients presented an increased level of lipoprotein (a) (>0.30 nmol/L), with an average level of 1.20 ± 0.84 nmol/L (range: 0.40–2.42 nmol/L, median: 1.1 nmol/L). The average body mass index was 26.99 ± 6.2 (range: 18.7–42.6, median: 26.35). Nine patients suffered from hypercholesterolemia of Fredrickson phenotype IIa. These patients were either genetically confirmed as homozygous patients with a defective LDLR gene or heterozygous FH patients. Three patients displayed abnormally high levels of cholesterol and triglycerides. Two patients had a phenotype consistent with Fredrickson IIb. One patient, who was simultaneously treated for type II diabetes mellitus, had a phenotype consistent with Fredrickson IIb-IV.

2.6. Statistical Evaluation. The significance of differences before and after extracorporeal therapy was examined by the Mann-Whitney U test, using the NCSS 2004 statistical software (Number Cruncher Statistical Systems, Kaysville, USA). Differences were considered significant for $P \leq 0.05$.

3. Results

Lipoprotein levels were relatively stable in all patients at the time of examination. The final measurements showed the average total cholesterol to be 7.49 ± 1.61 nmol/L, LDL-cholesterol to be 5.2 ± 1.39 nmol/L, and HDL-cholesterol to be 1.56 ± 0.43 nmol/L.

3.1. Preliminary Experiments. First, preliminary experiments were performed to verify the applicability of the UHPLC-MS/MS method in determining the influence of EE procedures on statin concentrations in biological fluids. In total, 36 samples were analyzed (before and after the EE procedure), including three samples taken from each of twelve patients at regular intervals. Eleven patients were treated with atorvastatin and one with simvastatin during the preliminary study. From the preliminary results, it was evident that intraindividual variability among the particular analyses was low. Therefore, the method was found to be convenient and reliable. The UHPLC-MS/MS method validation was

performed according to the International Conference on Harmonization (ICH) guidelines for validation [29].

3.2. Detailed Study. Plasma statin concentrations were measured regularly over the course of the study. In total, 170 samples (85 pairs, collected twice from each patient during 2009) were analyzed in all patients when they attended the procedures. For each patient, samples were always analyzed in pairs for concentrations of statins and their metabolites, with one sample taken before the procedure and the other sample taken after the procedure. Only eleven of the twelve patients were admitted to this study because one patient, who was treated with simvastatin, had previously switched to rosuvastatin therapy when it became available in the Czech Republic. In the following study, only patients treated by atorvastatin were observed.

The results from the study are shown in Table 1. After the EE procedures, clinically significant reductions of statins and their metabolites were observed. The concentration of atorvastatin in the serum decreased by an average of 47.2%, p-OH-atorvastatin by 50.0%, o-OH-atorvastatin by 37.9%, and atorvastatin lactone by 49.1%. The changes in concentrations of statins and their metabolites were evaluated statistically using the Wilcoxon nonparametric *t*-test. The difference in mean concentration before and after the procedures was significant for the patients as a group (see Table 1). However, the two EE procedures differ; LDL-apheresis is an APOB-specific treatment, and hemorheopheresis is a nonspecific filtration procedure. Therefore, it was necessary to evaluate the results separately. Upon individual evaluation of each EE procedure, we found that the statin and metabolite levels differed significantly before and after LDL-apheresis (Table 1). Results obtained for the hemorheopheresis group were also significant, except those for one metabolite (p-OH-atorvastatin). During LDL-apheresis, the atorvastatin serum concentration decreased by 60.8%, pOH-atorvastatin by 59.0%, o-OH-atorvastatin by 44.5%, and atorvastatin lactone by 58.9%. During hemorheopheresis, the atorvastatin serum concentration decreased by 48.8%, p-OH-atorvastatin by 43.60%, o-OH-atorvastatin by 44.0%, and atorvastatin lactone by 41.6%.

3.3. The Mechanism of the Statin Drop. Considering the relatively large decrease of serum statin concentrations in all patients studied, further experiments were performed to test the hypothesis that adsorbent columns retain statins during LDL-apheresis and that filters retain statins during hemorheopheresis. To test this hypothesis, 25 samples were collected and subjected to UHPLC-MS/MS analysis. In addition, 22 samples of waste liquids were examined to evaluate each patient twice. The results of this experiment are presented in Table 2. The average concentrations of statins in washing liquids were substantially lower than the serum levels (the atorvastatin concentration during LDL-apheresis was 0.28 ± 0.20 nmol/L, during hemorheopheresis was 3.04 ± 3.58 nmol/L, and for all patients was 1.34 ± 2.51 nmol/L). An analysis of LDL-apheresis was performed on the total amount of waste liquid; however, the waste liquid was only obtained from the first cycle (about 900 mL), during which the cholesterol retained in the adsorbent column was washed out with glycine. As stated above, both adsorbent columns worked together as a pair, and each column was typically filled and washed out four times. In total, 3600 mL of glycine was used, so measurements from all cycles were not technically possible. However, statins are considered to be present in the highest amounts in washing liquids during the first cycle. In contrast, during hemorheopheresis, it was possible to analyze all of the washing liquid (500–1500 mL of liquid contained in a bag). The volume of washing liquid during hemorheopheresis varied, depending on the number of times the Evaflux filter was washed, which was controlled by pressure feed-back. If the pressure increased above safe limits, the filter would wash back into the waste bag. The number of washing cycles depended mainly on the fibrinogen level of the patient.

The measurements for the filter matrix (three samples) revealed substantial retention of statins as well. From the matrix, the median atorvastatin level in the eluate was 7.83 nmol/L, the median p-OH-atorvastatin level was 1.20 nmol/L, the median o-OH-atorvastatin level was 7.71 nmol/L, and the median atorvastatin-lactone level was 3.83 nmol/L (Table 3). The measurements for the eluate from the filter matrix were performed in triplicate only, due to technical difficulties and economic demands.

The correlation between the changes of statin levels (atorvastatin, p-OH-atorvastatin, o-OH atorvastatin, and atorvastatin lactone) and changes of both the total cholesterol levels and LDL-cholesterol levels in the group of patients taking the statins 2–4 or 15 hours before the procedures was tested using Pearson's coefficient, and the same analysis was used to examine relative correlation (software STATISTICA 8). No significant correlation was found ($P > 0.05$).

4. Discussion

Advances in biotechnology bring new knowledge and new methods that can be used in many branches of medicine [30–34]. One such advance is extracorporeal therapy, which can be either non-specific (such as plasma exchange) or specific (such as immunoadsorption). This type of procedure can constitute an important and sometimes decisive component of FH therapy. EE with regard to medical, technical, and economic demands is indicated in cases when classical methods fail.

Our study revealed a significant decrease in atorvastatin and simvastatin levels after EE using LDL-apheresis and hemorheopheresis. The decrease was observed for the parent compound as well as for the metabolites (except for p-OH-atorvastatin in the hemorheopheresis group) and the interconversion products. After EE, the concentrations were about half of the initial concentrations. The question of clinical importance of statin level fluctuation after EE is very interesting. The notion that EE reduces plasma levels of statins is, at face value, an important issue. However, in reality the low bioavailability of statins and the fact that their LDL-lowering efficacy is not correlated with area under the curve concentrations from 0 to 24 hours after dosing [35]

Table 1: Atorvastatin and its metabolites in serum before and after the EE procedure.

Procedure	Compound	Number of samples	Before procedure			After procedure			p (Wilcoxon nonparametric T-test)
			Average (nmol/L)	Standard deviation	Median (nmol/l) (range)	Average (nmol/L)	Standard deviation	Median (nmol/l) (range)	
LDL-apheresis	AT S	16	8.83	5.41	6.86 (3.77–23.90)	3.46	1.76	3.64 (0.01–7.11)	0.000060
	p-OH-AT S	16	1.78	1.73	1.49 (0.00–7.59)	0.73	0.43	0.64 (0.00–1.53)	0.007436
	o-OH-AT S	13*	4.74	1.99	4.87 (2.18–7.58)	2.63	0.98	2.97 (1.19–4.18)	0.012861
	ATL S	16	4.74	2.27	4.34 (2.33–8.82)	1.95	1.49	1.68 (0.33–2.33)	0.000369
Haemorheopheresis	AT S	6	37.02	19.23	40.41 (6.46–59.20)	18.94	14.75	16.58 (1.93–44.26)	0.0092
	p-OH-AT S	6	2.89	3.12	1.63 (0.78–8.79)	1.63	1.54	0.80 (0.56–4.30)	0.126
	o-OH-AT S	6	19.98	9.50	23.71 (3.58–28.74)	11.19	6.32	12.35 (1.31–19.31)	0.0205
	ATL S	6	16.99	9.37	14.98 (2.66–27.85)	9.92	7.27	10.71 (1.02–20.30)	0.0475
Both procedure (LDL-apheresis +haemorhepheresis)	AT S	22	15.84	14.95	8.48 (3.77–49.73)	8.36	12.88	4.25 (0.01–59.20)	0.001150
	p-OH-AT S	22	2.04	2.15	1.49 (0.00–8.79)	1.02	1.04	0.69 (0.00–4.30)	0.009816
	o-OH-AT S	19	9.18	8.58	5.43 (2.18–28.74)	5.70	6.20	3.15 (1.19–21.97)	0.034282
	ATL S	22	8.09	7.46	4.95 (2.33–27.85)	4.12	5.23	1.95 (0.33–20.30)	0.002190

*Three other samples were obtained. However, it was technically impossible to measure the samples, as they blocked the extraction SPE supports. p-OH-AT: p-hydroxyatorvastatin. o-OH-AT: o-hydroxy-atorvastatin. AT: atorvastatin. S: serum. ATL: atorvastatin lactone.

Table 2: Atorvastatin and its metabolites in waste fluids.

Samples from the procedure	Compound	Number of samples	Average (nmol/L)	Standard deviation	Median (nmol/L) (range)
LDL-apheresis	AT	15	0.28	0.20	0.22 (0.00–0.77)
	p-OH-AT	15	0.07	0.07	0.06 (0.00–0.25)
	o-OH-AT	15	0.09	0.11	0.06 (0.00–0.39)
	ATL	15	0.15	0.18	0.12 (0.00–0.74)
Hemorheopheresis	AT	7	3.04	3.58	2.57 (0.21–12.05)
	p-OH-AT	7	0.37	0.37	0.29 (0.00–1.06)
	o-OH-AT	7	1.72	1.33	1.38 (0.05– 4.11)
	ATL	7	2.52	1.85	2.23 (0.96–6.38)
Both procedures (Hemorheopheresis +LDL-apheresis)	AT	22	1.34	2.51	0.34 (0.00–12.05)
	p-OH-AT	22	0.19	0.27	0.08 (0.00–1.06)
	o-OH-AT	22	0.71	1.13	0.12 (0.00–4.11)
	ATL	22	0.91	1.51	0.18 (0.00–6.38)

p-OH-AT: p-hydroxyatorvastatin, o-OH-AT: o-hydroxyatorvastatin, AT: atorvastatin, ATL: atorvastatin lactone.

Table 3: Atorvastatin and its metabolites in the matrix filter.

Compound	Number of samples	Average (nmol/L)	Standard deviation (SD)	Median (nmol/L) (range)
AT	3	11.07	12.32	7.83 (0.70–24.69)
p-OH-AT	3	1.18	0.98	1.20 (0.19–2.15)
o-OH-AT	3	8.28	8.25	7.71 (0.33–16.80)
ATL	3	6.32	7.51	3.83 (0.38–14.76)

p-OH-AT: p-hydroxyatorvastatin, o-OH-AT: o-hydroxyatorvastatin, AT: atorvastatin, ATL: atorvastatin lactone.

suggest that reducing plasma levels of statins by apheresis would not adversely affect plasma LDL levels. Also in our study the correlation was not found between statin levels and their LDL-lowering effect. But other data indicate that high plasma levels of statins are associated with myotoxicity, which implies that lowering statin levels with lipoprotein apheresis might be useful in that context. There are also data indicating that the effect of statins is not only hypolipidemic, but pleiotropic [36]. Statin dose/level lowering is in relation to the many unwanted pathophysiological mechanisms, such as in coronary heart disease complications [37], beginning of arrhythmias [38] statins can also influence some renal disorders [39] and play an important role in endothelial or enzymatic cascade activities inclusive of pathological chain "inflammation—thrombocyte activation—thrombogenesis" [40]. It can be concluded that maximum tolerated doses of statins and corresponding high and stable statin levels are desirable in patients with severe FH.

Statins and their metabolites, which may also be effective in lowering cholesterol levels (e.g., atorvastatin hydroxy-metabolites), bind to blood proteins, and several of these statin carriers can also be eliminated during an EE procedure. The levels of statins in biological fluids are very low because statins have a high first-pass metabolism. Following dosing with conventional statins widely used in therapy (atorvastatin, simvastatin, and lovastatin), only about 5% of the dosed statin reaches the systemic circulation. Typical plasma concentrations of statins are ng/mL levels. The concentrations of therapeutically effective metabolites of atorvastatin are even lower, typically 0.1–20 ng/mL [41, 42].

The differences in the statin decrease between LDL-apheresis and hemorheopheresis are likely related to the different treatment approaches and the duration of the procedures. Similarly, the concentrations of statins before the procedure were higher in the hemorheopheresis group. Patients treated by hemorheopheresis took atorvastatin in the morning, followed by EE within 2–4 hours. Atorvastatin is quickly absorbed after oral administration, and a maximum plasmatic concentration is reached within 1-2 hours [41]. Therefore, the samples from these patients were taken shortly after the maximum plasmatic concentration of statins was reached. Patients treated by LDL-apheresis took the statin dose the evening before EE, so the samples were obtained approximately 15 hours after dosing, which is longer than the mean elimination half-life of 14 hours [41, 42]. Only one patient treated by LDL-apheresis took the statin dose in the morning, which, in his daily regimen, meant at 4 A.M. For this patient, LDL-apheresis was performed within 5-6 hours of dosing. Therefore, the atorvastatin concentration before EE was found to be substantially higher in this patient (average of three measurements = 13.65 nmol/L) as compared with the average of all patients treated by LDL-apheresis (8.83 nmol/L). However, the atorvastatin concentration before EE was lower in this patient than in patients treated by hemorheopheresis (37.02 nmol/L). The time interval between dosage and EE

dramatically impacted the measurements of statin concentrations. Similarly, the duration of EE also affected the statin concentrations. The time interval between the two blood collections for the determination of statin concentration before and after EE was 3-4 hours in the hemorheopheresis group and 5-6 hours in the LDL-apheresis group.

The pharmacokinetic properties of statins are well known under standard conditions. Our finding of the importance of time interval between blood withdrawals (before and after EE) on the reduction of statin concentrations could be based on pharmacokinetics, unless the metabolism of statins differs between EE procedures. It would be possible to determine whether the time period is unimportant or decisive or if it is the only factor influencing the decrease of statin concentration. Unfortunately, no information about changes in metabolism during EE was found in the literature. However, it may be considered that the EE procedure is a type of stressful situation, which may change the pharmacokinetic decrease of statin concentrations over time. Therefore, a more precise evaluation of measurements of washing liquids and filters was undertaken in our work. LDL-apheresis is a specific type of EE, in which APOB becomes caught in adsorbent columns together with compounds bound to APOB. Our results revealed that there was only an average of 0.28 ± 0.20 nmol/L of atorvastatin in washing liquids. Taking into account an average decrease of atorvastatin of 5.37 nmol/L in serum after LDL-apheresis, the retention of statins in washing liquids was less important. It should be noted that washing liquids were examined only for the first cycle, although four cycles are typically performed. The concentration of statins in washing liquids after hemorheopheresis (on average, 3.04 nmol/L) was found to be higher than after LDL-apheresis, and the concentration was 11.07 nmol/L in the matrix of filters. The atorvastatin in the serum decreased by an average of 18.08 nmol/L following hemorheopheresis. The amount of atorvastatin retained in filters and washing liquids was therefore relatively more important than after LDL-apheresis. The absolute amount of retained statin following hemorheopheresis was higher as compared with LDL-apheresis. This result is in agreement with the nonspecificity of hemorheopheresis, which is actually a filtration procedure. The results can be summarized in the following: even though the period with lower levels of statins is not long (lasting only until the next dose of statin) and although these levels are not clinically dangerous (especially in LDL-apheresis), a certain amount of statins is retained during EE, and it is convenient to take a statin dose in the evening before EE (at least 15 hours before EE), especially in the case of hemorheopheresis. EE is then performed after the mean half-life elimination time of statin; thus, the absolute loss of statin is lower. We have shown (see above) that the absolute loss of statin is lower in the patients taking a statin dose at least 15 hours before EE than in patients taking the dose 2–4 hours before EE. It can be hypothesized that the reason why statin losses decrease with increasing the time interval between statin medication and EE is simple—they are practically cleared in a longer time after the mean life-time. Administration of medication in the morning followed by EE at the time of maximum statin plasma concentration is not ideal from this point of view. Exceptional cases that require the patient to take the drug in the morning on the day of EE can be resolved by the recommendation to take the drug after EE.

5. Conclusion

The UHPLC-MS/MS analytical method developed for the determination of statins present in biological materials appears to be adequately sensitive, precise, and highly selective for the purpose of studying patients with FH after EE. After EE, the concentrations of simvastatin and atorvastatin, as well as their metabolites and interconversion forms, were decreased. This outcome is conditioned by the decrease in concentration over time (according to pharmacokinetic properties), the type of procedure applied, and, most likely, other factors as well. However, we observed specific levels of statins in the washing liquids and filters. This amount was more important after hemorheopheresis. Therefore, it is convenient to prolong the interval between statin dosage and EE to greater than the half-life of the statins (dose ~6 P.M. the evening before the procedure). In contrast, a morning dose of statins on the day of EE is inconvenient, especially for hemorheopheresis.

Acknowledgments

This work was supported by a Grant from the Internal Grant Agency, Ministry of Health, Czech Republic, No. NS/9743-4, MZO 00179906, MSM 0021620820.

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PŘÍLOHA III

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Current trends and advances in modern bio-analytical methods: chromatography and sample preparation- a review

Analytica Chimica Acta, 2009, vol. 656, p. 8-35.

Analytica Chimica Acta 656 (2009) 8–35

Contents lists available at ScienceDirect

Analytica Chimica Acta

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

Review

A review of current trends and advances in modern bio-analytical methods: Chromatography and sample preparation

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ARTICLE INFO

Article history:
Received 10 July 2009
Received in revised form
29 September 2009
Accepted 1 October 2009
Available online 8 October 2009

Keywords:
UHPLC
HTLC
PHW-LC
HILIC
Monolith
Fused core columns
Bio-analytical method
Sample preparation
Microextraction
SPE
LLE
RAM
MIP
TFC
MEPS

ABSTRACT

Any bio-analytical method includes several steps, all of them being important in order to achieve reliable results. The first step is taking aliquots of samples for the analysis, followed by the extraction procedure and sample clean-up, chromatographic analysis and detection. Chromatographic methods, particularly liquid chromatography, are the methods of choice in bio-analytical laboratories. Current trends in fast liquid chromatographic separations involve monolith technology, fused core columns, high temperature liquid chromatography and ultra-high performance liquid chromatography (UHPLC). UHPLC has recently become a wide-spread analytical technique in many laboratories which focus on fast and sensitive bio-analytical assays. The key advantages of UHPLC are the increased speed of analysis, higher separation efficiency and resolution, higher sensitivity and much lower solvent consumption as compared to other analytical approaches. This is all enabled by specially designed instruments and sub-2-microne particle packed analytical columns.

There is a great contrast between ultra-fast chromatographic analysis and conventional sample preparation, which remains highly labor-intensive and time-consuming. Conventional sample preparation techniques including SPE, solid phase extraction; LLE, liquid–liquid extraction; PP, protein precipitation and many modern approaches (RAM, restricted access material; MIP, molecularly imprinted polymers; SPME, solid phase microextraction; LLME, liquid–liquid microextraction; MEPS, microextraction by packed sorbent and many others) have also been featured as fundamental and critical step of bio-analytical methods.

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doi:10.1016/j.aca.2009.10.004

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1. Introduction

Bio-analytical methods include analytical methods used in monitoring of drugs in biological materials. Such methods have different purposes: (1) to evaluate the pharmacokinetics and the metabolism in drug discovery of new candidates, (2) to compare the pharmacokinetic profiles of newly developed generic drug formulations, (3) to perform routine drug monitoring in order to establish appropriate dosage schemes, to reveal inter-individual metabolic variability and to minimize adverse effects, and (4) to determine drugs, drugs of abuse and their metabolites in forensic science applications. Requirement for such methods is often accompanied by an increasing number of biological samples needing fast quantitative analysis, together with a decrease in the desired quantitation levels, as the bioavailability of many drugs is at a low level and thus target concentrations are very low. Consequently, appropriately designed, fast, effective and sensitive bio-analytical methods are needed. In general, a reliable bio-analytical method, which is convenient for an intended purpose, should fulfill the requirements of validation guidelines, including accuracy, precision, selectivity, sensitivity, reproducibility and stability [1].

Nowadays, the main objective of bio-analytical laboratories is to develop reliable, fast and efficient procedures for performing qualitative and quantitative analyses. High performance liquid chromatography (HPLC) still remains a method of choice, as it is able to separate quite complicated mixtures of low and high molecular weight compounds, as well as different polarities and acid–base properties in various matrices. Unfortunately, conventional HPLC methods must sacrifice either time or resolution. Therefore, it is necessary to develop fast or ultra-fast methods without any loss of separation efficiency.

There are three main modern approaches in HPLC methods which enable the reduction of analytical time without compromising resolution and separation efficiency—the use of monolith columns, liquid chromatography at high temperatures and liquid chromatography at ultra-high pressures using sub-2-microne particle packed columns [2,3]. As a new trend, the application of fused core columns has come to be used in fast LC applications [4,5]. Another modern analytical approach which has become very popular in bio-analysis over the past few years is hydrophilic interaction liquid chromatography (HILIC). As drugs and their metabolites are often very polar molecules and HILIC is designed to address such molecules in a very efficient way, this method is able to solve many problematic retention/separation problems [6].

Samples obtained from biological materials are usually not directly compatible with HPLC analyses due to their complexity and protein content. Biological samples are problematic due to the irreversible adsorption of proteins in the stationary phase, resulting in a substantial loss of column efficiency and an increase in back-pressure. Sample preparation has conventionally been performed using protein precipitation (PPT), liquid–liquid extraction (LLE), or solid phase extraction (SPE). The manual operations associated with these processes are very labor-intensive and time-consuming, consisting of many steps. Using these methods, sample preparation time is far greater than with ultra-fast chromatographic techniques. Modern trends in sample preparation include parallel sample processing in 96-well plates and direct injection of the biological sample using on-line SPE or restricted access material (RAM) extraction. There are also a huge number of new sample preparation techniques being developed.

In this review, recent developments in bio-analytical applications of fast chromatographic separations, especially UHPLC technique, have been outlined and critically reviewed. The attention has been placed on instrumentation, stationary phases and detection possibilities. Sample preparation methods are discussed as initial and a critical part of bio-analytical methods, Conventional as well as modern approaches to sample preparation step are examined.

2. Advances and trends in chromatographic approaches

2.1. Monolith columns

Use of monolith sorbent instead of porous particles packed in columns has become popular in the field of bio-analytical applications in past few years. Monoliths can accept high flow-rates (up to 10 mL min^{-1}) in conventional column lengths without generating high back-pressures, which is their main advantage. The efficiency and resolution of monolith sorbents are comparable to silica par-

ticles of 3 μm in diameter. Monolithic rods are made by sol–gel technology, which enables the formation of highly porous material, containing both macropores and mesopores in its structure. Such an LC column consists of a single rod of silica or polymer-based material with two kinds of pores. The large pores (typically 2 μm) enable low flow resistance and therefore allow the application of high eluent flow-rates, while the small pores (about 12 nm) ensure sufficient surface area in order to reach high separation efficiency. Because of this, much higher flow-rates can be used, while the resolution of the monolith rod column is much less affected in regards to particulate materials. Column back-pressure remains low as well. Another practical advantage is the short-time needed for column equilibration when a mobile phase gradient is used. Monoliths allow the easy application of flow-rate gradients or the coupling of several columns together in order to increase separation efficiency [7]. On the other hand, there are several drawbacks to the use of monolith columns. One is the limited number of commercially available stationary phases (C8, C18, plain silica only). Another is the internal diameters of monolith columns (i.e., 4.6 and 3.0 mm, or 100 μm i.d. are typical; however, 2.0 or 3.0 have not as yet been manufactured in all common column lengths). These two disadvantages reduce their application domains substantially. Large internal column diameters, which are more readily available in all column lengths, are not fully compatible with MS (mass spectrometry) and induce a high consumption of organic solvent, especially with flow-rates up to 10 mL min^{-1}. Finally, monoliths made of silica possess a limited chemical stability (pH range 2–8) [2], which again limits their applicability. Recent applications of monolith technology in bio-analytical methods are demonstrated in Table 1 [8–13]. Monolith technology enables a substantial reduction in analysis time and has been proven to be suitable for bio-analytical measurement. Up to the present time, *Chromolith* commercial monolith columns have been for the most part used for routine analyses of biological fluids, including plasma, urine, serum and blood. Typically, a 100 mm column length is used in order to enhance separation efficiency. Monoliths are often used in connection with UV [8,12,13] or FD (fluorescence detection) [10]. Due apparently to the extremely high flow-rates applied in monoliths, they have generally not been used in mass spectrometry detection.

2.2. High temperature liquid chromatography (HTLC)

High temperature liquid chromatography (HTLC) ($T > 60$ C) can also be used to perform rapid analysis using standard column lengths, since mobile phase viscosity and back-pressures are decreased. Efficiency, mass transfer, and optimal velocity increases simultaneously with temperature, permitting the application of high mobile phase velocity. The low viscosity and high diffusivity of a mobile phase at high temperatures produce much lower mass transfer resistance, which leads to flatter van Deemter curves. Therefore, HTLC can be faster and more efficient. The term used for HTLC, in which pure water is used as an eluent is pressurized hot water liquid chromatography (PHW-LC, water > 100 C). This is distinguished from conventional HPLC in that solvent strength is increased by temperature rather than by organic solvents. The absence of an organic solvent makes HTLC an environmentally friendly technique, therefore it is sometimes called green chromatography [2,14].

However, even with the afore-mentioned advantages, HTLC is not routinely used, since it has some drawbacks. First, the limited availability of stable high temperature-resistant packing materials is a problem. Second, a potential degradation of unstable com-

Table 1
Use of monolith stationary phases in bio-analytical applications.

Determined substances	Matrix sample prep.	Method/stationary phase	Mobile phase	Detection	Analysis time	Validation data	Reference
L-Ascorbic acid, isoascorbic acid; L-dehydroascorbic acid (subtraction)	Blood	Chromolith performance C18 (100 mm × 4.6 mm)	2.5 mM NaH_2PO_4; 2.5 mM DTMACl; 1.25 mM Na_2EDTA; 2% ACN	UV 264 nm	0.8 min	$r^2 = 0.999$; R.S.D. = 4.2–4.5%; R.S.D. = 4.3–4.6%; LOD = 1.5 μmol L^{-1}; LOQ = 4.95 μmol L^{-1}	[8]
Captopril	Human plasma PP	Cromolith performance RP 18e (100 mm × 4.6 mm)	ACN: 0.1% (v/v) FA v H_2O (40:60) (v/v)	ESI-MS/MS $[M+H]^+$ derivatization	NA	$r^2 = 0.993$; LOQ = 10 ng mL^{-1}; CV = 9.9%; R.S.D. = 3.9%	[9]
Tramadol; O-desmethyltramadol; N-desmethyltramadol; O,N-didesmethyltramadol	Human plasma, saliva, urine LLE	Chromolith performance RP18e (100 mm × 4.6 mm)	MeOH:H_2O, 19:81 (v/v)	FD; excitation: 200 nm; emmision: 301 nm	7 min	$r^2 = 0.995–0.998$; R.S.D. = 1.1–5.2% (in plasma); LOQ = 2.5 ng mL^{-1}	[10]
Cyclosporin A	Human plasma PP, on-line SPE	Chromolith performance RP18e (10 mm × 4.6 mm)	ACN:ammonium acetate, 90:10	ESI-MS/MS $[M+H]^+$	3 min	R.S.D. = 1.9–4.7%; LOD = 0.4 ng mL^{-1}; LOQ = 0.015 μg mL^{-1}	[11]
Retinol, α-tocopherol	Blood serum LLE	Chromolith performance RP18e (100 mm × 4.6 mm)	100% MeOH	DAD 325 nm	1.8 min	$r^2 = 0.9997–0.9999$; R.S.D. = 5.93%; LOD = 0.02 μmol L^{-1}; LOQ = 0.07 μmol L^{-1}	[12]
				295 nm		R.S.D. = 5.58%, LOD = 0.1 μmol L^{-1}; LOQ = 0.3 μmol L^{-1}	
Retinol, α-tocopherol, retiny-palmitate retinyl-stearate	Blood serum LLE	Chromolith performance RP18e (100 mm × 4.6 mm)	MeOH:H_2O, 95:5 (v/v)	DAD 325 nm	6 min	$r = 0.9995–0.9998$	[13]
			MeOH:2-propanol, 60:40 (v/v)	295 nm		R.S.D. = 5.3–8.1%; R.S.D. = 0.32–1.92%	
				330 nm		LOD = 10 nmol L^{-1} to 0.5 μmol L^{-1}; LOQ = 0.02–1.1 μmol L^{-1}	

pounds could occur. This despite the fact that several authors have claimed that compounds do not decompose at high temperature, since the analysis time is very short and analytes are on chromatographic support. Finally, a particular set-up is required for, respectively, heating, then cooling the mobile phase before and after travel through the chromatographic column. Heat transfer inside the column is slow therefore possible radial and axial temperature gradients could occur [2,14]. As a result, only a few applications of HTLC in bio-analytical approaches have been published so far [15–17].

In some articles, the abbreviation HTLC was used for completely different techniques—high-throughput liquid chromatography [18,19], or high turbulence liquid chromatography [20], the latter actually referring to turbulent-flow chromatography (a sample preparation step for biological samples using a narrow-bore, large particle size column at high flow-rates, see Section 3.3.7). This could cause confusion, especially in the use of scientific literature databases.

2.3. Ultra-high performance liquid chromatography (UHPLC)

The use of sub-2-microne particles in ultra-high pressure systems presents another option in performing rapid analyses. Initially based on the theories of van Deemter et al. [21], then Giddings [22] and finally Knox [23], the use of small particles is one of the best solutions in the quest to improve chromatographic performances. Optimal separations are also achieved at higher linear velocities and over a wider range of linear velocities because of the low mass transfer resistance of these supports. Thus, better resolution and shorter analysis time could be attained by reducing the particle size. These factors have been taken advantage of through the increasingly used technique of ultra-high performance liquid chromatography (UHPLC), also termed ultra-high pressure liquid chromatography. The UHPLC approach will be discussed in detail below, as its use has become the most pervasive (Table 2).

2.3.1. Use of small particles at ultra-high pressures

The use of small particles in ultra-high pressure systems is one of the best solutions for the speeding up of analyses without compromising efficiency or resolution. Based on the van Deemter theory, then on Giddings, later on Knox and further interpretations, efficiency expressed as the height equivalent to a theoretical plate H is proportional to particle size squared—Eq. (1):

$$H = A + \frac{B}{u} + Cu = 2\lambda d_p + \frac{2\gamma D_M}{u} + \frac{f(k)d_p^2 u}{D_M} \quad (1)$$

where u is the linear velocity of mobile phase, and A, B, C are constants related to eddy diffusion (A), longitudinal diffusion (B) and mass transfer in mobile and stationary phase (C), d_p is the particle diameter of column packing material, D_M is the analyte diffusion coefficient, λ is the structure factor of the packing material, γ is a constant termed tortuosity or obstruction factor and k is the retention factor for an analyte [21]. Recently, Desmet et al. [24,25] has further described the optimization of efficiency in much more detail using kinetic plots. Kinetic plots are advantageous in that the performance of different HPLC supports can be directly compared, regardless of their length, particle size or pressure drop. The influence of maximum back-pressure on N_{max} or t_0 is described following by Eqs. (2) and (3):

$$N_{max} = \frac{\Delta P_{max}}{\eta}\left[\frac{K_{V_0}}{u_0 \times H}\right] \quad (2)$$

$$t_0 = \frac{\Delta P_{max}}{\eta}\left[\frac{K_{V0}}{u_0^2}\right] \quad (3)$$

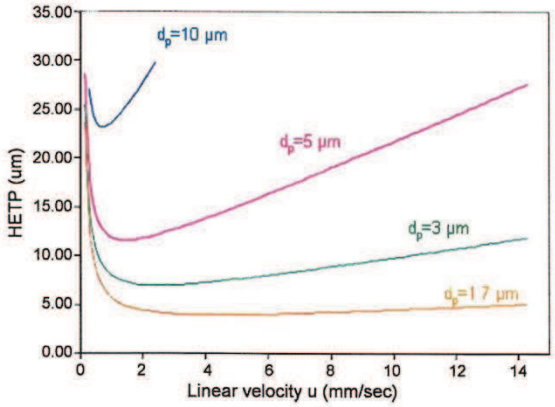

Fig. 1. Theoretical van Deemter curves plotted for 10, 5, 3 and 1.7 µm particles.

where K_{V_0} is the column permeability, u_0 is the velocity of permeating nonretained solute, η is the mobile phase viscosity and H is the height equivalent to the theoretical plate. Both equations use a scaling value to describe the drop in pressure ΔP and the viscosity η. A *Free Excel* spreadsheet can be used to draw kinetic plots [26]. Kinetic plots may in this way be used as a selection tool for the best particle type, column temperature and length in order to achieve a given number of theoretical plates.

The plot height equivalent to the theoretical plate H versus linear velocity u (van Deemter plot) describes the performance of various particles. Sub-2-microne particles demonstrate lower values of H and thus higher efficiency. At the same time, optimal separations are achieved at higher linear velocities and over a wider range of linear velocities because of the low mass transfer resistance of these supports. Therefore, sub-2-microne particles may be used to obtain better resolution and reduced analysis time [2]. The curve is much steeper for larger particles at high linear velocities because of the C term of Eq. (1), where the particle size is squared—see Fig. 1.

However, small particles induce a high pressure drop, and according to Darcy's law, the pressure drop is inversely proportional to particle size at the optimum linear velocity—Eq. (4):

$$\Delta P = \Phi \frac{\eta L u}{d_p^2} \quad (4)$$

where Φ is the flow resistance, η is the mobile phase viscosity, L is the column length, u is the mobile phase linear velocity and d_p is the particle size. As u is inversely proportional to particle size, pressure drop is inversely proportional to the cube of particle diameter. This means that under optimal flow velocity a 1.7 µm particle packed column will generate 27 times higher pressure than a 5 µm particle packed column. Therefore, new ultra-high pressure resistant systems had to be developed in order to profit fully from the advantages of the use of sub-2-microne particles. A comprehensive review of ultra-high pressure liquid chromatography techniques for fast separations was published by Wu and co-workers [3].

2.3.2. Instrumentation

In UHPLC, the most challenging part of the system is sample introduction at very high pressures in a miniaturized volume. This was studied most importantly by Jorgenson et al. and by Lee et al., who developed the first static split injection and later the pressure balanced valve [27–29]. UHPLC systems had been originally used in academic research laboratories for use with capillary columns. In 2004 the *Waters* corporation introduced the first commercially available UHPLC system, which was followed at first modestly and later more extensively by other important manufacturers (Table 3).

Table 2
Applications of UHPLC in bio-analysis.

Determined substances	Matrix sample preparation	Method/stationary phase	Mobile phase	Detection	Analysis time	Validation data	Reference
Lansoprazol	Human plasma LLE	ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm)	0.5% FAc in H_2O:0.5% FAc in ACN 30:70	API-MS/MS $[M+H]^+$	0.4 min	r^2 = 0.9999; R.S.D. = 0.51–8.61%; LOQ = 50 pg mL^{-1}	[36]
5-Hydroxytryptophol glucuronid (GTOL)	Urine SPE	ACQUITY HSS C18 (100 mm × 2.1 mm, 1.8 μm)	0.1% FAc: ACN 97:3, gradient	ESI, APCI-MS/MS $[M-H]^-$	2.4 min	r^2 = 0.999; R.S.D. = 5.8%; LOQ = 6.7 nmol L^{-1} R.S.D. = 3.3%; LOQ = 0.02 μmol L^{-1}	[37]
5-Hydroxyindoleacetic acid (HIAA), IS = GTOL-H_4, 5-HIAA-H_2							
6 metabolites of acetaminofen S-adenosyl-L-methionine	Rat urine ultracentrifugation	ACQUITY UPLC BEH C18 (100 mm × 2.1 mm, 1.7 μm)	0.1% FAc in H_2O: 0.1% FAc in ACN 0:100, gradient	ESI-MS/MS $[M+H]^+$ $[M-H]^-$	11 min		[38]
Acarviostatins—6 groups (I03, II03, II13, II23, III03, IV03)	Filtrate of culture *S. coelicoflavus ZG0656*	ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm)	ACN:1.5 mM aqueous ammonia 1.5:98.5, gradient	ESI-MS/MS $[M+H]^+$ $[M+2H]^{2+}$ $[M-OH]^+$	11 min		[39]
Diastereoizomers of drug candidate	Rat plasma, blood LLE	ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm) comparison with HPLC	A: 0.1% FAc in H_2O B: ACN:MeOH (20:80) + 0.1% FAc 39:61, gradient	ESI-MS/MS $[M+H]^+$	3 min 8 min	r > 0.99; R.S.D. = 3.8–10.9%; LOQ = 1 ng mL^{-1}	[40]
Amphetamine methamphetamine and derivatives, ketamine ephedrine,pseudoephedrine, phentermine	Blood SPE	ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm)	Pyrrolidin:MeOH 50:50	ESI-MS/MS $[M+H]^+$	3 min		[41]
Apolipoproteins A1, A2, A4, C1, C2, C3, D, F, M	Human plasma LLE	ACQUITY UPLC BEH C18 (100 mm × 2.1 mm, 1.7 μm) comparison with HPLC	A: 2% ACN v 0.1% FAc B: 90% ACN v 0.1% FAc 90:10, gradient	ESI-MS/MS $[M+H]^+$	3 min 65 min	r^2 = 0.9989 (apo A1); R.S.D. = 3.5–7.6%	[42]
Warfarin IS = coumachlor	Human plasma SPE	ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm)	0.1% FAc:ACN 50:50	ESI-MS/MS $[M-H]^-$	3 min	$r \geq$ 0.9999; R.S.D. = 3.0–10.2%; LOQ = 0.25 ng mL^{-1}	[43]
2-Thiouracile (TU), 6-methyl-2-TU, 6-propyl-2-TU, 6-phenyl-2-TU, 1-methyl-2-mercaptoimidazole, 2-mercaptobenzimidazole (MBI) IS = dimethyl-TU, MBI-d_4	Thyroid tissues SPE	ACQUITY UPLC BEH C18 (100 mm × 2.1 mm, 1.7 μm)	0.1% FAc in H_2O:0.1% FAc in ACN 100:0, gradient	ESI-MS/MS $[M+H]^+$	6.5 min	r = 0.999; R.S.D. = 6%; LOQ = 0.05 μg mL^{-1}	[44]
Doxazosine IS = tamsulosine	Human plasma	ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm)	A: 0.05% pentadecafluorooctanoic acid in ACN B: 0.05% pentadecafluorooctanoic acid in H_2O 10:90, gradient	ESI-MS/MS $[M+H]^+$	1.8 min	$r \geq$ 0.999; R.S.D. = 8%; LOD = 0.02 ng mL^{-1}	[45]
Anandamide (AEA) virodhamine IS = AEA-d_8	Human plasma LLE	ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm)	A: 2 mM AmAc + 0.1% FAc B: 0.1% AmAc in ACN 80:20, gradient	ESI-MS/MS $[M+H]^+$	1.8 min	r^2 = 0.999; R.S.D. = 3.7–4.8%; LOD = 18.75 fmol mL^{-1}; LOQ = 25 fmol mL^{-1}	[46]

Lovastatin IS = mycophenolate mofetil	Human plasma LLE	ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm)	H_2O:ACN 20:80	ESI-MS/MS $[M+H]^+$	2.5 min	R.S.D. = 15%; LOQ = 0.08 ng mL^{-1}	[47]
Ethinyl estradiol, 19-norethindrone, levonorgestrel IS = deuterium labeled	Human plasma semi-automated LLE derivatization	ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm) comparison with HPLC	A: 0.1% FAc v ACN B: ACN:H_2O (50:50) gradient	ESI-MS/MS $[M+H]^+$	2.7 min 6 min	r^2 = 0.9988–0.9993; R.S.D. = 10.7–13.4%; LOQ = 0.01 ng mL^{-1}; LOQ = 0.1 ng mL^{-1}	[48]
Coenzyme Q10	Rat serum LLE	ACQUITY UPLC BEH C18 (50 mm × 1.0 mm, 1.7 μm)	ACN:2-propanol:FAc 90:10:0.1	APCI-MS/MS $[M+H]^+$ $[M-H]^-$	4.5 min	r = 0.9991; R.S.D. = 10%; LOQ = 50 ng mL^{-1}	[49]
IS = coenzyme Q9 melatonin, nicotin, bupropion, repaglinid losartan, omeprazol, dextromethorphan chlorzoxazon, midazolam and cytochrome P450 specific metabolites	Human urine LLE	ACQUITY BEH Shield RP18 (50 mm × 2.1 mm, 1.7 μm) comparison with HPLC	0.1% AcAc:ACN 95:5, gradient	ESI-MS/MS $[M+H]^+$ $[M-H]^-$	3.25 min 12 min	R.S.D. = 1–14%; LOD = 0.05–1.0 ng mL^{-1}	[50]
Methoxalen	Human plasma automated LLE	ACQUITY UPLC BEH C18 (50 mm × 1.0 mm, 1.7 μm)	10 mM AmF:ACN 60:40, gradient	ESI-MS/MS $[M+H]^+$	1.0 min	r = 0.9988; R.S.D. = 3.0–7.7%; LOQ = 1.1 ng mL^{-1}	[51]
IS = ketokonazole hydrochloride ethyl glucuronide (Et-G)	Hair (post-morten) incubate in water	ACQUITY HSS T3 (100 mm × 2.1 mm, 1.8 μm)	0.1% FAc:ACN 99:1, gradient	ESI-MS/MS $[M-H]^-$	1.6 min	r^2 = 0.999; R.S.D. < 3.5%; LOQ = 2.5 pg mg^{-1}	[52]
IS = Et-G-d5 cladribine, clofarabine	Mouse plasma PP	ACQUITY UPLC BEH C18 (50 mm × 1.0 mm, 1.7 μm) comparison with HPLC	A: 4 mM AmAc + 0.1% FAc in H_2O B: 4 mM AmAc 0.1% FAc in ACN, 95:5, gradient	APCI (APPI)-MS/MS	0.9 min 2.0 min	r^2 > 0.99; R.S.D. < 15%	[53]
IS = ketoconazole baicalin	Rat cerebrospinal liquid blood in vivo microdialysis	ACQUITY UPLC BEH C18 (50 mm × 1.0 mm, 1.7 μm)	ACN:0.1% FAc 10:90, gradient	ESI-MS/MS $[M+H]^+$	3 min	r = 0.9986; R.S.D. = 1.8–8.1%; LOD = 0.1 ng mL^{-1}; LOQ = 2.37 ng mL^{-1}	[54]
Tauroursodeoxycholic, glycoursodeoxycholic, taurocholic, glycocholic, taurochenodeoxycholic, ursodeoxycholic, taurodeoxycholic, glycochenodeoxycholic, cholic, glycodeoxycholic chenodeoxycholic, deoxycholic, lithocholic acids	Human plasma on-line RAM	ACQUITY UPLC BEH C18 (100 mm × 2.1 mm, 1.7 μm)	0.1% FAc in H_2O:0.1% FAc in MeOH 50:50, gradient	ESI-MS/MS $[M-H]^-$	28 min	r^2 = 0.995–0.999; R.S.D. = 0.9–9.9%; LOD = 11–427 pg; LOQ = 38–473 pg	[55]
Nimodipine IS = nitrendipine	Human plasma LLE	ACQUITY UPLC BEH C18 (50 mm × 1.0 mm, 1.7 μm)	0.1% FAc in H_2O:0.1% FAc in ACN 50:50, gradient	ESI-MS/MS $[M+H]^+$	1.5 min	r = 0.9948; R.S.D. = 3.1–7.2%; LOQ = 0.2 ng mL^{-1}	[56]
Morphine, codeine, 6-monoacetylmorphine pholcodine, oxycodone, ethylmorphine, cocaine, benzoylecgonine	Urine SPE	ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm)	5 mM ammonium bicarbonate:MeOH 95:5, gradient	ESI-MS/MS $[M+H]^+$	4.6 min	R.S.D. = 0.1–0.6%; LOD = 0.001–0.02 μg mL^{-1}; LOQ = 0.003–0.06 μg mL^{-1}	[57]
Celecoxibe etoricoxibe (COX-2 inhibitors)	Serum, synovial liquid LLE	ACQUITY UPLC BEH C18 (150 mm × 2.1 mm, 1.7 μm)	0.1% FAc in H_2O:0.1% FAc in MeOH 90:10, gradient	ESI-TOF-MS ICP-MS $[M+H]^+$	8 min	r^2 = 0.91–0.996; R.S.D. = 2.8–3.4%; LOD = 0.55 ng	[58]

Table 2 (*Continued*)

Determined substances	Matrix sample preparation	Method/stationary phase	Mobile phase	Detection	Analysis time	Validation data	Reference
Testosterone (TE), 5α-dihydrotestosteron (DHT), IS = [$^2H_3$]-TE, [$^2H_3$]-DHT	Human serum LLE	ACQUITY UPLC BEH C18 (100 mm × 2.1 mm, 1.7 μm)	H_2O:2 mM AmAc in ACN/H_2O (98:2) 100:0, gradient	ESI-MS/MS $[M-H]^-$	4.5 min	r = 0.9983; R.S.D. = 15%; LOQ = 0.01 ng mL^{-1}	[59]
Azithromycine	Human plasma LLE	ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm)	50 mM AmAc in H_2O:ACN 40:60, gradient	ESI-MS/MS $[M+H]^+$	2 min	r = 0.9991; R.S.D. = 15%; LOQ = 1 ng mL^{-1}	[60]
Mitiglinide IS = nateglinide	Human plasma LLE	ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm)	MeOH:10 mmol L^{-1} AmAc 65:35	ESI-MS/MS $[M+H]^+$	2 min	r = 0.9973; R.S.D. = 4.0–14.0%; LOQ = 1.080 ng mL^{-1}	[61]
16α-, 2α-, 7α, 6α-, 2α-, 6α-, and 16α-hydroxyl testosterone, IS = corticosterone	Microsoms of rat hepatic cells PP	ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm)	A: 5% ACN + 0.1% FAc in H_2O B: 0.1% FAc in MeOH 95:5, gradient	Turbospray-MS/MS $[M+H]^+$	2.5 min	r^2 > 0.99; R.S.D. = 0.8–7.7%; LOQ = 0.005 μg mL^{-1}	[62]
Ketoprofen, IS = ibuprofen	Dermal microdialysis sample LLE	ACQUITY UPLC BEH C18 (100 mm × 2.1 mm, 1.7 μm)	ACN:MeOH:H_2O 60:20:20	ESI-MS/MS $[M-H]^-$	2 min	r > 0.999; R.S.D. < 2%; LOD = 0.5 ng mL^{-1}; LOQ = 0.1 ng mL^{-1}	[63]
17 ginsenosides, IS = dioxine	Rat urine SPE	ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm)	H_2O:ACN 95:5, gradient	ESI-MS/MS $[M+H]^+$ $[M+Na]^+$ $[M+K]^+$ $[M-H]^-$	13 min	r^2 = 0.993–0.999; R.S.D. = 5.7–11.2%; LOD = 2–18 ng mL^{-1}; LOQ = 8–55 ng mL^{-1}	[64]
Icariin, icariside II, osthole, IS = carbamazepine	Rat plasma PP	ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm)	0.1% AcAc:ACN 75:25, gradient	ESI-MS/MS $[M+H]^+$	7.2 min	r = 0.9975–0.9991; R.S.D. < 8.0%; LOQ = 2.00 ng mL^{-1}	[65]
Bufalin, cinobufagin, resibufogenin, IS = diphenhydramin	Rat plasma LLE	ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm)	ACN:0.1% FAc in H_2O 10:90, gradient	ESI-MS/MS $[M+H]^+$	3 min	r = 0.9915–0.9953; R.S.D. = 1.41–11.35%; LOQ = 1.0 ng mL^{-1}	[66]
Akonitine, mesaconitine, hypaconitine, benzoylhypacone	Human plasma SPE	ACQUITY UPLC BEH C18 (100 mm × 2.1 mm, 1.7 μm)	0.1% FAc:MeOH 60:40, gradient	ESI-MS/MS $[M+H]^+$	5 min	r^2 = 0.996–0.998; R.S.D. = 2.22–8.61%; LOD = 0.033 ng mL^{-1}; LOQ = 0.1 ng mL^{-1}	[67]
Pravastatin	Rat urine	ACQUITY UPLC BEH C18 (100 mm × 2.1 mm, 1.7 μm)	A: 0.1% FAc in H_2O B: 0.1% FAc in CAN	ESI-MS/MS $[M+H]^+$	13 min		[68]
Pregnenolon, 17-hydroxypregnenolon dehydroepiandrosteron, progesterone, 16-hydroxyprogesteron, 17-hydroxyprogesteron androstenedion	Human plasma LLE	ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm) ACQUITY UPLC BEH C18 (100 mm × 2.1 mm, 1.7 μm)	0.1% F Ac:ACN 15:85, gradient A: 0.1% FAc B: ACN:MeOH (3:1) + 1% isopropanol 66:44, gradient	APCI-MS/MS $[M+H]^+$ $[M-H_2O+H]^+$ $[M-2H_2O+H]^+$	8 min	R.S.D. = 2.4–19.4%; LOD = 5–40 ng mL^{-1}; LOQ = 10–110 ng mL^{-1}	[69]
Multiresidual screening veterinary drug: quinolones (13), sulfonamids (32), nitroimidazols (6), penicillins (12), macrolids (20), tetracyclines (5), anthelmintic/benzimidazols (9) tranquliziers (7)	Urine	ACQUITY (50 mm × 2.1 mm, 1.7 μm)	A: H_2O:FAc:ACN (47:3:50) B: H_2O:FAc:ACN (50:3:47) 100:0 gradient	ESI-MS/MS $[M+H]^+$	6 min		[70]
Artemisinin IS = B-arteeher	Rat serum LLE	ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm)	MeOH:0.3% FAc 80:20	ESI-MS/MS	2.8 min	r = 0.9981; R.S.D. = 2.6–15.8%; LOQ = 4 ng mL^{-1}	[71]

Diastereoisomers of SCH 503034 2H9-SCH 503034	Monkey plasma LLE	ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm) comparison with HPLC	A: MeOH/H_2O (5:95) + 40 mM perfluopenthanic acid (PFPA) B: MeOH:H_2O (95:5) + 40 mM PFPA 50:50	APCI-MS/MS $[M+H]^+$	6 min 14 or 18 min	$r^2 > 0.99$; R.S.D. = −1.3 to 6.8%; LOQ = 1 ng mL^{-1}	[72]
Lacidipine IS = nifedipine	Human plasma LLE	ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm)	30 mM AmAc:ACN 18:82	ESI-MS/MS $[M+H]^+$	2 min	$r^2 = 0.9956$; R.S.D. = 3.3–11.3%; LOQ = 0.025 ng mL^{-1}	[73]
Alverine, para-hydroxy alverine, IS = mebeverine	Human plasma SPE	ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm)	ACN:10 mM AmF 60:40	ESI-MS/MS $[M+H]^+$	4 min	$r = 0.998$; R.S.D. = 1.91–6.26%; LOQ = 100 pg mL^{-1}	[74]
21 compounds: 19 compounds from preparation Yin Chen Hao Tang and 2 metabolites 45 in total with nonidentified	Rat plasma SPE	ACQUITY UPLC BEH C18 (100 mm × 2.1 mm, 1.7 μm)	A: 0.1% FAc in H_2O B: 0.1% FAc in ACN 99:1, gradient	ESI-MS/MS	18 min	LOD = 4.7–301.8 ng mL^{-1}	[75]
21 endogenous corticosteroids	Urine SPE	Hypersil Gold C18 (50 mm × 2.1 mm, 1.9 μm)	A: 0.1% AmAc in 5% ACN B: 0.06% AmAc in 95% ACN 90:10, gradient	ESI-MS/MS	12 min	$r^2 = 0.9903$–0.9992; R.S.D. = 1.7–7.4%; LOD = 0.5–1.0 ng mL^{-1}; LOQ = 0.5–2.0 ng mL^{-1}	[76]
Profiling of bile acids—6: cholic acid, chenodeoxycholic, muricholic acid, ursodeoxycholic, deoxycholic acid, lithocholic acid + their conjugates (taurin, glycin)	Hepatic tissues plasma PP, bile, urine SPE	ACQUITY UPLC BEH C18 (100 mm × 2.1 mm, 1.7 μm)	5% ACN in MeOH:7.5 mM AmAc	ESI-MS/MS	22 min	R.S.D. = 1.7–16.03%; LOD = 2–5 ng mL^{-1}	[77]
Puerarin, daidzein, baicalin, wogonoside, liquiritin, IS = carbamazepine	Rat plasma LLE	ACQUITY UPLC BEH C18 (100 mm × 2.1 mm, 1.7 μm)	MeOH:H_2O with 0.1% FAc + 5 mM AmAc, 35:65, gradient	ESI-MS $[M+H]^+$ $[M+Na]^+$ $[M-gluc+H]^+$	6 min	$r = 0.995$–0.999; R.S.D. = 3.2–11.4%; LOQ = 2.5–10.2 ng mL^{-1}	[78]
8-Oxo-guanosine (8-oxoGuo), 8-oxo-2′deoxy-guanosine (8-oxodG) IS = ^{15}N5-8-oxodG, ^{15}N5-8-oxoGuo	Human urine	Acquity UPLC BEH Shield RP18 (2.1 mm × 100 mm, 1.7 μm)	ACN:2.5 mM AmAc 0:100%, gradient	ESI-MS/MS $[M+H]^+$	23 min	R.S.D. = 1.4–8.3%; R.S.D. = 1.8–5.7%; LOQ = 1 nM	[79]
Gemcitabine IS = $[^{13}C_1,^{15}N_2]$-gemcitabine	Human plasma SPE	ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm)	ACN:0.1% FAc in H_2O 30:70, gradient	MS/MS $[M+H]^+$	1.4 min	$r = 0.9991$; R.S.D. = 11.3%; LOQ = 20 ng mL^{-1}	[80]
2,2-Difluoro-2-deoxyuridine (dFdU) IS = $[^{13}C_1,^{15}N_2]$-dFdU						$r = 0.9986$; R.S.D. = 5.6%; LOQ = 100 ng mL^{-1}	
16 β-agonists: metaproterenol, cimaterol, terbutaline, salbutamol, cimbuterol, fenoterol, clencyclohexerol, clenproperol, ractopamine, clenbuterol, formoterol, tulobuterol, mabuterol, clenpenterol, clenisopenterol, mapenterol, IS = salbuterol-d_3, clenbuterol-d_9	Pig liver, kidney, muscle, homogenisation sonification, SPE	ACQUITY UPLC BEH C18 (100 mm × 2.1 mm, 1.7 μm)	MeOH: 0.1% FAc 5:95, gradient	ESI-MS/MS $[M+H]^+$	10 min	$r^2 > 0.99$; R.S.D. = 3.4–12.0%; R.S.D. = 8.2–14.8%	[81]
43 benzodiazepine, zolpidem, zopiclone	Human plasma SPE	ACQUITY UPLC BEH C18 (100 mm × 2.1 mm, 1.7 μm)	0.05% FAc in H_2O:0.05% FAc in ACN 75:25, gradient	ESI-MS/MS $[M+H]^+$	12 min	$r^2 > 0.99$; R.S.D. = 0.6–10.1%; R.S.D. = 0.6–16.7%; LOD = 0.2–8.0 ng mL^{-1}	[82]

Table 2 (*Continued*)

Determined substances	Matrix sample preparation	Method/stationary phase	Mobile phase	Detection	Analysis time	Validation data	Reference
Alprostadil IS = diphenhydramine	Rat plasma LLE	ACQUITY UPLC BEH C18 (2.1 mm × 50 mm, 1.7 μm)	ACN:0.1% FAc 30:70, gradient	ESI-MS/MS $[M+H]^+$	2 min	$r^2 > 0.99$; R.S.D. = 2.4–8.5%; LOQ = 0.4 ng mL^{-1}	[83]
Lopinavir ritonavir IS = d_8-lopinavir, d_6-ritonavir	Human plasma SPE	ACQUITY UPLC BEH C18 (2.1 mm × 50 mm, 1.7 μm)	10 mM AmF with FAc:MeOH 10:90	ESI-MS/MS $[M+H]+$	1.2 min	R.S.D. = 1.3–2.3%; R.S.D. = 1.7–4.7%	[84]
α-Fluoro-β-alanine (FBAL), 5-fluorouracil (FU), capecitabine (cape), IS = [$^{13}C_3$]-FBAL, [$^{13}C_1$,$^{15}N_2$]-FU, [$^2H_{11}$]-Cape	Human plasma	ACQUITY UPLC BEH C18 (2.1 mm × 50 mm, 1.7 μm)	ACN:10 mM AmF 10:90, gradient	TSI-MS/MS $[M-H]^-$ $[M+H]^+$	4 min	r = 0.9979–0.9991; R.S.D. = 9.9–10.7%	[85]
Procyanidins and metabolites: catechin glucuronide, epicatechin glucuronide, catechin methylglucuronide epicatechin methyl-glucuronide, catechin methyl-sulphate, epicatechin methyl-sulphate, catechin, epicatechin, dimer, trimer, IS = catechol	Plasma SPE	ACQUITY HSS T3 (100 mm × 2.1 mm, 1.8 μm)	A: H_2O:AcA (99.8:0.2) B:ACN, 95:5, gradient	ESI-MS/MS $[M-H]^-$	12 min	R.S.D. = 3.5–4.3%; LOD = 0.003–0.8 μM; LOQ = 0.01–0.98 μM	[86]
Thiouracil, methylthiouracil, propylthiouracil, phenylthiouracil, tapazole, mercaptobenzimidazole, IS = tapazole-d_3, propylthiouracil-d_5	Urine, thyroid gland, homogenisation sonification, SPE	ACQUITY UPLC BEH C18 (100 mm × 2.1 mm, 1.7 μm)	A: H_2O:ACN:AcAc (80:20:0.1) B: H_2O:ACN:AcAc (10:90:0.1), gradient	ESI-MS/MS $[M-H]^-$ $[M+H]^+$	6 min		[87]
Trantinterol enantiomers, IS = diphenhydramine	Rat plasma, derivatization, LLE	ACQUITY UPLC BEH C18 (2.1 mm × 50 mm, 1.7 μm)	3 mM AmAc:ACN, 80:20, gradient	ESI-MS/MS $[M+H]^+$	7 min	r = 0.9981–0.9986; R.S.D. = 8.7–9.6%; LOQ = 1 ng mL^{-1}	[88]
Diltiazem, 2 metabolites: N-desmethyldiltiazem, O-desacetyldiltiazem, IS = ziprasidone	Human plasma LLE	ACQUITY UPLC BEH C18 (100 mm × 2.1 mm, 1.7 μm)	10 mM AmAc:ACN, 25:75	ESI-MS/MS	1.5 min	r = 0.99; R.S.D. = 1.2–9.4%; LOQ = 0.48 ng mL^{-1}; LOQ = 0.24 ng mL^{-1}	[89]
Glycyl-sarcosine, IS = isoniazid	Cell homogenate PP	ACQUITY UPLC BEH HILIC (50 mm × 2.1 mm, 1.7 μm)	0.1% FAc:ACN 10:90, gradient	ESI-MS/MS $[M+H]^+$	2.5 min	r = 0.9991; R.S.D. = 7.1%; LOQ = 1 ng mL^{-1}	[90]
Tocol, δ-tocopherol, γ-tocopherol, α-tocopherol, α-tocopherol acetate	Human kolostrum, human milk, homogenisation, sonification LLE	Zorbax Eclipse Plus C18 RRHT (2.1 mm × 50 mm, 1.8 μm) ACQUITY UPLC BEH C18 (2.1 mm × 50 mm, 1.7 μm)	ACN:MeOH 60:40	FD exc: 297 nm em: 328 nm PDA 292 nm	2 min	r = 0.999–1.000; R.S.D. = 6%; LOD = 0.021–0.034 μg mL^{-1}; LOQ = 0.07–0.119 μg mL^{-1} r = 0.990–0.999; R.S.D. = 3%; LOD = 0.09–0.022 ng mL^{-1}; LOQ = 0.27–0.73 ng mL^{-1}	[91]

IS = internal standard; LLE = liquid–liquid extraction; SPE = solid phase extraction; PP = protein precipitation; RAM = restricted access material; UPLC = ultra performance liquid chromatography; ACN = acetonitrile; MeOH = methanol; FAc = formic acid; AcAc = acetic acid; AmAc = amonium acetate; AmF = amonium formate; R.S.D. = relative standard deviation; LOQ = limit of quantitation; LOD = limit of detection; ESI = electrospray ionization; APCI = atmospheric pressure chemical ionization; MS/MS = tandem mass spectrometry; TOF = time of flight; ICP = inductively coupled plasma; FD = fluorescence detection; PDA = photo diode array.

Table 3
An overview of currently available commercial UHPLC instruments and their specifications.

Instrument name	Introduction on market	Maximum back-pressure	Flow-rate range	Temperature capability	Injection volume	Dead volume	Injection time	Acquisition rate	Manufacturer
Acquity UPLC	2004	100 MPa	Up to 2 mL min^{-1}	Up to 90 °C	0.1–50 μL	80 μL	15 s	Up to 80 Hz	Waters
1200 RRLC	2006	60 MPa	Up to 5 mL min^{-1}	Up to 100 °C	0.1–100 μL	260 μL	<30 s	Up to 80 Hz	Agilent
Acella High Speed	2006	100 MPa	Up to 2 mL min^{-1}	Up to 100 °C	1–1000 μL	65 μL	30 s	Up to 80 Hz	ThermoElectron
X-treme LC	2006	100 MPa	Up to 10 mL min^{-1}	Up to 65 °C	0.1–100 μL	Low[a]	30 s	Up to 100 Hz	Jasco
PromimenceUFLC	2007	35 MPa	Up to 10 mL min^{-1}	Up to 85 °C	0.1–100 μL	[b]	10 s	Up to 100 Hz	Shimadzu
LaChrom Ultra	2007	60 MPa	Up to 5 mL min^{-1}	Up to 85 °C	0.1–50 μL	Low[a]	30 s	Up to 100 Hz	Hitachi
Ultra HP	2007	120 MPa	Up to 5 mL min^{-1}	Only pump specified					Scientific Systems
RSLC UltiMate 3000	2008	80 MPa	Up to 5 mL min^{-1}	Up to 110 °C	0.01–500 μL	35 μL	15 s	Up to 100 Hz	Dionex
Platin Blue	2008	100 MPa	Up to 2 mL min^{-1}	Up to 140 °C	1–500 μL	110 μL	15 s	Up to 200 Hz	Knauer
Flexar (FX-15)	2009	100 MPa		Up to 90 °C	Min 1 μL		8 s	Up to 100 Hz	Perkin Elmer
Infinity	2009	120 MPa	Up to 5 mL min^{-1}	Up to 100 °C	0.1–100 μL	55–110 μL	<19 s	Up to 160 Hz	Agilent

[a] The manufacturer did not specify exact number.
[b] Depends on configuration.

Individual UHPLC systems differ in their amounts of maximum reachable back-pressure, flow-rate range possibilities, dead volume and other parameters, compared in Table 3.

Generally, a UHPLC system must primarily withstand high back-pressures, but this is not the only requirement. It must also be adapted to operate in fast and ultra-fast mode with reduced column diameters such as 2.1 mm i.d., limiting frictional heating and substantially reducing solvent consumption [2]. Simultaneously, such systems need low volume, detection cells to provide sensitive detection, small extra-column volumes, narrow tubing and low injection volume, as only 1% of column volume should be injected in order to prevent overload. Nguyen et al. summarized the requirements for UHPLC system as follows [2]:

- robust pumping and injection modules;
- fast injection cycles;
- small gradient delay volume;
- fast detection constant, high detector sampling rate (acquisition > 20 Hz);
- low extra-columns volumes, tubing and low volume detection cell;
- appropriate columns and stationary phases.

2.3.3. Stationary phases and columns

The key imperative for performing efficient separation at ultra-high pressure is the quality and stability of the packed stationary phases. Recently, many new stationary phases and new column geometries have been introduced in order to reduce the analysis time while maintaining high resolution and selectivity. Developments in stationary phases have led toward high chemical (a wide range of pH) and mechanical stability (high back-pressures) and also the introduction of new selectivity chemistries, including, e.g., for use in the HILIC stationary phase (Amide and Glycan being introduced by *Waters* in 2009).

Particle size has decreased since the 1950s, when 100 μm particle packed columns were used, through 10, 5, and 3 μm up to today sub-2-microne particles, which is the most basic way to acheive reduced analysis time without loss of efficiency and resolution. The first small nonporous supports containing sub-2-microne particles were introduced in 1988 by Horvath and co-worker [30]. Because of the lack of availability of concomitant instrumentation, small nonporous supports were commercialized much later, in 2004. Most stationary phases used for early capillary UHPLC featured 1–1.5 μm silica-based nonporous particles. Wu et al. [31] compared the efficiencies of columns packed with 1.5 and 3.0 μm nonporous and porous particles in UHPLC. It was observed that nonporous particles provided overall higher efficiency at high linear velocities. However, the difference in efficiency diminished significantly when the particle size was reduced from 3 to 1.5 μm. Further, the sample loading capacity for porous C18 particles was 15 times higher than that for nonporous C18 particles. In addition, the average retention factors for a 1.7 μm porous C18 column were significantly higher than those for a 1.5 μm nonporous C18 column. Pressure drops for nonporous particles were higher in practice than for porous particles, because mobile phases with a higher aqueous content were required to obtain similar retention factors. The majority of commercial packings are made of porous particles because of their higher sample loading capacity. Typically, 10 cm columns packed with 1.7–2.0 μm particles generate pressures compatible with the pressure limits of current commercial instruments [3].

A huge number of new stationary phases based on sub-2-microne particles using 1.5–2.0 μm particle sizes have been developed over past 5 years (Table 4). Both hybrid and silica materials were used as stationary phase supports and they were in addition modified by various chemistries including C8, C18, Phenyl,

Table 4
An overview of currently available sub-2-microne analytical columns and their properties.

Column name	Stationary phase support	Particle size [μm]	Limitations			Manufacturer
			pH	Column chemistry	Temperature	
Acquity BEH	Hybrid	1.7	1–12 2–11 1–8 2–11	(C8, C18, Phenyl) (Shield) (Silica for HILIC) (Amide, Glycan—HILIC phases)	20–90	Waters
Acquity HSS	Silica	1.8	2–8 1–8	(T3, C18 SB) (C18)	20–45	Waters
Alltima HP	Hybrid	1.5	1–10	(HILIC)	20–60	Alltech
Platinum	Silica	1.5	2–8	(C8, C18)	20–60	Alltech
GP Series	Silica	1.8	2–8.5	(C8, C18, C4)	20–60	Sepax
HP Series	Silica	1.8	2–8.5	(PHE, CN, NH_2 SCX, SAX, silica, HILIC)	20–60	Sepax
Poly-RP	Hybrid (PS/DVB)	1.0/1.7	1–14	(PHE)		
Hypersil Gold	Silica	1.9	1–11 2–9 2–8	(C18) (C8, Q) (PFP)	25–60	ThermoElectron
Nucleodur	Silica	1.8	1–11 1–10 1–9	(C8,C18) (C18 isis, Sphinx RP) (C18 pyramid)	Up to 85	Machery Nagel
Pathfinder	Hybrid	1.5	1–12	(AS, AP, PS, MR)	Up to 250	Shimadzu
Pinnacle DB	Silica	1.9	2.5–7.5	(C18, PFP-propyl, silica, acqueous C18, CN, C8, PAH, X3-C18)	Up to 80	Restek
Pronto Pearl		1.8 totally porous 1.5 nonporous	2–8	(C18, C8, aminopropyl) (C18)	20–60	Bischoff
TSKgel Super ODS	Silica	2.0	2.0–7.5	(C18, C8, PHE)	20–60	Tosoh
YMC ultra-fast	Silica	2.0	2–8	(C18, Hydro C18)	20–60	YMC
Zorbax	Silica	1.8	2–9 2–8 1–6 2–11.6	(Eclipse plus C8, C18, Eclipse XDB-C18, C8, PHE) (Eclipse phenyl-hexyl, PAH, Elipse XDB-CN) (StableBond) (Extend-C18)	Up to 60 80–100 StableBond	Agilent

Cyano, Shield and other polarities. One of the key advantages of hybrid sorbent is its exceptionally high chemical stability, which allows it to be used throughout almost the entire pH range, typically 1–12 (Acquity BEH columns or Pathfinder columns). This allows for the successful analysis of basic compounds at pH values far above their pK_a values. Some manufacturers have put their efforts into developing high stability silica which is able to work beyond the conventional pH range of silica columns (2–8). Some examples include *Zorbax Extent* and *Hypersil Gold* columns, see Table 4 for details. It is no longer true that UHPLC stationary phases are not readily available and that special chemistries are lacking. Today reversed phase, HILIC, ion-exchange and normal phases may all be found in UHPLC.

In UHPLC it is suggested to minimize frictional heating effects from the radial temperature gradient by reducing the column diameter [3], so that typical UHPLC column diameters are 2.1 or 1.0. It was observed that the effluent of conventional columns operated at high pressures is warmer than in the mobile phase entering the column. In addition, the temperature at the column centre was warmer than near the wall. These longitudinal and radial temperature gradients across the column can be attributed to frictional heating between the mobile phase and the small particles, along with poor heat dissipation in packed columns. Radial temperature gradients can decrease column efficiency due to the resulting heterogeneous radial distribution of the mobile phase linear velocity. A longitudinal temperature gradient can cause a decrease in the retention factor of analytes [3]. These effects may be prevented by using narrower column diameters, as stated above.

2.3.4. Detection approaches

The narrow peaks produced by fast UHPLC require a small detection volume and fast acquisition rate to ensure high efficiency.

In capillary UHPLC the easiest detection approach is to use on-column detection. A detection window is usually created for on-column UV detection by removing an approximately 1 mm wide band of the polymeric coating from the fused silica tubing immediately after the outlet frit of the capillary column. The drawback of this detection mode is low sensitivity, as the path-lengths for UV detection are equivalent to the column internal diameters (30–150 μm) of capillaries. On-column detection was used in connection with amperometric [32], UV [33], TOF-MS (time of flight mass spectrometry) [33] or inductively coupled plasma MS (ICP-MS) detection [34].

Most commercial UHPLC instruments are equipped with a modified UV detector to ensure the optimal peak capture. The flow cell volume is usually much lower than that for conventional HPLC to minimize the extra-column volume, typically 0.5–2.0 μL. However, smaller flow cells would reduce the path-length upon which the signal depends (as predicted by Lambert–Beer's law). Additionally, a reduction in cross-section means the light path is reduced, transmission drops and noise increases. Therefore, a conventional flow cell SS cylinder cannot provide the performance needed for UHPLC detection. In order to maintain the path-length, light guided flow cells may be used. These are actually teflon (*Waters*) or fused silica (*Agilent*) capillaries, which prolong the path-length of light transmission based on the difference of refractive indexes of fluid and cladding.

The detection software must be capable of achieving both a fast detector time constant (<0.1 s) and a high data acquisition rate to ensure enough data points for a narrow peak (typically >20, 200 Hz being maximum acquisition rate). The same applies for MS detectors. Low dwell times and low inter-channel and inter-scan delays are required in order to obtain a sufficient amount of data points per peak. Other detectors, including FD or ELSD (evaporative light scattering detector), are currently available for coupling with UHPLC systems.

2.3.5. UHPLC at elevated temperatures

Increasing the column temperature can significantly reduce mobile phase viscosity as well as simultaneously increase mass transfer efficiency, allowing operation at higher flow-rate for the same pressure [14]. An elevated temperature can further improve speed and efficiency in UHPLC, since the advantage of using higher temperatures in HPLC is a significantly shortened separation time with minimal loss in column efficiency. Commercially available column thermostats already enable working at higher temperatures (*Acquity UPLC* and *Flexar* up to 90 °C, 1200 *RRLC* and *Infinity* up to 100 C, RSLC *Ultimate* up to 110 C and *PLATINblue* up to 140 C—Table 3).

Plumb et al. [35] used a combination of UHPLC at 11,000 Psi with a narrow-bore sub-2-microne particle packed column at 90 °C. By elevating temperature, back-pressure was reduced while maintaining high flow-rates and chromatographic efficiency with peaks 1–3 s at base. In such analytical conditions extremely high resolution chromatograms were obtained for the metabolite profiling of urine samples. The stability of Acquity BEH column was found to be sufficient even at 90 °C, as 1700 injections of precipitated plasma were conducted without loss in performance retention.

The *Acquity UPLC* system was used in connection with a GC oven in order to heat the column up to 180 C [17]. Thermal gradients (50–180 C within 6 min) were utilized for metabonomic/metabolomic UHPLC–MS investigations. Their performance was found to be superior to conventional RP-HPLC, with an increased number of ions being detected. The maintenance of both minimum back-pressures and post-column cooling was found to be critical in maximising the chromatographic performance of the system. The BEH columns used in this study showed satisfactory stability, as no loss of performance was observed after 250 injections of urine samples at a designated thermal gradient.

This shows that UHPLC at elevated temperatures could be an option to speed-up analysis and to further increase efficiency. However, all factors related to HTLC must be taken into consideration (see Section 2.2 for details).

2.3.6. Applications of UHPLC in bio-analytical methods

UHPLC has recently become a widely used analytical technique in analytical laboratories of all specializations, in conventional laboratories as well as research centres. To date approximately 700 papers have been published on the subject of UHPLC and sub-2-microne particles (Web of Science). Major applications of UHPLC include pharmaceutical analyses (drug discovery and development, quality control of drug products, bioequvivalence studies, therapeutic drug monitoring, etc.), proteomics, metabolomics, environmental analysis, food analysis, plant analysis and chiral separations. In bio-analytical applications, UHPLC has demonstrated that separations 2–21 times faster can be achieved while maintaining or improving resolution and sensitivity. A summary of recent bio-analytical applications is displayed in Table 2.

UHPLC is an ideal fast-separation tool for complex mixture analysis in both isocratic and gradient modes, since this technology has been demonstrated to be capable of achieving higher peak capacity, speed, and sensitivity than conventional HPLC through the UHPLC use of sub-2-microne particles and optimized instrumentation [3]. Since biological samples can be complex and the sample numbers are usually large, fast separations with high resolution and high sensitivity are often required.

UHPLC coupled to ESI tandem mass spectrometry seems to be a method of choice in bio-analytical applications [36–90]. Only one work referred to the utilization of FD and PDA (photo diode array) detection [91]. Most methods make use of *Acquity UPLC* systems in connection with *BEH* C18 analytical columns. A typical UHPLC analysis of a single compound in a biological matrix using an internal standard requires 1–2 min [43,45,51,52,56,60,61,63,73,83]. This high rate of analyses is extremely important for high-throughput laboratories. One ultra-high performance was found using the multiresidual screening method, in which more than 100 veterinary drugs were determined within 6 min [70]. A typical analysis of multi-component mixtures (2–43 compounds) is possible in around 5–12 min (Table 2). Examples of this are given in Figs. 2 and 3. A longer analytical run was necessary only for the analysis of derivatives of oxo-guanosine (23 min) [79] and cholic acids (28 min) [55] and (22 min) [77], respectively.

The speed of fast and ultra-fast analytical runs may be contrasted with labor-intensive and time-consuming sample preparation, as procedures including SPE and LLE were prevalently used as pre-treatment step. On-line RAM sample preparation was applied only in one case of analysis of cholic acids [55]. In some cases semi-automated [48] or automated LLE [51] was applied in order to increase throughput.

2.4. Fused core columns

Separation efficiency and speed of analysis may also be increased using superficially porous particles, often termed also fused core columns. The use of superficially porous particles was first reported in 1960s [92] with the aim of reducing the diffusion distance of analytes to minimize mass transfer. In 1992 a new process of producing tiny superficially porous particles was introduced by Kirkland [93]. Later this process was commercialized under the brand name *HALO*. *HALO* silica particles contain a 1.7 μm fused core and 0.5 μm layer of porous silica coating, creating a total particle diameter of 2.7 μm. A similar technology was later used in commercial *Ascentis* fused core silica columns (Sigma–Aldrich) and *Kinetex* (Phenomenex). The use of fused core silica particles have dramatically improved chromatographic peak efficiencies over fully porous particles in reversed phase [4,94,95] as well as in HILIC separation mode [5], both gradient and isocratic elution. These properties might be explained using the HETP equation (1): by the combination of a 25% lower axial diffusion (due to the solid core of the particle) and a 20% lower eddy dispersion term (due to narrow particle size distribution). Specifically, the reduction in axial diffusion allows for higher flow-rates to be used without a detriment to chromatographic performance [96].

Many articles published on experimental work comparing sub-2-microne particles with fused core columns have drawn similar conclusions [97–100]. Cabooter et al. [97] compared sub-2-microne *Acquity BEH* C18 at 1000 bar and *HALO* fused core C18 columns at 600 bar. Results have shown that both sub-2-microne and 2.7 μm porous shell particles coupled columns systems achieved 100,000 plates at equal rates of speed, with only small dissimilarities. These differences depended generally on the nature of pharmaceutical compounds tested. Fused core particles proved a bit more expedient, as required efficiency was reached at lower back-pressure, thus some margin for improvement seems to be available. Similarly, Cunliffe and Maloney [98] compared the performance of several commercial sub-2-microne particle columns with porous shell particle columns under isocratic and gradient conditions. The results have shown that porous shell particles had about 80% of the efficiency of the *Acquity* columns at 45% of the back-pressure.

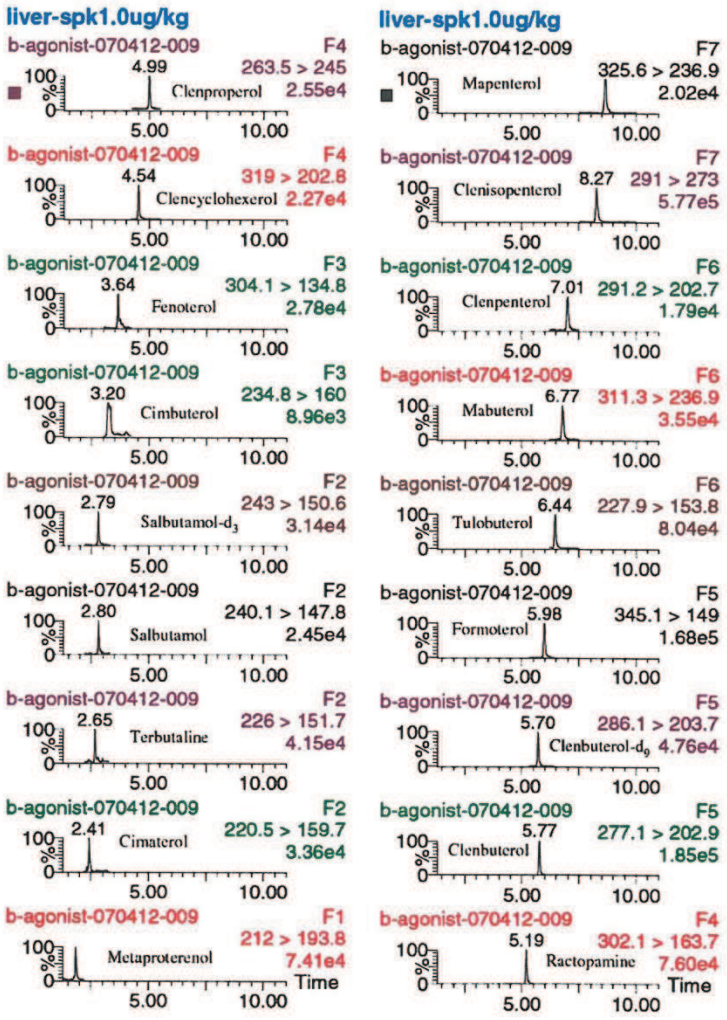

Fig. 3. An example of high-throughput UPLC–MS/MS analysis: MRM chromatograms of the 16 agonists fortified at 1.00 μg kg^{-1} (7.00 μg kg^{-1} for clenproperol and cimbuterol) using a blank liver sample. Reprinted from [81], copyright Elsevier.

typical in UHPLC and requires for special LC equipment [103]. An *Ascentis Expresss* C18 analytical column was employed in the UHPLC–MS/MS analysis of pharmaceuticals in plasma. The superficially porous stationary phase demonstrated a resolving power equivalent to sub-2-microne material under ballistic gradient chromatography conditions. Fused core columns were confirmed to be valuable tools for bio-analytical purposes, as thousands of protein precipitated plasma extracts could be measured with acceptable precision and accuracy [104].

2.5. Hydrophilic interaction liquid chromatography (HILIC)

HILIC is an alternative to conventional RP-HPLC (reversed phase HPLC) or NP-HPLC (normal phase HPLC). HILIC has proven quite convenient for the analysis of small polar molecules weakly retained or eluted with dead volume in conventional RP-HPLC systems. This method is often substituted for normal phase chromatography because of the latter's bad reproducibility, low solubility of polar compounds in NP mobile phases as well as the great difficulties encountered when connection with MS detection is required. In HILIC, analyte retention is believed to be caused by the partitioning of the analyte between a water-enriched layer of stagnant eluent on a hydrophilic stationary phase and a relatively hydrophobic bulk eluent, with the main components usually being 5–40% water in ACN. This is perhaps the most rational way to address compounds which are very hydrophilic and uncharged. The mobile phase is composed of a high percentage of organic solvent (typically acetonitrile); this is complemented by a small percentage of water/volatile buffer. The water-enriched liquid layer is established within the stationary phase, thus the partitioning of solutes from the mobile phase into the hydrophilic layer occurs. Under the HILIC conditions, the stationary phase is of polar character, usually containing hydroxyl-ethyl, diole and amino groups, or it could be, among other possibilities, a special kind of "zwitterionic" stationary phase. The primary mechanism of separation is partitioning based on hydrogen bonding; the secondary mechanism, possibly influencing selectivity, is electrostatic interaction with charged stationary phases. Elution is enabled by increasing the polarity of the mobile phase, thus the content of water component. The applications now encompass most

Table 5
Use of HILIC in bio-analytical applications.

Determined substances	Matrix sample prep.	Method/stationary phase	Mobile phase	Detection	Analysis time	Validation data	Reference
Mitragynine ajmalicine (IS)	Human urine LLE	Atlantis HILIC (50 mm × 3.0 mm, 3 μm) Waters	MeOH:5 mM ammonium acetate, 90:10 gradient	ESI-MS/MS $[M+H]^+$	7 min	$r > 0.995$; LOD = 0.01 ng mL^{-1}; LOQ = 0.1 ng mL^{-1}	[105]
Metoclopramide levosulpiride (IS)	Human plasma LLE	Atlantis HILIC (50 mm × 3.0 mm, 3 μm) Waters	ACN:100 mM ammonium formate, 85:15 (v/v)	ESI-MS/MS $[M+H]^+$	3.5 min	$r^2 = 0.998$; LOQ = 2.00 ng mL^{-1}; R.S.D. = 1.8–7.7%	[106]
GABA	Microdialysis symplex	ZIC-HILIC (20 mm × 2.1 mm, 3.5 μm) SeQuant AB	ACN:0.1% formic acid in H_2O, 85:15 gradient	ESI-MS/MS $[M+H]^+$	3 min	$r = 0.9981$; R.S.D. = 2.7%; LOQ = 1 nm	[107]
Glutamate						$r = 0.9940$; R.S.D. = 4.0%; LOQ = 10 nM	
Miglustat	Human plasma cerebrospinal fluid PP	Atlantis HILIC (150 mm × 2.1 mm, 3 μm) Waters	ACN:H_2O:ammonium acetate buffer, 75:10:15 (v/v/v)	ESI-MS/MS $[M+H]^+$	5 min	$r > 0.996$ R.S.D. = 1.3–7.8%	[108]
Acetylcholine	Human liver tissues	PolyHYDROXYETHYL A (35 mm × 2.1 mm, 5 μm) PolyLC Inc.	ACN:20 mM ammonium formate with 0.1% formic acid, 97:3 gradient	MS/MS $[M+H]^+$	17 min	$r > 0.999$; R.S.D. = 2.2–8.3%; LOD = 0.2 ng mL^{-1}; LOQ = 0.6 ng mL^{-1}	[109]
Chopine						LOD = 30 ng mL^{-1}; LOQ = 80 ng mL^{-1}	
Butyrobetaine						LOD = 2.0 ng mL^{-1}; LOQ = 15.0 ng mL^{-1}	
Metformin prodrug 1, 2	Human blood rat blood PP	Supelcosil LC-Si (4.6 mm × 250 mm, 5 μm) Supelco	ACN:0.01 M ammonium acetate, 60:40 (v/v)	UV 235 nm	15 min	$r = 0.994–0.997$; R.S.D. = 2.01–11.05%	[110]

categories of polar compounds, charged as well as uncharged, although HILIC is particularly well-suited for solutes lacking charge, during which coulombic interactions cannot be used to mediate retention. Recently this approach has gained great attention because of the increased need to analyze polar compounds in complicated mixtures. Another reason for its popularity is the wide-spread use of MS coupled to HPLC, as HILIC mobile phases are well compatible and give high sensitivity [6]. Recently, HILIC has become very popular in bio-analytical applications because drugs and their metabolites are often polar structures [105–110]. Some detailed examples are given in Table 5. Most methods of HILIC enhanced the properties of HILIC mobile phases (volatility, high content of acetonitrile/methanol) in the coupling with electrospray mass spectrometry detection. *Atlantis* HILIC based on plain silica was the most widely used analytical column in bio-analysis.

3. Sample preparation

Sample preparation impacts nearly all the later assay steps and is hence critical for unequivocal identification, confirmation and quantification of analytes. It includes both the isolation and/or preconcentration of compounds of interest from various matrices as well as making the analytes more suitable for separation and detection. Sample preparation typically takes 80% of the total analysis time. While chromatographic methods are preferred in the analysis of organic molecules, sample preparation in bio-analytical methods regularly employs liquid–liquid extraction and solid phase extraction. In contrast with ultra-fast chromatographic analyses, conventional sample preparation approaches are still highly labor-intensive and time-consuming, consisting of many steps. For this reason, many new sample preparation techniques have been developed over the last decade.

Besides wide-spread conventional and automatic SPE, LLE and PP technique, newly developed sample preparation techniques include solid phase microextraction (SPME), liquid–liquid microextraction (LLME), pressurized liquid extraction (PLE), extraction using restricted access material (RAM), microextraction by packed sorbent (MEPS), molecularly imprinted polymer (MIP), monolith spin extraction, turbulent chromatography (TFC), salting-out liquid–liquid extraction (SALLE), stir bar sorptive extraction (SBSE) and others. Recent investigations have focused on the development of methods to reduce the sample volume required, the analytical time, the cost and the solvent consumption and even the elimination of chlorinated solvents. Further, miniaturization and automation using on-line coupling of analytical methods is a very important development as well.

A complete review of the current status and recent advances in sample preparation techniques was conducted by Chen et al. [111], in which the detailed classification of all sample preparation techniques is outlined and selected techniques are discussed in detail.

In our review, the most commonly used automatic and routine sample preparation techniques in bio-analytical methods will be discussed. Further, new trends and challenges in the sample preparation of biological fluids will be also presented. The first consideration in sample preparation, the stability of biological samples, will now be discussed.

3.1. Stability of biological samples

Because of the complex nature of biological matrices, sample preparation steps are the most important integral part of bio-analytical methods. One of the key problems of biological samples is the instability of drugs, metabolites and prodrugs in these kinds of samples. The stability of drugs in biological materials may be affected by storage temperature, enzymes as well as the pH of the biological samples, anticoagulants and freeze–thaw cycles. Moreover, instability may occur during any of the numerous steps of bio-analytical methods: in the biological matrix before taking aliquots of samples for the analysis, during the extraction step, during the evaporation to dryness or reconstitution, in the solution inside injection vials and, in the case of mass spectrometry, in the ion source as well. Therefore, short-time, long-time and freeze–thaw stability studies should be performed for standard solutions as well as for real samples.

The degradation of a drug during sample treatment can cause an underestimation of drug concentration, in contrast to the

degradation of the metabolite or prodrug, which may cause an overestimation. Generally, degradation occurs naturally [112], could be caused by exposure to light [113], or may be a result of a reaction with the biological fluid [114]. The drug may also be absorbed into the surface of containers or synthetic barriers, such as polymers or separation gels [115].

There are some groups of compounds which undergo interconversion reactions, thus special precautions must be taken when analyzing acyglucuronides [116], lactone and open-hydroxy acid compounds [117,118], or samples which contain a thiol group and a corresponding disulfide [119]. Minimizing interconversion will depend on conditions during the bio-analytical procedure, pH being one of the most important factors, together with temperature. Other reasons for sample instability could be epimerization [120,121] or E to Z isomerisation reactions [122,123], influenced again by pH or light exposure.

Highly unstable metabolites or drugs may be stabilized by the addition of stabilizing agents such as citric acid to blood samples, or citrate or phosphate buffers to a plasma sample. These can be used to maintain the pH of plasma during storage or processing, as the pH of biological samples changes during storage, ultrafiltration, centrifugation and extraction [124–126].

The type of anticoagulant which is used during the collection of the blood sample, may also affect the stability of drugs tested for or their metabolites. A variety of chemical agents, such as EDTA [20] [126,127], formic acid [20], acetic acid [20], sodium fluoride [126,127], lithium heparin [127], potassium oxalate [127] and methyl acrylate [128], have been used to stabilize analytes in biological matrices.

3.2. Liquid–liquid extraction-based approaches

3.2.1. Liquid–liquid extraction (LLE)

Liquid–liquid extraction was one of the first sample preparation techniques and continues to be widely used for biological sample analysis. LLE is based on a transfer of analyte from the aqueous sample to a water-immiscible solvent based on the octanol–water partition coefficient. Nevertheless, some shortcomings, such as emulsion formation, the use of large sample volumes and toxic organic solvents and above all, the production of a large volume of environmental pollutants make LLE expensive, time-consuming and environmentally harmful. Another drawback of LLE is its unsuitability for hydrophilic compounds.

Despite this, LLE is still widely used in the sample preparation of biological fluids (Table 2). Newly developed UHPLC techniques still employ this approach in sample preparation. As an advance, LLE has become semi-automated for drug analysis applications, wherein multi-well plates may be used for the simultaneous preparation of 96 samples [129]. Peng et al. reported a fully automated high-throughput LLE method for the preparation of biological samples using a 96-well plate and 96-channel robotic liquid-handling workstation in order to completely automate the entire process. Extraction time was substantially shortened using this approach [130].

3.2.2. Extrelut liquid–liquid extraction

During the 1980s Extrelut columns were developed as a solid phase replacement for traditional liquid–liquid extractions done with a separatory funnel. Classical extraction using a separation funnel is often associated with following disadvantages: the formation of emulsion, poor phase separation, high solvent consumption as well as a low degree of automation, resulting in high personnel costs. Extrelut columns (manufactured by *Merck*) contain specially processed wide-pore diatomaceous earth, a chemically inert matrix for use within a pH range of 1–13. After the sample is pH adjusted or salt is added (as in the salting-out of the sample) and applied to the Extrelut column, the matrix acts as a holder for the sample. Next, an immiscible organic solvent is passed through the Extrelut column to extract the desired components, which are then analyzed. Elution is carried out with 2–3 times the sample volume. The residue is subsequently evaporated to dryness, reconstituted and injected onto the chromatographic system, or the liquid may simply be allowed to run through the column. The capacity of Extrelut prepacked columns for aqueous samples is variable, and they can hold up to a maximum of 1–20 mL of aqueous sample.

Extrelut liquid–liquid extraction was commonly used in bioanalytical laboratories as sample preparation, e.g., in the analysis of acetylgestagens in plasma, followed by SPE and by HPLC–MS analysis (elution by 13 mL of ethyl acetate) [131]; in the analysis of ticlopidine in plasma, followed by capillary GC–MS (elution by 6.5 mL of hexane) [132]; in the analysis of synthetic corticosteroids in urine samples, followed by HPLC–MS (elution by 12 mL of toluene/diethyl ether) [133]; in the analysis of rifabutin in plasma, followed by HPLC-PDA (elution 2× by 3 mL of hexane/ethyl acetate) [134] or in the analysis of lornoxicam and its metabolite in plasma and synovial fluid, followed by HPLC-PDA (elution 2× by 5 mL dichloromethane) [135].

Thus Extrelut LLE brings some clear advantages, including, e.g., the prevention of the formation of emulsions and the possibility of automation. However, in principle the procedure still remains LLE with the main drawbacks such as use of toxic organic solvents (often more than 10 mL for the elution of one sample) and above all, production of large volume of environmental pollutants, which makes LLE expensive and environmentally harmful. The technique is also quite time-consuming, as anywhere from 5 to 20 min are needed after sample loading until an extraction can be completed.

3.2.3. Liquid–liquid microextraction (LLME)

Conventional LLE is widely used as a pre-treatment technique for the sample preparation and pre-concentration of biological samples. Nevertheless, because of its disadvantages, such as the formation of emulsions and the use of large volumes of toxic organic solvents, the miniaturization has led to the development of a number of new methodologies based on LLE. Microextraction LLE techniques should use negligible volumes of extracting solvent and take a minimum number of steps. This advancement has resulted in new miniaturized technologies, such as single-drop microextraction (SDME), hollow fibre liquid phase microextraction (HF-LPME) and dispersive liquid–liquid microextraction (DLLME) [111,136,137]. SDME can be further sub-divided into direct immersion single-drop microextraction (DI-SDME), head-space single-drop microextraction (HS-SDME), liquid–liquid–liquid microextraction (LLLME) and continuous-flow microextraction (CFME) [136]. Developments and applications of SDME have recently been reviewed by Xu et al. [138].

SDME was introduced in 1996 as a pre-concentration technique, based on the use of a micro-drop (1–10 μL) of extracting solvent immiscible with water [139]. DI-SDME, a static mode of LLME, is based on the exposure of a microdrop of water-immiscible extracting phase suspended in a stirred aqueous solution from the tip of a micro-syringe needle. After extracting for a prescribed period of time, the drop is retracted back into the micro-syringe needle and finally injected into the chromatographic system. DI-SDME requires the execution of a water-immiscible extracting phase and the use of analytes more soluble in the extracting phase than in sample solution [138,136].

In CFME the extraction is carried out in a glass extraction chamber instead of a vial. Instead of being stirred, the sample is pumped "around" continuously at a constant flow-rate. When the extraction chamber is full of sample, a drop is formed at the tip of a micro-syringe needle. In contrast with other SDME, the solvent drop makes contact with fresh and flowing sample solution

[140,141]. Due to the continuous contact with the flowing fresh sample solution, the extraction efficiency and concentration factor are higher than in static extraction. The sample flow-rate should ensure an effective microextraction of analytes without drop dislodgement or bubble formation, which is the main problem of DI-SDME [136].

LLLME is a mode of microextraction suitable for ionizable analytes. This process is based on the extraction of analytes from aqueous stirred samples into an organic layer or membrane with a lower density than water and simultaneous back-extraction into an aqueous microdrop. The pH of the aqueous solution and the aqueous microdrop can be adjusted to obtain firstly the neutral form of the analyte, extractable by organic solvent and then ionize it to be extracted into aqueous drop. LLLME is more difficult to execute in comparison to other forms of SDME, however, it is more convenient for coupling with RP-HPLC or CE [136].

The use of HS-SDME allows for the pre-concentration of volatile or semi-volatile analytes. Microextraction, pre-concentration and derivatization can be performed in one single step. The extracting solvent (which may also contain a derivatizing agent) is exposed to the head-space (gaseous phase) above a sample of a volatile or semi-volatile nature. The headspace or hanging-over SDME allows the use of both organic and aqueous solvents as receiving phase to extract volatile or semi-volatile compounds since the droplet does not come into direct contact with the sample solution. This type of extraction is mostly preferred by GC, some works reported also coupling with HPLC, CE and MS [111,136,138].

In HF-LPME, a hydrophobic porous hollow fibre is used to protect and expose a certain volume of extracting solvent to the sample. Here, an organic solvent is immobilized in the wall pores of the hollow fibre, providing a supported liquid membrane (SLM), and an aqueous acceptor solution is held within its lumen [142]. Therefore, this technique is sometimes called SLM extraction or hollow fibre membrane liquid–liquid extraction or hollow fibre supported liquid membrane extraction [143] and it is classified as a membrane liquid-phase technique [142,144]. SLM extraction is the most important of the membrane extraction techniques (among others such as dialysis, electrodialysis, membrane extraction with sorbent interface (MESI), etc.), which attempt to automate LLE [137,145]. The HF-LPME extraction process occurs in the pores of a hollow fibre in an immobilized solvent. This is easily accomplished by dipping the hollow fibre for a few seconds into the organic solvent, which immediately flows into the pores by capillary forces. The lumen of the hollow fibre is then filled with a μL volume of acceptor solution and the whole assembly is placed in a sample solution for the extraction of target analytes. The target analytes are extracted from the sample (aqueous) through a supported liquid membrane, then trapped in the acceptor solution (aqueous or organic) in the lumen of the hollow fibre. After extraction, the acceptor solution is subjected to a final chemical analysis, typically by chromatographic methods. When the acceptor solution is an organic solvent, HF-LPME is two-phase sampling mode, which is directly compatible with GC. Alternatively, in three-phase sampling mode (HF-LLLME), the analytes are extracted into an organic layer, which fills the pores of the hollow fibre and is then back-extracted into an aqueous phase placed inside the fibre. Thus the acceptor solution is aqueous, which is compatible with HPLC or CE. The sample-to-acceptor ratio is very high, so analyte enrichments are also very high without the need for evaporation and reconstitution [136,146].

DLLME is based on a ternary component solvent system in which an appropriate mixture of extraction solvent and disperser solvent is quickly injected into the aqueous sample with a syringe. The mixture is then gently shaken and a cloudy solution is formed. After centrifugation, the fine particles of extraction solvent are sedimented in the bottom of a conical test tube. The resultant sedimented phase is taken out with a micro-syringe and injected into GC for analysis. The water-immiscible extracting solvent should have a higher density than water, while the disperser solvent should be miscible in the extracting solvent and the aqueous sample. The fine droplets of extraction solvent are dispersed throughout the aqueous sample, allowing interaction with the analyte [136,147].

Liquid–liquid microextraction techniques are quite commonly used in the modern sample preparation of biological fluids. Basic schematics of their principles are shown in Fig. 4. The most widely used LLME technique was unequivocally HF-LPME. Its principles and applications have of late been revised by Pedersen-Bjergaard and Rasmussen [146]. 34% of summarized applications were bio-analytical methods for the determination of drugs in various biological materials aside from environmental and food applications. Recently, HF-LPME has been employed in the analysis of xenobiotics nitrophenols in human plasma [148], the anti-diabetic drug pioglitazone in plasma [149], the antidepressants amitryptilin, imipramine and sertraline in urine and plasma samples [150], flunitrazepam in plasma and urine samples [151], progesterone in human serum [152] as well as β-agonists and β-antagonists in urine [153] and many others. Both HPLC and GC methods were used for the final analysis.

LLLME has been used less commonly in the analysis of biological fluids, probably because of the difficulty involved in the process. The analysis of local anesthetics in human plasma coupled to HPLC [154] or the analysis of nonsteroidal anti-inflammatory drugs combined with HPLC–UV was performed [155]. Methamphetamine and amphetamine were determined in urine by LLLME combined with HPLC–UV [156] and phentanyl was determined in water samples, plasma and urine samples followed by HPLC [157]. Other SDME techniques (DI-SDME, CFME and HS-SDME) were mostly reserved for the determinations of environmental samples and trace elements.

Another LLME sample preparation technique much less used in bio-analysis was DLLME, the subject of recent review of Zang et al. [147]. This technique is typically reserved for environmental applications, therefore only few bio-analytical methods dealt with DLLME. These include the analysis of the psychotropic drugs amytriptylin, clomipramine, and thioridazine in urine, followed by HPLC [158], the determination of 7-aminoflunitrazepam in urine in connection with LC–MS/MS [159] and the determination of amitryptyline and nortriptyline, followed by GC-FID [160].

Generally, some advantages of LLME methods are simplicity, cost effectiveness and negligible solvent consumption. There are also some drawbacks of individual techniques. DI-SDME requires careful and elaborate manual operation because of the problem of drop dislodgement and instability, especially at high stirring rates or temperatures and when samples are not perfectly clean. Moreover, solvents with relatively high water solubility and low boiling points are not suitable for DI-SDME because of their high rate of dissolution and evaporation. In addition, an extra filtration step is usually necessary for the sample solutions with complex matrices (except for HS-SDME). On a top of that, the sensitivity and precision of DI-SDME still need to be improved. The main problem is prolonged extraction time, which could lead to drop dissolution and/or dislodgement. Therefore, SDME is not yet suitable as a routinely applicable on-line pre-concentration technique and it should be studied further [111,136,138,161].

HF-LPME is a useful technique because of the high analyte enrichments involved (>100-fold) for organic and inorganic compounds in a wide range of polarity, extremely low solvent consumption, high efficiency of sample clean-up and ease of automation. However, in HF-LPME there are difficulties involved in the manipulation of the hollow fibre during its placement at the tip of the micro-syringe needle before the microextraction process. This could be a source of contamination. For the

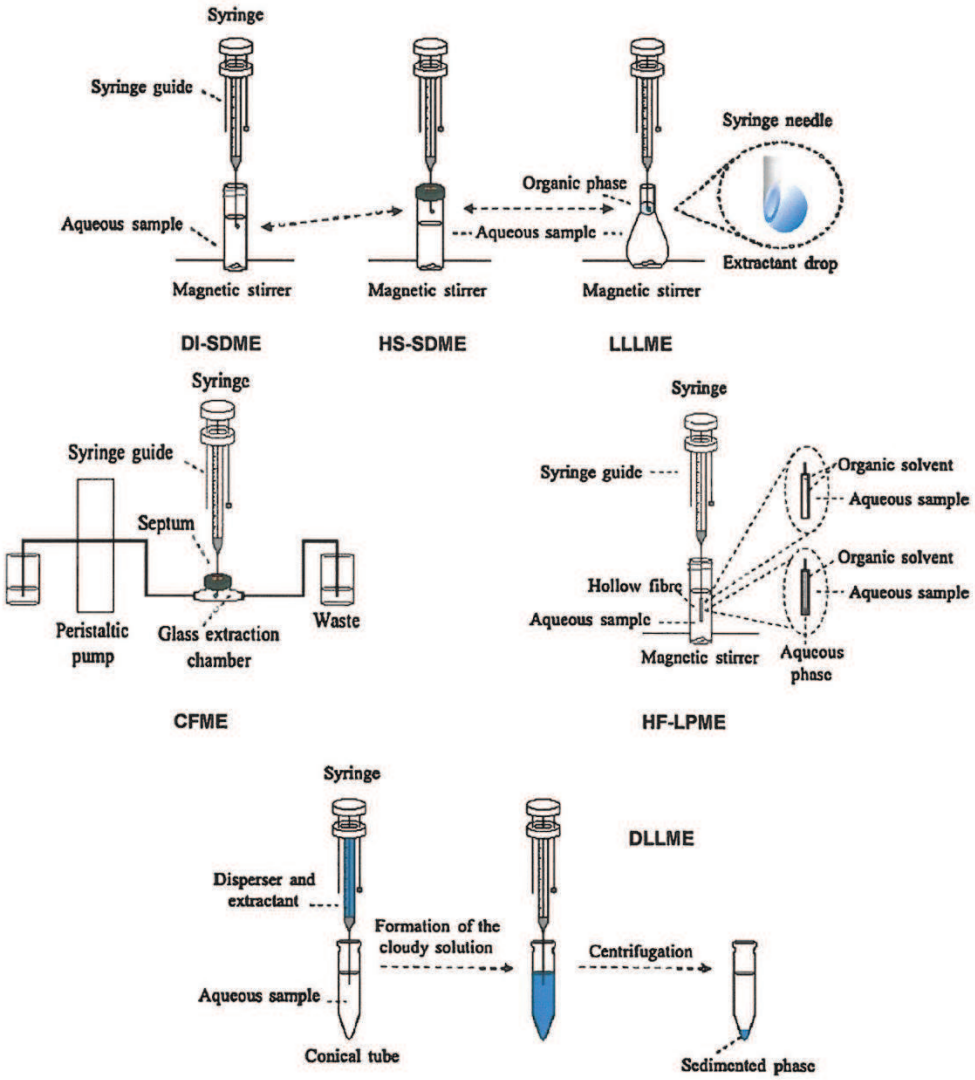

Fig. 4. Schematics of direct immersion single-drop microextraction (DI-SDME), headspace single-drop microextraction (HS-SDME), liquid–liquid–liquid microextraction (LLLME), continuous-flow microextraction (CFME), hollow fiber liquid phase microextraction (HF-LPME), hollow fiber liquid–liquid–liquid microextraction (HF–LLLME) and dispersive liquid–liquid microextraction (DLLME), reprinted from [136], copyright Elsevier.

moment, HF-LPME has been proven for the implementation in routine analytical laboratories, but so far it has had to be done with home-built equipment. Its utilization across the board is currently limited by the unavailability of commercial equipment [111,136,137,146].

The main advantages of DLLME are its simplicity of operation, rapidity (equilibrium is reached very quickly, as opposed to LLME), low-cost, high-recovery, a high enrichment factor and environmental friendliness. Nevertheless, this technique is limited to the use with only a small number of extracting solvents. These should efficiently extract the analyte of interest meeting all criteria—having a higher density than water, forming a stable cloudy solution and being easily removed from the bottom of the conical vial after centrifugation. As these criteria are met by only a few organic solvents and the method is not easy to automate, DLLME will not become a wide-spread technique in bio-analytical laboratories in near future [147].

3.2.4. Pressurized liquid extraction (PLE)

In pressurized liquid extraction, rapid extraction of solid matrices is performed using organic solvents by applying high temperatures (up to 200 C) as well as applying high pressures (up to 20,000 kPa) to maintain the solvent in liquid state in a flow-through system. The utilization of elevated pressures allows solvents to be used above their atmospheric boiling points to increase solvation power and extraction kinetics. Increased temperatures can also disrupt the frequently strong solute–matrix interactions. This increases the extraction efficiency and rate as well as simultaneously reduces the consumption of organic solvents along with operating time [111,145]. A subclass of PLE is pressurized hot water extraction (PHWE), where pure water is used as a solvent [162,163]. The applications of PLE in the analysis of food and biological samples have been summarized by Carabias-Martínez et al. [164].

PLE was commercialised in an automated version as accelerated solvent extraction (ASE). Both static and flow-through designs

may be used. In the latter, fresh solvent is continuously introduced to the sample, improving the extraction but diluting the sample. As a consequence of the principles PLE, extraction procedures which would have taken many hours of Soxhlet refluxing can be carried out in minutes on a smaller sample. The extracts are generally more concentrated than those from conventional extractions. They may be analyzed directly, or the solvent can be cooled and the analyte trapped on glass beads or in a cartridge and subsequently extracted into smaller solvent volumes [165]. PLE has advantages over other extraction methods, including better reproducibility, the reduced use of extraction solvent and reduced time for sample preparation. Its disadvantages are related to thermal stability and extraction selectivity. Problems of PLE may be overcome using different solvents and thus its use could be further developed [111].

As PLE is used exclusively with solid samples, in bio-analysis it could be applied to the analysis of tissues or blood, as described in the developed methods for PCBs in blood [165], tetracyclines in animal tissues [166] and paroxetine, fluoxetine and norfluoxetine in fish tissues [167]. However, in bio-analysis extraction is typically conducted on fluids, such as urine, serum, blood and plasma. Therefore, PLE is definitely not a technique of choice in bio-analytical laboratories.

3.2.5. Salting-out assisted liquid–liquid extraction (SALLE)

SALLE is essentially liquid–liquid extraction. The concept of salting-out by acetonitrile in bio-analysis was introduced in 1989 by Rustum et al. [168]. Salting-out was initially used in the extraction of metal-chelates, ion-pairs and organic materials prior to atomic absorption spectrophotometry, HPLC, polarography and absorption spectrophotometry [169].

The salt-induced phase separation between acetonitrile and aqueous solution was induced by the addition of a variety of inorganic and organic electrolytes. Water-miscible organic solvents have important disadvantages over water-immiscible ones, because their phase volumes depend on added amounts of the salting-out agent. A constant and potentially large amount of salt must be added to obtain constant recovered-volumes during the two phases [170]. Salting-out was used for the determination of metals, including copper, aluminium, iron and manganese in biological samples using a relatively small amount of TBAP (tetrabutylammonium perchlorate, 0.17 g) together with ammonium sulphate (2.5 g) to achieve rapid and clear phase separation between acetonitrile and aqueous solution. The determination was performed in bovine liver and citrus leaves [170].

An alternative LLE using subzero temperatures was measured against the salting-out approach in the analysis of thiamylal, a barbiturate, in serum samples. Subzero LLE approaches included an addition of acetonitrile and a cooling down to −20 °C. Salting-out was performed using acetonitrile for the extraction along with sodium chloride. pH adjustment of the sample was not necessary, as salting-out was described to be less affected by pH, while pH is one of the key factors in both LLE and SPE. Subzero temperature LLE has been designated as time-consuming, due to the need to refrigerate samples, while salting-out is a less time-consuming approach. However, the acetonitrile phase separated by salting-out might contain salts as well as water, which would not be convenient for mass spectrometry, thus for LC–MS applications subzero temperature LLE was found to be more convenient [171].

γ-Hydroxybutyrate and its precursors have been determined in blood and urine samples using the salting-out method with sodium chloride. Salting-out was found to be suitable for this purpose, as was proven by method validation [172]. Advantages of salting-out with acetonitrile have been demonstrated, including the ease of biological sample clean-up as well as analyte enrichment. However, the use of magnesium sulphate, ammonium sulphate, sodium chloride, calcium chloride, potassium carbonate and calcium sulphate can cause problems for subsequent LC–MS analysis.

Recently, Wu et al. reported the utilization of salting-out assisted liquid–liquid extraction using mass spectrometry-compatible salts as a sample preparation step prior to HPLC–MS analysis [173]. SALLE performed with mass spectrometry-compatible salt ammonium acetate could open a new application area of this technique in LC–MS analyses. According to the authors, a small portion of the salt may dissolve into the acetonitrile phase, however, the impact of the salts on subsequent LC–MS analysis should be insignificant. Additionally this approach can easily be implemented through automation by simply adding a concentrated ammonium acetate solution instead of salt. The concentration of salt (1 M) in final plasma samples was sufficient to produce clear and clean phase separations [173].

It is questionable if a 1 M concentration of ammonium acetate does not induce an excessive concentration of this salt in the acetonitrile phase. It has been previously published that relatively high concentrations of ammonium acetate (>10 mM) can cause a substantial decrease in the response of mass spectrometers [174,175]. Therefore, further investigations of this factor should be undertaken.

3.2.6. Protein precipitation (PP)

Protein precipitation is a traditional sample preparation technique for the treatment of plasma. Miscible organic solvents (acetonitrile, methanol) have been for the most part used for these purposes. Protein precipitation is considered to be the fastest and the simplest extraction approach applicable for both hydrophilic and hydrophobic compounds. Further, both development requirements and costs are minimal. Protein precipitation is highly convenient when fast method development is desired. However, the extract – the supernatant – is relatively unclean, since it might still contain a significant amount of un-precipitated plasma components. Therefore selectivity is low and can induce analyte co-precipitation or mass spectrometry signal suppression. Additional centrifugation in order to separate the resultant protein precipitates from the analyte provides sufficient cleaning for most HPLC–MS analyses [176].

In spite of above stated problems, protein precipitation has been used in quite recent applications [177–179]. Over the years, the method has become semi-automated [180] and later fully automated. A 96-well plate format was used for automatic protein precipitation in connection with HPLC–MS/MS, as was first demonstrated by Ma et al. [176]. A vital step was the integration of on-deck plate shakers, centrifuge, plate sealer and plate seal piercing stations, all of which enabled the automation of both the liquid-handling and plate handling steps. The most recent applications of protein precipitation have utilized a semi-automatic 96-well plate format approach in the analysis of both clarithromycin [181] and fudosteine [182] in human plasma as well as in protein precipitation followed by centrifuge filtration for the analysis of vitamin B_6 in serum [183].

3.3. Solid phase extraction-based approaches

3.3.1. Solid phase extraction (SPE)

SPE is the most popular sample pre-treatment approach nowadays due to following advantages: high recovery, effective pre-concentration, the need for less organic solvent (compared to LLE), no foaming in the formation of emulsions, ease of operation and greater possibility of automation [129]. The applicability of SPE in bio-analysis in connection with modern chromatographic approaches is documented in Table 2. SPE is unequivocally the leading sample preparation method used in routine bio-analytical

laboratories. There were a few articles revising method developments and especially new materials for use in SPE [184,185].

In SPE, analytes to be extracted are partitioned between a solid phase and a liquid phase (sample). These analytes must have greater affinity for the solid phase than for the sample matrix. Retention may involve nonpolar, polar or ionic interactions. A wide range of currently available SPE sorbents ensures various selectivity, this in various product form including column cartridges, discs and well-plates [129] and pipette tips with fixed SPE support [186,187], sometimes called disposable pipette tip extraction (DPX) [188].

SPE is chiefly used to prepare liquid samples and extracts of semi-volatile or nonvolatile analytes, but may also be used for solids pre-extracted into solvents. The choice of sorbent is the key factor in SPE, because this can control parameters such as selectivity, affinity and capacity. This choice depends strongly on the analytes and their physically chemical properties, which should define the interactions with the chosen sorbent. However, results also depend on the kind of sample matrix and interactions with both the sorbent and the analyte. SPE sorbents range from chemically bonded silica of the C8 and C18 organic group, grafitized carbon and ion-exchange materials up to polymeric materials (PS-DVB, cross-linked styrene-divinylbenzene, PMA, cross-linked methacrylate, MA-DVB and many others), mixed-mode sorbents (containing both nonpolar and strong cation or anion), immunosorbents, molecularly imprinted polymers (details in Section 3.4.1) as well as restricted access materials (details in Section 3.3.6) and also recently developed monolith sorbents [189]. Silica sorbents have several disadvantages as compared with polymeric sorbents. Silica sorbents are unstable in a broader pH range and contain silanols, which can cause the irreversible binding of some groups of compounds, e.g., tetracyclines [184,185].

SPE method development has many similarities with HPLC. SPE may be performed either on-line or off-line. Conventional SPE cartridges are easy to handle by using vacuum or positive-pressure manifold. However, it is not easy to control the flow-rate, and in addition care should be taken to prevent the column from drying out prior to sample application. This is still a drawback of many commercial cartridges except, e.g., *Oasis* (Waters) or *Abselut* (Varian). As it could be difficult to elute the analyte of interest from conventional SPE cartridges using minimal solvent volume unless organic solvent composition rises up to 100%, special SPE discs are typically used for these purposes. This approach is much quicker as no more evaporation to dryness and reconstitution is no longer necessary because elution can be performed directly by mobile phase. The on-line configuration of SPE utilizes a 96-well plate format for SPE automated with a robotic liquid-handling system, facilitating high-throughput analyses of biological samples [129,184].

In spite of all advantages stated above, SPE is time-consuming and relatively expensive, as SPE cartridges are manufactured for single use only. They sometimes have poor batch-to-batch reproducibility and still require a relatively large amount of organic solvent. These disadvantages do not prevent SPE to be the most widely used sample preparation technique in bio-analytical laboratories.

3.3.2. *Monolith spin extraction*

A new tool for the sample preparation of compounds in biological material was introduced by Namera et al. in 2008 [190]. Monolithic silica disks manufactured in laboratory and chemically modified by C18 according to previously published works [191,192] was packed into spin columns. This device was then used for sample preparation in several works of this research group [190,193,194]. In such columns, the structure of monolithic silica comprises the support body with a surface area per unit volume that is large in comparison with particle-based silica support. Typical sample preparation steps of SPE including sample loading, washing and elution of target compounds can be all achieved by the centrifugation of the spin column. Furthermore, this extraction column can prepare many samples simultaneously using only centrifugation, without the need for evaporation and reconstitution. For sample loading, washing and elution of analytes, the spin column was installed into a siliconized microtube (2 mL), which was replaced before the elution of analytes. Thus, the typical elution protocol comprises the following steps [190,193]:

1. Conditioning by 0.2 mL (or 0.5) of acetonitrile + centrifugation.
2. Conditioning of distilled water 0.2 mL (or 0.5) + centrifugation.
3. Application of sample—0.2 mL of serum (or urine 0.5), buffer and IS + centrifugation.
4. Addition of distilled water 0.2 mL + centrifugation.
5. Washing by 0.2 mL 5% methanol (or 0.5 of another suitable solvent) + centrifugation.
6. Elution by 100 μL (or 200) of acetonitrile + centrifugation.

In a monolith spin column the flow-rate may be easily controlled by monitoring the rotation speed and time. The time required is reduced as the rotational speed increases. The amount of the drug that is adsorbed from the samples depends on the duration for which the sample is in contact with the sorbent as well as on sample pH. This is based on properties of hydrophobic sorbents such as C18, which require analytes to be in molecular form instead of ion form in order to be retained [190].

Monolith spin extraction has been applied for the analysis of amitraz and its metabolite in human serum [193], for the analysis of dibucaine and naphazoline in human serum [194] and for the analysis of amphetamins and methylenedioxyamphetamines in urine [190]. Extraction recovery was within the range 91.2–105% for amitraz [193] and 70.2–78.6% for dibucaine and naphazoline [194].

The main advantages of monolithic spin column extraction are the possibility of its use with low sample volumes as well as the miminal requirements of elution solvent. The elution solvent can be injected directly into chromatographic system without the need for evaporation. The operation is very simple. This approach is, however, quite new, thus its wider applicability must be further verified.

3.3.3. *Microextraction by packed sorbent (MEPS)*

Microextraction by packed sorbent is a new technique for sample preparation that can be connected on-line with LC or GC. In MEPS, approximately 1–2 mg of solid packing material is either inserted into the barrel of a syringe (100–250 μL) as a plug with polyethylene filters on both sides, or between the syringe barrel and the injection needle as a cartridge (Fig. 5). Sample preparation takes place on the packed bed. The bed can be packed or coated to provide selective and suitable sampling conditions. MEPS was recently invented and developed in the laboratories of Astra Zeneca [195,196] and this new method seems very promising for the extraction of drugs and metabolites from biological samples [197]. The key factor in MEPS is that the volume of solvent used to elute the analytes from the extraction process is of a suitable order of magnitude to be injected directly into an LC or GC system. MEPS can thus be described as a short LC column in a syringe. The bed dimensions are scaled from a conventional SPE bed and in this way MEPS can be adapted to most existing SPE methods by simply scaling the reagents and sample volumes from the conventional device to the MEPS.

The extraction is performed as follows: the plasma sample (50–1000 μL) is drawn through the syringe manually or by an autosampler (which pumps the sample up and down). When the plasma has passed through the solid support, the analytes have

been adsorbed to the solid phase. The solid phase is then washed once by water (50 μL) to remove the proteins and other interfering material. The analytes are then eluted with an organic solvent such as methanol or LC mobile phase (20–50 μL) directly into the instrument's injector (Fig. 5). The process can be fully automated. In MEPS, any sorbent material can be used either as packing bed or as a coating. Typical examples include silica-based (C2, C8, C18), RAM or MIPs sorbents [195]. MEPS can be connected on-line to an LC [196,198–204] or a GC [195] without any modification of the instrument. Connection to a robot makes the method fully automated.

The MEPS technique has been used to extract a wide range of analytes. Thus, several drugs such as local anaesthetics and their metabolites [195,196]; the anticancer drugs roscovitine [198], olomoucine [199], cyclophosphamide [200] and busulfan [201]; the β-blocker drugs acebutolol and metoprolol [202]; the antidepressant drugs dopamine and serotonine [203] as well as anti-addictive methadone [204] have been extracted successfully from biological samples such as plasma, urine or blood.

The MEPS technique differs from commercial SPE in that the packing is inserted directly into the syringe, not into a separate column. Thus, there is no need for a separate robot to apply the sample into the solid phase as with conventional SPE if automation is required. The packed syringe may also be used several times. A single syringe has been used more than 100 times with plasma or urine samples [205] and more than 400 times for water samples. This is contradictory to [197], where less then 100 extractions were possible due to the fact that the plasma matrix can result in the changing of the sorbent surface chemistry and sorption properties of the solid phase [197]. Therefore, for each application re-use must be verified. These are still very promising results compared to conventional SPE cartridges, which are recommended for single use only. Experimental studies using SPE report the re-use of SPE cartridges only a few times (3–4×) [206,207].

MEPS can handle small sample volumes (10 μL of plasma, urine or water) as well as large volumes (1000 μL) and can be used for GC, LC or CEC applications. Compared with liquid LLE and SPE, MEPS reduces sample preparation time and organic solvent consumption. MEPS may be fully automated and the extraction procedure takes only couple of minutes for each sample. Compared to SPME, the new technique is more robust. In SPME the sampling fiber of SPME is quite sensitive to the nature of the sample matrix. MEPS can be used without major problems for complex matrices (such as plasma, urine and organic solvents). This is not the case with SPME. Concerning some drawbacks of MEPS technique, they include the possibility of bubble formation and some difficulties connected to off-line arrangement as on-line coupling is not possible with every liquid chromatographic system. For off-line MEPS the speed of plunger movement is crucial for the recovery of analytes. Too high speed of movement does not enable adsorption of the analyte to MEPS support and leads to misleading recovery results and irrepeatability.

3.3.4. Solid phase microextraction (SPME)

SPME was developed in 1989 by Pawliszyn and co-workers [208,209] and introduced in early-1990s as a simple and effective adsorption/absorption and desorption technique which eliminates the need for solvents. Applications of SPME in the analysis of drugs have been widely revised in the past [210,211] as well as the possibilities of interfacing SPME with HPLC [212]. SPME may be performed in two arrangements: fiber SPME and in-tube SPME [111,137].

Fiber SPME is based on a modified syringe which contains stainless steel microtubing within its needle. Inside there is a fused-silica fiber tip which is coated with organic polymer, typically polydimethylsiloxane (PDMS). This coated fiber can be moved inside and outside by a plunger. A significant effort was put into the development and optimization of these fibers over the years. This has recently been revised by Gaurav et al. [213]. The extraction and preconcentration of the analyte is completed by coating in the position outside. The penetration of the septum of a GC injection port is performed with the fiber in inside position. Then, the desorption of analyte and transfer to a capillary column requires moving the fiber outside. Using such simple equipment, all steps—extraction, preconcentration, derivatization and transfer to the chromatograph are integrated in one device. Therefore, the main advantage of fibre SPME is the simplicity and automation of sample preparation procedures [210].

In bio-analytical methods either direct immersion (DI-SPME) or head-space fibre (HS-SPME) was applied with or without a derivatization step. Thus using the direct immersion approach, which means to expose the fibre to the sample in solution, e.g., antidepressants (mirtazapine, citalopram, paroxetine, duloxetine, fluoxetine and sertraline) were determined in plasma samples using poly(pyrrole) film SPME and HPLC–UV [214]. Further, clenbuterol in human urine and serum [215] as well as citalopram, fluoxetine and their main metabolites in urine [216] were determined without a derivatization step, then followed by subsequent

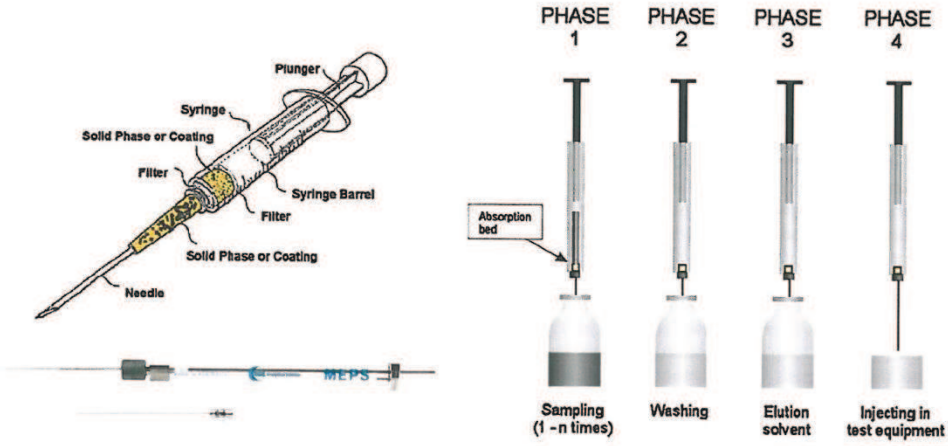

Fig. 5. Schematic of microextraction by packed sorbent, reprinted from [195,197], copyright Elsevier.

HPLC analysis. The development has proceeded further, when, e.g., MIPs were used as SPME fibre for the analysis of β-blockers in urine and plasma samples in connection with HPLC [217]. The application of fibre SPME is more advantageous when derivatization is needed. The process can be performed *in situ* prior to analysis, directly in SPME. Using *in situ* derivatization, 29 organic acids were analyzed in urine—derivatization using trimethyloxonium tetrafluoroborate [218] or benzodiazepines after the acid hydrolysis of the corresponding glucuronides in urine [219].

The outstanding advantage of HS-SPME in bio-analytical methods is the prevention of direct contact of the fibre with the sample, and therefore the prevention of contamination of the surface of the fibre with organic polymers. On the other hand, fibre HS-SPME is limited to special analytes because of the requirements of a high vapour pressure of the analyte. Furthermore, the transfer of fibres to the GC as well as desorption should be performed immediately after extraction because of the high vapour pressure of analytes in the coating along with the risk of analyte loss during storage of the loaded fibre. No derivatization was necessary in case of HS-SPME GC assay of metaldehyde in human serum [220], amphetamine and methamphetamine in human urine [221] or captopril in human serum and pharmaceutical preparations, in which ion mobility spectrometry was employed for the detection [222]. *In situ* derivatization HS-SPME GC was of benefit in analysis of so-called club drugs (ketamine, methamphetamine, gamma-hydroxybutyrate and methylenedioxymethamphetamine) in human urine using pyridine and hexyl chloroformate for the derivatization prior to GC–MS analysis [223] or analysis of the 12 fatty acids in feces after derivatization with 1-pyrenyldiazomethane [224].

To sum up, fibre SPME has several advantages, such as ease of use, nonusage of solvents and minimal equipment requirements. It is fast and easy to automate as well as provides good linearity and high sensitivity. However, as was stated above, these advantages are of use only in some areas of bio-analysis. Thus, the matrix and volatility of the target analyte must be taken into consideration. The combination of the low volatility of analyte with a complex matrix (containing polymer components including proteins in plasma or cell cultures) considerably limits the application of fibre SPME. Another drawback is the longer time needed for extraction, which is critical for some analytes and can become unacceptable. Generally, also the recoveries are considerably lower than those of LLE and SPE. Sample clean-up for fibre DI-SPME is not optimal, therefore interferences from endogenous trace substances could occur. On the other hand, the determination of volatile analytes which may be examined by HS-SPME is more favorable. Apart from all of this, there are some principal disadvantages of the use of fibre SPME: the capacity of SPME fibre is limited, temperature programs for GC require very low initial temperature because of the necessity of cryofocusing of the analyte, prolonging GC analysis. In addition desorption takes more time than in the injection of LLE or SPE extracts, and carryover effects occur very easily. As SPME is by nature dirty extraction, quantitation is more prone to errors due to changes in the matrix even when using internal standard. Finally, SPME fibre is quite fragile. Because of these restrictions and limitations, fibre SPME is not a universal sample preparation method, especially not in bio-analytical laboratories, and will not become so in the future [210].

At a later time, in-tube SPME, which uses an open tubular fused-silica capillary instead of a fibre to facilitate on-line hyphenation with chromatography, was explored. A fused-silica capillary column is placed as the injection loop in a standard autosampler. The column length and the thickness of extractant coating are tunable. During in-tube SPME the sample is repeatedly drawn and ejected through an internally coated capillary for extraction, and this is followed by the direct desorption of the extracted analytes from the capillary by a mobile phase for transport to HPLC column. This approach requires more complex instrumentation than regular SPME, but higher sensitivity can be obtained by using a longer tube and, consequently, more sorbent is exposed [111,225,226]. Typically, commercially available GC columns were used for in-tube SPME such as a polypyrrole [227] or 5% phenylpolydimethyl siloxane capillary [228]. Later, monolithic poly(methacrylic acid-ethylene glycol dimethacrylate) [229], β-cyclodextrine [230], RAM alkyl-diol-silica [231] or MIP packed into the capillary were used in order to improve the selectivity of extraction.

In-tube SPME was firstly introduced by Eisert and Pawlyszin in 1997 [226]. A mixture of thermally labile phenylurea pesticides was determined in aqueous samples using a piece of ordinary capillary GC column with Omegawax 250 coating, using subsequent HPLC analysis. The same capillary was used for the determination of β-blockers and their metabolites in urine and serum samples, followed by HPLC–MS analysis [232]. Later, a monolithic capillary column was used for the in-tube SPME of antagonists of angiotensin II receptor from human plasma and urine [233] as well as amphetamines from urine [229]. Recently, automated in tube SPME has been used for the determination of nicotine, cotinine and related alkaloids in human urine and saliva [234]; nontricyclic antidepressants (mirtazapine, citalopram, paroxetine, duloxetine, fluoxetine and sertraline) in human plasma [235] and for the immunoaffinity determination of fluoxetine in serum samples [236]. HPLC–UV or HPLC–MS was typically used as the accompanying chromatographic technique.

In-tube solid phase microextraction allows the convenient automation of the extraction process. This not only reduces analysis time, but also provides better precision and sensitivity than manual off-line techniques. The main advantages are ease of automation, fast and solvent-free operation and low cost of such an approach. In-tube SPME also presents minor exposition of the analyst to the biological samples and organic solvents. However, the applications published so far have employed laboratory-made devices only, which could limit the wide-spread application of in-tube SPME in routine bio-analysis [225].

3.3.5. *Stir bar sorptive extraction (SBSE)*

This sorptive and solvent-less extraction technique, introduced in 1999, is based on the same principles as SPME. Instead of a polymer-coated fibre, a large amount of extracting phase is coated on a stir bar. The most widely used sorptive extraction phase is polydimethylsiloxane (PDMS). The extraction of an analyte from the aqueous phase into an extraction medium is controlled by the partitioning coefficient of the analyte between the silicone phase and the aqueous phase. For a PDMS coating and aqueous samples this partitioning coefficient resembles the octanol-water partitioning coefficient. The amount of extraction phase in SBSE is 50–250 times greater than in SPME (typically 0.5 μL of extraction phase for 100 μL volume PDMS fibre). After extraction and thermal desorption, the analyte can be introduced quantitatively into the analytical system. This process provides high sensitivity, since the complete extract can be analyzed. In contrast to SPME, desorption process is slower because the extraction phase is extended, thus desorption needs to be combined with cold trapping and reconcentration [137].

Applications of SBSE in environmental and biomedical analysis have recently been revised by Kawaguchi et al. [237] and by David and Sandra [238]. Stir bar sorptive extraction of a liquid sample is performed by placing a suitable amount of sample in a headspace vial or a container. A PDMS-coated stir bar is added and the sample is stirred for 30–240 min. Controlled kinetically, the extraction time is determined by the sample volume, the stirring speed, the stir bar dimensions and also must be optimized for given applications. After the extraction, the stir bar is removed and wiped very gently with ling-free tissue to remove water droplets. Two desorption methods

may be used—thermal desorption (TD) [239] or liquid desorption (LD) [240] for subsequent connection with HPLC or GC.

Since the PDMS phase is a nonpolar liquid phase it should extract preferably low polarity analytes. High polarity compounds are not well recovered. Therefore, the possibility of *in situ* derivatization, where derivatization and SBSE are performed at the same time, was described for fatty acids and phenols [241] as well as for barbiturates and benzodiazepines [242]. Similarly, metabolites with glucuronic acid or sulphate conjugates are often determined together with parent drugs. In order to determine such compounds in biological matrices, *in situ* de-conjugation can be performed, in which SBSE and de-conjugation is performed at the same time [243].

In bio-analytical applications, various types of samples including urine, plasma, saliva can be analyzed using SBSE. Special attention should be paid to the possibilities *in situ* derivatization combined with SBSE, since often target compounds are quite polar, e.g., metabolites. Recently, SBSE has been employed in the analysis of barbiturates and benzodiazepines as well as their metabolites in urine and blood [244]. RAM sorbent was employed for SBSE of caffeine and its metabolites from rat plasma [243]. Oxidative stress marker 4-hydroxynoneal was determined in urine using SBSE with derivatization [245].

SBSE may be successfully applied for bio-analytical purposes. Since only PDMS coating is available as an extraction phase, SBSE was prevalently used for low polarity analytes, but the problems of polar compounds may be solved by *in situ* derivatization. It is clear that further developments in stir bar coatings and designs could extend the applicability of the method. The main drawback of this method is the duration of extraction, typically 30–150 min [238]. For this reason SBSE may be impractical for routine high-throughput laboratories.

3.3.6. Restricted access materials (RAM)

Restricted access materials are biocompatible sample preparation supports which enable the direct injection of biological fluids into a chromatographic system. The technique was introduced in 1991 by Desilets et al. [246], who also established the term RAM. RAM sorbents represent a special class of materials that are able to fractionate a biological sample into a protein matrix and analyte fraction, based on molecular weight cut-off. Macromolecules are excluded and interact only with the outer surface of the particle support, which is coated with hydrophilic groups. This minimises the adsorption of matrix proteins. Applications of RAMs have recently been revised by several research groups [225,247–249].

The basis of RAMs is the simultaneous size-exclusion of macromolecules and extraction/enrichment of low-molecular compounds into interior phase via partition. The outer surface of the particles, which is in contact with biological matrix components such as proteins and nucleic acids, possesses a special chemistry to prevent adsorption of these molecules. Macromolecules can be excluded by a physical barrier by means of the pore diameter or by a chemical diffusion barrier created by a protein (or polymer) network at the outer surface of the particle. RAMs can be classified according to the protein exclusion mechanism into the following two groups—RAM with a physical barrier (reverse phase, alkyl-diol-silica material, porous silica with combined ligand) or RAM with a chemical barrier (semi-permeable surface, protein-coated silica, mixed-functional materials or shielded hydrophobic phase) [247]. Also during the development of RAM supports, enantioselective RAMs using glycopeptides antibiotics as chiral selector [250] or weak cation-exchange RAM were introduced [251].

The application of RAM to direct and repeated analyses of drugs in biological fluids has become well established, especially with the commercialization of such supports. The robustness of RAM supports for direct injection of biological fluids has been described in literature, e.g., 200–2000 repeated injections of plasma samples were performed by Schafer et al. [252] without a decrease in RAM column extraction performance. Recent applications include, e.g., the determination of enrofloxacin in nasal secretions and in the plasma of pigs [253] as well as the determination of antidepressant drugs in plasma and urine [254]. As a new trend, a completely new sorbent combining restricted access material together with molecularly imprinted polymer (RAM-MIP, Fig. 6) with a hydrophilic external layer was synthesised. The applicability was verified on analysis focused on the selective recognition of p-acetamimophenol [255]. RAM-MIP material was found more suitable for employment in the treatment of biological material because of its reduction of nonspecific interactions. Its applicability was also evaluated as drug delivery system. RAM-MIP has been found very promising for the selective and controlled release of drugs in gastrointestinal simulating fluids.

3.3.7. Turbulent-flow chromatography (TFC)

The use of turbulent-flow for the direct injection of drugs made of biological fluids to perform on-line sample clean-up has seen consistent growth since its introduction in late-1990s, as revised by Mullet et al. [225]. TFC was introduced by cohesive technologies in 1997 [256].

The principle of turbulent-flow chromatography applications in sample preparation is the separation of small analyte molecules from the macro-molecular matrix due to the large diffusion coefficients of proteins. Such enhanced separation efficiency is the result of the onset of turbulence, which is responsible for an increase of the mass transfer rate through formation of "eddies" in the mobile phase [257]. This eddy formation results in a plug profile for the eluent and thus facilitates reduced band broadening as opposed to the parabolic profile of typical chromatographic laminar flow. The generation of turbulent-flow in a packed column depends on the particle size and on the nature and linear velocity of the mobile phase. Short, narrow columns, packed with large-size particles (typically 50 mm × 1.0 mm, 50–60 μm), are required to generate turbulent-flow while maintaining manageable system back-pressures. Applied flow-rates are typically in the range of 4–5 mL min^{-1}. The retained analytes are subsequently eluted from the extraction column using organic mobile phase onto an analytical column for the chromatographic separation [18].

TFC has been recently employed in the analysis of loratadin and desloratadin in human plasma [19], sitagliptin in human urine and hemodialysate [258], immunosuppressants in blood [259], antidepressants in serum [260], dextromethorphan and its metabolites in plasma [261,262] and some others [225].

TFC has the potential to eliminate off-line sample clean-up procedures for clinical analysis and may be easily connected to HPLC. On-line coupling requires some modifications to the off-line extraction techniques. The coupling is most commonly performed with the help of multiport valves and one or more pumps for the dynamic extraction or transfer of the extract to the chromatographic system. In an on-line system the whole extract is transferred to the chromatographic column, in contrast to traditional off-line techniques, where only a small part is injected. This means that the sensitivity of the on-line method is much greater. However, the high sensitivity may easily lead to the overloading of the analytical column. Therefore, miniaturization of the extraction system is often required to avoid this problem [19]. One of the drawbacks of TFC could be the reduced lifetime of the extraction column. Zeng et al. utilized 15% aqueous solution of acetic acid and 90% THF in order to extend the lifetime of extraction columns [18]. Acetic acid

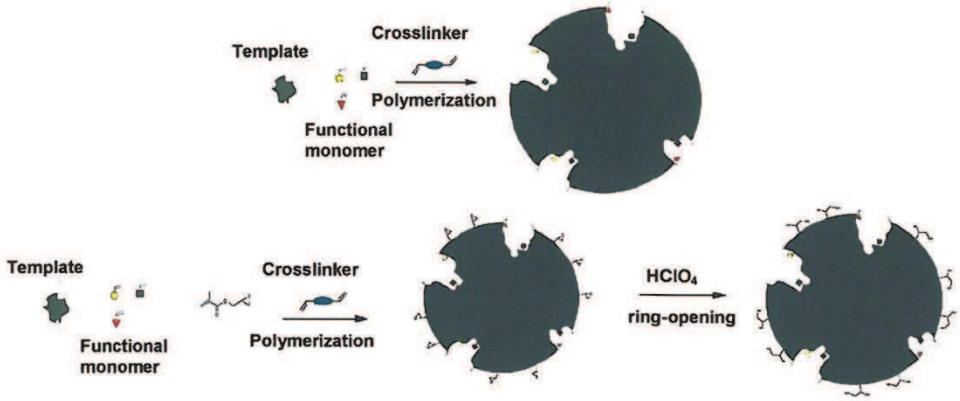

Fig. 6. Schematic of synthesis of traditional molecularly imprinted polymer and restricted access material-molecularly imprinted polymer, reprinted from [255], copyright Elsevier.

used as mobile phase removed proteins, and THF solution was used to remove lipids from the extraction and analytical column. Using this approach, 2000 injections of biological samples were possible.

3.4. Solid phase extraction-based selective approaches

3.4.1. Molecularly imprinted polymers (MIPs)

Molecularly imprinted polymers are selective materials used for solid phase extraction. MIPs can not only concentrate, but also selectively separate the target analytes from real samples, which is crucial for the quantitative, sensitive and selective determination of analytes in complex matrices. The main benefit of MIPs is the possibility to prepare selective sorbents pre-determined for a particular substance or a group of structural analogues. MIPs are synthetic polymers with highly specific recognition ability for target molecules [263]. Details on the classification and preparation of MIPs have been given in recently published article [264]. MIPs used for SPE may be synthesized by three imprinting techniques which enable the formation of complex template-functional monomer: (1) covalent imprinting, (2) noncovalent imprinting and (3) hybridization of both. The imprinting molecule complexes one or several functional monomers. The next is polymerization. As a result, imprints possess a steric (size and shape) and chemical (special arrangement of complementary functionality) memory for a template [263,265]. The first application of MIP in SPE was introduced by Sellergren in 1994 [266], in which pentamidine was determined in urine using a pentaimidine-imprinted dispersion polymer in SPE column.

SPE using MIP can be performed either on-line or off-line. In biological applications MIP is typically used in off-line mode for extracting analytes from different matrices, such as biological fluids (urine, serum and plasma) or tissue samples (liver, kidney and muscle). MIPs for bio-analytical applications were typically prepared by noncovalent imprinting technique in connection with bulk polymerization [263]. MIPs were widely used in the analysis of cephalosporin antibiotics in human plasma and serum [267–270], fluoroquinolone antibiotics in human urine and pig tissue [271] and in the analysis of β-blockers [272–274]. MIPs used in immunoassay analysis are termed MIAs (molecularly imprinted sorbent assays), however, they have not become wide-spread because of their many drawbacks [275].

Due to substantial advantages of using MIPs, including high selectivity, stability, reusability, ease of use and low cost preparation, they have been used effectively as sorbents in the clean-up and selective enrichment of analytes from different samples. However, some features still need improvement. Currently, the MIPs used for SPE are typically prepared by a noncovalent imprinting technique, which gives quite low yields of specific binding sites. This results in low sample load capacity and high nonspecific binding. So the main advances expected in development of new MIP synthesis methods is the improvement of their capacity and selectivity. Another possible problem of MIPs used for SPE is the undesirable leakage of templates, which can seriously interfere with the quantification of trace compounds in complex matrices [263]. This drawback could be resolved by use of stable isotope-labelled compounds as templates for the preparation of MIPs [276]. Another approach is to first heat MIPs and then elute them with a strongly polar solvent. This can reduce or even eliminate the leakage of template [277].

3.4.2. Aptamers

Aptamers are oligonucleotides (DNA or RNA) that bind with high affinity and specificity to a wide range of target molecules, such as drugs, proteins and other organic or inorganic molecules. Aptamers are generated by an *in vitro* selection process called systematic evolution of ligands by exponential enrichment (SELEX) which was first reported in 1990 [278,279]. The SELEX method has permitted the identification of unique RNA/DNA molecules from very large populations of random sequence oligomers (DNA or RNA libraries). These molecules bind to the target molecule with very high affinity and specificity. Aptamers show a very high affinity for their targets, with dissociation constants typically ranging from the micromolar to low picomolar, comparable to those of some monoclonal antibodies, sometimes even better [280].

Aptamers were also reported to be used for the selective isolation of some compounds, e.g., cocaine, from biological fluids by means of high selectivity binding [281]. This could be a future trend in sample preparation, however, there is a need to improve the capacity of the necessary highly selective supports [282].

4. Conclusions and future perspectives

4.1. Fast analytical approaches

The approaches in fast liquid chromatographic analyses discussed in this review are summarized in Table 6. The advantages and drawbacks of individual techniques – HTLC, monolith columns, fused core columns and UHPLC – are pointed out. The main advantages of HTLC are, firstly, practically no use of organic solvent, which makes HTLC a green approach and secondly, the possibility to com-

Table 6
Comparison of fast analytical approaches.

Fast chromatographic approach	Advantages	Drawbacks
Monolith columns	Low back-pressure Fast analyses High efficiency Possibility to couple many columns Compatibility with conventional instruments	Only few stationary phases commercially available High solvent consumption Low pH resistance of silica monoliths Low temperature stability Lack of narrow-bore columns Method transfer is not straightforward
High temperature liquid chromatography	Low mobile phase viscosity and high mass transfer Low back-pressure Low concentration of organic modifier (green chromatography) Fast analysis Temperature as a variable to change method selectivity Can be used also at ultra-high pressure	Heating and cooling of mobile phase Lack of thermally stable stationary phases Thermal stability of compounds must be evaluated Difficulty with method transfer (selectivity!)
Ultra-high performance liquid chromatography	Substantial decrease in analysis time Very high efficiency and resolution Low solvent consumption High mechanical and chemical stability of UHPLC stationary phases Easy method transfer Can use temperature as variable to optimize method Already, high variety of stationary phases available	Dedicated instrumentation and stationary phases Solvent compressibility and frictional heating Lower sample loading capacity
Fused core columns	Decrease in analysis time Very high efficiency and resolution Low back-pressure No need for specialized instrumentation Various stationary phases commercially available High sample loading capacity	Relatively higher solvent consumption comparing to UHPLC Lower pH range (2–8)

pletely change selectivity. However, the drawbacks listed in Table 6 still limit the wide-spread utilization of this technique in routine laboratories.

Monoliths seem to be more convenient for routine use in bio-analytical applications, but their application still remains limited due to the restricted availability of stationary phase chemistries and the low pH stability of silica ones, if the laboratory is not able to synthetise these. Some advances have been made in the commercial development of column geometries, as now i.d. 3.0 mm × 100 mm and i.d. 2.0 mm × 50 mm have been made newly available. This, however, still does not satisfy the requirements of various MS applications, in which a 2.0 i.d. in various column lengths would be desired in order to, first, be compatible with ESI and, second, to reduce solvent consumption. The high solvent consumption iduced by the high flow-rates applied is one of the main drawbacks of monolith columns.

As a result, fused core columns and UHPLC seem to be the most convenient approaches for modern, high-throughput, efficient, economic and ultra-fast analysis. They provide the most substantial reduction in analysis time and very high efficiency. Further, various stationary phase selectivity in both sub-2-microne and 2.7 μm porous shell particle scale are now available. With regard to the stability of stationary phases, UPHLC seems to be slightly more advantageous, because chemically more stable stationary phases are available. Commercially available fused core columns are manufactured from silica, which is applicable within the conventionally limited pH range of 2–8. UHPLC in its design requires special instrumental equipment. This may be a drawback when compared to the utilization of fused core columns, which can be connected to any HPLC or UHPLC instrument. Similarly, due to low system volume and low volume of analytical columns in UHPLC, precautions must be taken in order to prevent column overload. The problem of frictional heating in UHPLC may be solved by the use of appropriately narrow column diameters (2.1 or 1.0). This also ensures low solvent consumption and makes fast approaches environmentally friendly. However, the low volume, sub-2-microne narrow-bore arrangement induces a higher pressure drop in UHPLC, a drop which is substantially lower when using fused core columns. Thus from this point of view, fused core columns appear to be more advantageous. Concerning practical applications, UHPLC is unequivocally leading fast chromatographic approach.

4.2. Sample preparation

Sample preparation techniques must be chosen and optimized with the regard to method purpose. As is stated in the ICH guidelines, the validated method must be appropriate for the intended purposes [1]. Sample preparation procedures used in bio-analytical applications and their important features are displayed in Table 7. An appropriate technique should be chosen with the regard to extraction time, selectivity, the number of steps, solvent consumption and the possibility of the use of on-line methods arrangement.

Current sample preparation techniques employ small amounts of sample as well as simpler methods which are "just enough" prior to analysis, as more steps could introduce more errors. New developments have been attempted to enhance selectivity (immunoaffinity, MIP and aptamers) as well as to reduce solvent consumption, thus making sample preparation environmentally friendly (microextraciton approaches). Finally, such methods also feature high-throughput automated techniques.

As is shown in Table 7, the fastest approaches are MEPS, on-line RAM and TFC. The highest selectivity is obtained using MIP or aptamers. Among solvent-less techniques, microextractions such as SPME and SBSE or on-line RAM and TFC, which both use mobile phase for sample elution, are the most environmentally friendly. However, most microextraction techniques, including LLME, SPME and SBSE, must be excluded from use with high-throughput applications, as a long-time for the establishment of equlibrium is necessary. Therefore these will never be widely used in modern analytical laboratories. Overall, on-line RAM, TFC and MEPS appear to be the most convenient in regards to extraction time, ease of use, possibility of automation and solvent consumption. However,

Table 7
Comparison of discussed sample preparation approaches.

Sample preparation technique	Extraction time [min]	Selectivity	Multi-step	On-line possibility	Solvent consumption
LLE	15–25[a]	Medium	Yes	+	High
Extrelut LLE	15–30	Medium	Yes	+	High
LLME	5–60[b]	Medium	Adsorption/desorption	–	Very low
PLE	10–15	Medium	NO	+	Low
SALLE	<10	Low	NO (centrifugation)	+	High
SPE	15–25[a]	Medium	Yes	+	Relatively high
Monolith spin extraction	<10	Medium	Yes	–	Low
MEPS	1–5	Medium	Yes	+	Very low
SPME	10–60[c]	Medium	Adsorption/desorption	+	NO
SBSE	30–240	Medium	Adsorption/desorption	–	NO
MIP	15–20[a]	High	Yes	+	Relatively high
RAM	<5	Medium	NO (centrifugation)	+	NO
TFC	<5	Medium	NO	+	NO
PP	<10	Low	NO (centrifugation)	+	High

[a] On-line automatic approaches enable faster extractions, typically up to 10 min.
[b] Except of DLLME, where reaching of equilibrium is quick.
[c] In-tube SPME arrangement is much faster.

more experimental work and development is necessary before the wide-spread implemention of these for use as sample preparation techniques in modern high-throughput laboratories.

Conventional sample preparation techniques such as SPE, LLE and PP are still the most widely used in routine laboratories. However, their performance should be surpassed by new modern approaches and their development should proceed further, following high-throughput, low volume, ease of use, automated and environmental trends in order to reduce the contrast with fast LC approaches. So far, this has not happened, because their automation and on-line connection with chromatographic techniques (especially SPE) enables effective and reliable sample preparation in many analytical laboratories. This will probably not change in the near future.

Acknowledgements

The authors gratefully acknowledge the financial support of GAČR 203/07/P370 and MSM 0021620822.

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PŘÍLOHA IV

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Microextraction by packed sorbent as sample preparation step for atorvastatin and its metabolites in biological samples - critical evaluation

Journal of Pharmaceutical and Biomedical Analysis, 2011, vol. 55, p. 301–308.

Journal of Pharmaceutical and Biomedical Analysis 55 (2011) 301–308

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Microextraction by packed sorbent as sample preparation step for atorvastatin and its metabolites in biological samples—Critical evaluation

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ARTICLE INFO

Article history:
Received 30 September 2010
Received in revised form 20 January 2011
Accepted 22 January 2011
Available online 28 January 2011

Keywords:
Atorvastatin
UHPLC
Tandem mass spectrometry
MEPS
Bio-analytical method

ABSTRACT

Atorvastatin belongs to the group of lipid-lowering drugs known as statins. They significantly reduce the levels of total cholesterol, low-density cholesterol and plasma triglycerides therefore they are widely used in the treatment of hypercholesterolemia. Recently developed methods for the determination of atorvastatin and its metabolites in plasma used SPE (solid phase extraction) or LLE (liquid–liquid extraction) as the sample preparation step. However, both procedures are quite time-consuming and need relatively high volume of solvent/sample, which is impractical for the routine analyses of many biological samples.

The aim of this work was to develop and validate more suitable sample preparation method for the determination of atorvastatin and its metabolites in biological samples using MEPS (microextraction by packed sorbent). The optimal conditions of MEPS extraction were using C8 sorbent and only 50 μl of the sample. The analytes were eluted by 100 μl of the mixture of acetonitrile:0.1 M ammonium acetate pH 4.5 (95:5, v:v). The analytical method was validated and demonstrated good linearity ($r^2 > 0.9990$), recovery (89–115%) and intra-day precision (RSD < 10%). Total time of the sample preparation was three times shorter (7 min) compared to SPE. The volume of sample was twenty times lower and the volume of solvents about ten times lower compared to SPE. Combination of fast MEPS method together with quick UHPLC–MS/MS was used for the determination of atorvastatin and its two metabolites in serum obtained from patients with familiar hypercholesterolemia.

1. Introduction

Recently, UHPLC–MS/MS becomes a leading trend in modern bio-analytical methods [1]. There is however a great contrast between ultra-fast chromatographic analysis and conventional sample preparation, which remains highly labour-intensive and time-consuming. Conventional sample preparation techniques such as SPE (solid phase extraction), LLE (liquid–liquid extraction) and PP (protein precipitation) are still dominating in sample preparation area even though many modern approaches including MIP (molecularly imprinted polymers, a method with enhanced selectivity), various microextractions, such as SPME (solid phase microextraction), LLME (liquid–liquid microextraction), MEPS (microextraction by packed sorbent), and an on-line sample preparation techniques using RAM (restricted access material) or in-tube SPME and many others have been developed and introduced in practical use [1]. All these modern sample preparation approaches have gained an attention however, for the moment they have not replaced conventional sample preparation techniques.

Microextraction by packed sorbent is one of the quite new sample preparation techniques developed in the laboratories of Astra Zeneca in 2004 [2,3]. Actually, MEPS is a miniaturization of conventional SPE. Sample preparation there takes place on the packed bed. MEPS can thus be described as a short "LC column in a syringe". The bed dimensions are scaled from a conventional SPE bed and in this way MEPS can be adapted to the most existing SPE methods by simply scaling the reagents and sample volumes from the conventional device to MEPS.

In MEPS, approximately 1–2 mg of solid packing material is either inserted into the barrel of a syringe (100–250 μl) as a plug with polyethylene filters on both sides, or between the syringe barrel and the injection needle as a cartridge (Fig. 1). The bed can be packed or coated to provide selective and suitable sampling conditions. In MEPS, any sorbent material can be used either as packing bed or as a coating. Commercially available sorbents include silica based C2, C8, C18 and M1 (mixed C8 and SCX) [2]. The key factor in MEPS is that the volume of solvent used to elute the analytes from the extraction process is of a suitable order of magnitude to be injected directly on-line into an LC [3–5] or GC [2]

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doi:10.1016/j.jpba.2011.01.025

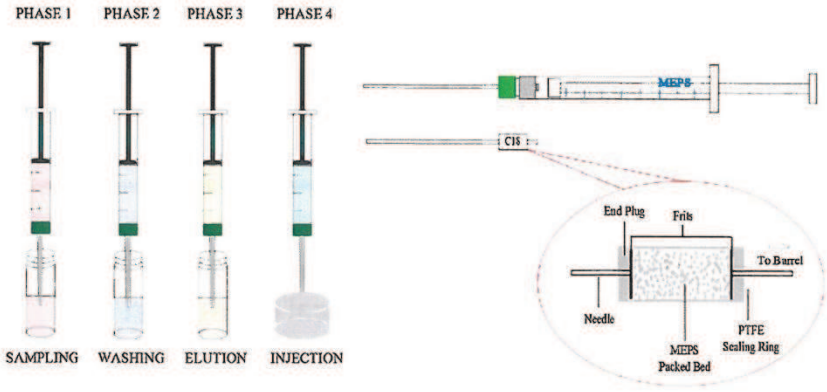

Fig. 1. Schematic of microextraction by packed sorbent [1].

system without any modification of the instrument. Connection to a robot makes the method fully automated. MEPS can handle small sample volumes (10 μl of plasma, urine or water) as well as relatively large volumes (1000 μl). MEPS technique differs from commercial SPE in that the packing is inserted directly into the syringe, not into a separate column. Thus, there is no need for a separate robot to apply the sample on the solid phase, which is on the other hand necessary in case of conventional SPE if automation is required.

The MEPS technique has been used to extract various analytes from biological samples. Several drugs such as local anaesthetics and their metabolites [2,3]; the anticancer drugs roscovitine [4], olomoucine [5], cyclophosphamide [6] and busulfan [7]; the β-blocker drugs acebutolol and metoprolol [8]; the anti-depressant drugs dopamine and serotonine [9] as well as anti-addictive methadone [10] have been successfully extracted by MEPS from biological samples such as plasma, urine or blood.

Statins are drugs widely used for the treatment of hypercholesterolemia and also of its severe forms such as familiar hypercholesterolemia. They have potent cholesterol-lowering effect and they significantly reduce morbidity and mortality associated with coronary heart disease as it was proved by many clinical trials [11–13]. Therapeutic range of statins is typically 10–80 mg/day. High doses might be used with caution in the elderly, in patients with renal or hepatic insufficiency, hypothyroidism or diabetes. Therapeutic drug monitoring is not routinely done in patients treated by statins. In order to establish and control appropriate dosage scheme, which would minimize adverse effects and keep the cholesterol lowering effect, it would be very helpful to monitor the levels of statins in biological materials. Moreover, the method would be very useful for the determination of possible loses of statins during extracorporeal elimination procedures such as hemodialysis, LDL-apheresis.

Statin molecules exist in two forms, lactone and open-ring hydroxy acid form [14,15]. In vivo, the hydroxy acid forms are the active drugs to lower plasma cholesterol while the lactone forms are inactive prodrugs. Atorvastatin is one of the drugs worldwide the most commonly occurring in commercially available pharmaceutical formulations used in the clinical treatment of hypercholesterolemia. It is administered in the open-ring hydroxy acid form – the active form. It is absorbed from the gastrointestinal tract and it undergoes an extensive first-pass metabolism in the liver, which produces two active hydroxy metabolites, ortho-hydroxyatorvastatin and para-hydroxyatorvastatin and three inactive lactones (Fig. 2). More than 90% of atorvastatin is bound to plasma proteins. About 70% of the total plasma HMG-CoA activity is attributed to active metabolites of atorvastatin, even if their concentrations are very low [16–18]. As it figures out from the information above, the levels of statins in biological fluids are very low, probably because only about 5% of dosed statin reaches the systemic circulation. Typical plasma concentrations are in ng/ml levels. The typical plasma concentration range of active metabolites of atorvastatin is between 0.1 and 20 ng/ml.

The methods for the determination of simvastatin and atorvastatin were recently reviewed by our group [19]. In clinical applications HPLC–MS/MS was unequivocally the method of choice in analysis of atorvastatin together with its metabolites [20–25] using typically ESI (electrospray ionization) in positive ion mode. SRM (selected reaction monitoring) transition 559 › 440 was monitored for atorvastatin molecule. Recently only four new methods for the determination of atorvastatin were published. Developed HPLC-UV method for the determination of atorvastatin in human plasma however did not determine metabolites together with atorvastatin and used liquid–liquid–liquid microextraction (LLLME) as the sample preparation technique [26]. Another new method for the determination of atorvastatin together with fenofibrate used the UHPLC with UV detection. It was developed and validated for the analysis of atorvastatin, fenofibrate and their degradation products in tablets but not in biological samples therefore the sensitivity of UV detection was insufficient for clinical applications [27]. For the determination of atorvastatin, simvastatin and lovastatin HPLC method with CAD (charged aerosol detector) was applied [28] and the sensitivity of UV and CAD was compared. UHPLC–MS/MS method for the determination of atorvastatin and simvastatin, their metabolites and interconversion products in biological samples was developed and validated with good reproducibility, sensitivity and selectivity and it was applied to serum and lipoprotein fractions in our laboratory [29]. Sample preparation step in bio-analytical assays of atorvastatin and its metabolites employed mostly LLE [21–24] or SPE [20,25,29]. Both approaches are multi-step, time-consuming and also the consumption of sample and organic solvent is quite high, especially in case of LLE. That is not suitable for the routine analyses of huge number of samples therefore new preferable sample preparation procedure would be convenient.

Over the last year only one article presented another sample preparation technique than LLE or SPE. Farahani et al. [26] presented a rapid and economical liquid–liquid–liquid microextraction method for the determination of atorvastatin in human plasma for screening purposes, however no metabolites were determined.

The aim of the work was to develop fast, easy and low-volume (for both – sample and organic solvent volume) sample preparation technique convenient for routine preparation of biological samples containing atorvastatin and its metabolites. On-line MEPS extrac-

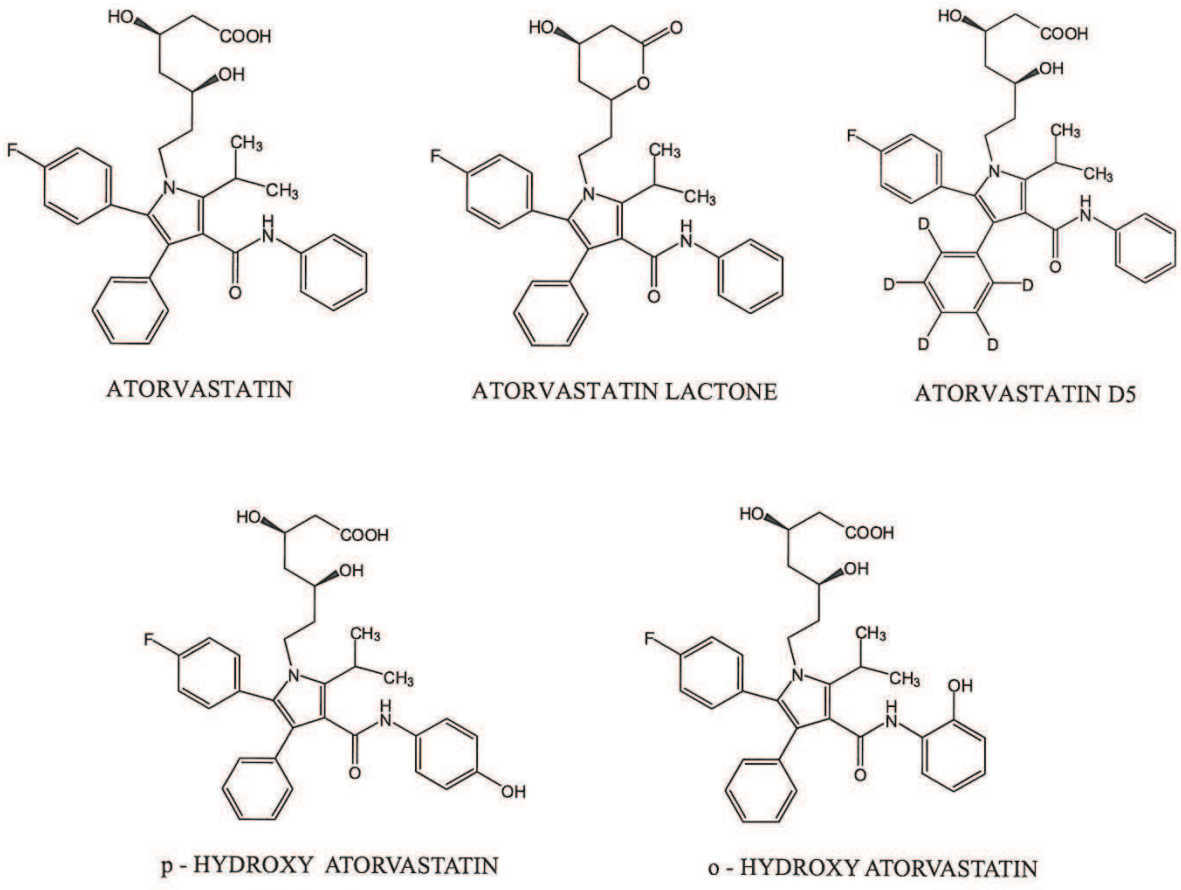

Fig. 2. Chemical structure of atorvastatin, atorvastatin hydroxy metabolites and atorvastatin lactone.

tion was previously described as a method convenient for these purposes [2–10] however on-line coupling is not possible with every HPLC or UHPLC system. Therefore the evaluation of MEPS procedure in off-line arrangement is highly valuable.

2. Experimental

2.1. Chemicals and reagents

Working standards of atorvastatin, atorvastatin lactone, p-hydroxyatorvastatin, o-hydroxyatorvastatin and atorvastatin deuterium labeled (D5 – phenyl ring) were purchased from Toronto Research Chemicals (Ontario, Canada).

Acetic acid, reagent grade, ammonia, reagent grade and acetonitrile, LC–MS grade, were purchased from Sigma–Aldrich. HPLC grade water was prepared by Milli-Q reverse osmosis Millipore (Bedford, MA, USA) and it meets European Pharmacopoeia requirements.

2.2. Chromatography and mass spectrometry

UHPLC system Acquity UPLC (Waters, Prague, Czech Republic) was used for the purpose of this study. It consisted of ACQ-binary solvent manager and ACQ-sample manager. UHPLC–MS/MS analysis was performed according to the previously published method [29] with only slight modification. UHPLC analyses were performed on BEH C_{18} analytical column (100 mm × 2.1 mm, 1.7 μm, Waters, Prague, Czech Republic) based on bridged ethyl hybrid (BEH) particles. Mobile phase was composed of acetonitrile and 0.5 mM ammonium acetate pH 4.0 using gradient elution with initial mobile phase composition acetonitrile:ammonium acetate (30:70). Thereafter the concentration was changed within 1.5 min to 70% of acetonitrile and subsequently to 95% of acetonitrile within 5.25 min. Mobile phase flow rate was 0.25 ml/min. The analytical column was kept at 35 °C. The solutions were stored in the autosampler at 4 °C.

The MS/MS triple quadrupole system was used in this study. Quattro Micro (Micromass, Manchester, GB) was equipped with a multi-mode ionization source (ESCI). Ion source was set-up in ESI positive mode according to [29] as follows: capillary voltage: 3500 V, ion source temperature: 130 °C, extractor: 3.0 V, RF lens: 0.5 V. The desolvation gas was nitrogen at flow 500 l/h and at the temperature 375 °C. Nitrogen was used also as a cone gas (120 l/h). Cone voltage (CV) was set up individually for each analyte. Quantitation of all analytes was performed using SRM (selected reaction monitoring) experiment. Two specific transitions were optimized for each molecule and secondary ion ratio was calculated in order to increase selectivity of the method. Argon was used as collision gas and collision energy (CE) was optimized for each analyte individually – see [29]. The MassLynx 4.1 Data System was used for MS control and data gathering. QuanLynx software was used for data processing and quantitation – regression anal-

ysis of standard and matrix calibration curves and calculation of concentrations.

2.3. Preparation of standard solutions

The stock solutions of standards were prepared by dissolving of the amount corresponding to 1.0 mM of appropriate working standard into 1.0 ml of dissolution media according to the solubility properties, because the molecules differ significantly in solubility. The stock solutions of atorvastatin lactone were prepared in pure acetonitrile. The stock solutions of atorvastatin, atorvastatin D5 and atorvastatin hydroxy-metabolites were prepared in mobile phase used at initial step of gradient elution – acetonitrile:ammonium acetate 0.5 mM, pH 4.0 (30:70, v:v). Stock solutions were further diluted by mobile phase (keeping the pH of solution between 4.0 and 5.0 in order to prevent the interconversion) to achieve individual points of calibration curve in the range 0.1–100 nM, using seven calibration points (100, 50, 10, 5.0, 1.0, 0.5 and 0.1 nM). Stock solutions of all tested compounds were stable for two weeks at 20, 4 and −18 °C with a mean percentage change of <3%. Thereafter, fresh stock solutions were prepared.

2.4. Sample preparation – MEPS and SPE

Serum samples were prepared using both methods: newly developed MEPS technique and SPE procedure described previously [29]. Briefly, in SPE internal standard atorvastatin D5 (100 μl) was added to 900 μl of the serum samples containing the analytes. 500 μl of sample was loaded on Discovery DSC-18 sorbent previously activated with 1 ml of acetonitrile and conditioned with 1 ml of 0.1 M ammonium acetate pH 4.5. The SPE cartridge with loaded sample was washed with 2 ml of 0.1 M ammonium acetate pH 4.5 and 1 ml of mixture of acetonitrile:0.01 M ammonium acetate pH 4.5 (15:85, v:v), and subsequently the analytes were eluted with 1 ml of acetonitrile:0.1 M ammonium acetate pH 4.5 (95:5, v:v). The eluate was filtered via 0.20 μm PTFE filter and sample was injected onto UHPLC system.

In MEPS C8 sorbent packed in bin, which is inserted into a needle assembly connected to 100 μl syringe was used (SGE Analytical Science, Germany). The sorbent was activated three times with 100 μl of acetonitrile and conditioned three times with 100 μl of 0.1 M ammonium acetate pH 4.5. 50 μl of sample was aspirated through the syringe. The sorbent was washed two times with 100 μl of 0.1 M ammonium acetate pH 4.5 and consequently with 100 μl of mixture acetonitrile:0.01 M ammonium acetate pH 4.5 (15:85, v:v). The analytes were eluted with 100 μl of mixture of acetonitrile:0.1 M ammonium acetate pH 4.5 (95:5, v:v). The eluate was filtrated via PTFE microfilter (4 mm × 0.2 μm) and the sample was transferred into the micro insert of vial and injected onto UHPLC system.

Serum samples were kept at −80 °C and after the thaw cycle they were processed immediately by MEPS procedure and analyzed by UHPLC–MS/MS. The change in serum samples concentration at 4 and −18 °C was not greater than 15% within the period of two weeks, which is in agreement with the results of method precision and accuracy.

2.5. Method validation

The newly developed MEPS–UHPLC–MS/MS method was validated in terms of linearity, accuracy, precision, selectivity and sensitivity (limits of detection and quantitation) according to the requirements of ICH (International Conference on Harmonization) [30]. For the determination of linearity, two calibration curves of all analytes were prepared: matrix calibration curve using blank serum sample, which was spiked and then treated by MEPS procedure (1) in the concentration range 0.5–100 nM and standard calibration curve (2) where stock standard solutions were diluted by mobile phase in the concentration range 0.1–100 nM.

For method precision, spiked blank serum treated by MEPS at three different concentration levels were measured in three replicates in order to calculate % of RSD, which describes the closeness of agreement between series of measurements. Inter-day and intra-day precision was measured.

Method accuracy was described as the recovery experiment. Recovery was determined via a comparison of the response of serum samples spiked prior to MEPS extraction with that of blank serum samples that were first treated by MEPS procedure and then it was spiked with the analytes. It was complemented at three different levels in three replicates to establish the closeness of agreement between the true and measured value as it corresponds to ICH requirements [30]. QC samples were prepared at the same concentrations as were the concentration levels prepared for precision and accuracy experiments. Lyophilized standard serum samples were used for the purposes of method validation. Matrix effect was evaluated using blank serum samples, which were first treated by MEPS procedure and than spiked by standard solution at three concentration levels within the calibration range. The results were compared with the measurement of standard calibration curves (2) and matrix effects were calculated. Limits of detection and quantitation were established based on signal-to-noise (S/N) ratio approach. Limit of detection was expressed as S/N = 3, limit of quantitation was expressed as S/N = 10.

The stability of samples in standard stock solutions was evaluated at 20, 4 and −18 C and in serum samples at 4 and −18 C in a short-term and long-term measure.

2.6. Patients

The long-term patients included 2 men in the age of 44 and 65 years, regularly treated for 7.7 and 9.6 years with extracorporeal elimination procedures – one with LDL-apheresis (Adsorbers Lipopak 400, Pocard, Moscow, Russia) and the second with hemorheopheresis (filters Evaflux 4A, Kuraray, Osaka, Japan). The clinical phenotype of familiar hypercholesterolemia was characterised by an increased level of total plasmatic cholesterol and LDL-cholesterol, and by the occurrence of xanthomas and premature symptoms of ischemic heart disease. The patients were treated with high-doses of statins (maximally tolerated dose – 40 and 80 mg of atorvastatin daily) and with ezetimib (both with 10 mg daily). The use of patient data and samples for the research purposes was approved by an Ethical Committee and patients gave their written consent.

For the evaluation of atorvastatin levels and its metabolites, two blood samples were taken: one just before the start of the extracorporeal elimination procedure, the second just after extracorporeal elimination procedure (LDL-apheresis, hemorheopheresis). The blood was drawn from needle inserted into two peripheral veins before the start of procedures at 9.00 a.m., and the second blood sample was drawn just after procedures i.e. after 4–5 h later. After separation, serum aliquots were stored at −80 °C until analysis. The samples were assayed in random order. All samples were analyzed by personnel who had no knowledge of the subjects' clinical data.

3. Results and discussion

3.1. Development of MEPS procedure

The sample preparation procedure was optimized using off-line MEPS arrangement and two different MEPS cartridges – C18 and C8. Although C18 sorbent was used for the SPE extraction, C8 was chosen for the MEPS extraction, because it provided better results for recovery and precision during method validation especially for

Table 1
Validation results – linearity, method accuracy, precision, recovery, LOD and LOQ.

Method validation	p-OH AT	o-OH AT	AT	ATL
Linearity (r^2) – matrix calibration curve	0.9992	0.9990	0.9993	0.9993
Linearity (r^2) – standard calibration curve	0.9997	0.9998	0.9996	0.9997
Method accuracy [%]				
L1	106.38	97.09	104.55	95.01
L2	98.54	93.32	101.45	89.10
L3	103.99	103.80	115.81	111.24
Method precision [RSD %]				
L1	6.37	2.73	1.99	4.60
L2	1.95	9.78	1.43	4.42
L3	7.75	7.78	1.50	2.90
Matrix effect [%]				
L1	89.30	101.38	98.39	94.69
L2	94.30	99.00	95.58	93.29
L3	98.88	100.55	101.64	105.63
LOD [nM]	0.33	0.15	0.03	0.15
LOQ [nM]	0.66	0.49	0.08	0.18

p-OH-AT: p-hydroxyatorvastatin, o-OH-AT: o-hydroxyatorvastatin, AT: atorvastatin, ATL: atorvastatin lactone, L1, L2, L3: concentration 100, 10 and 5 nM, LOD, LOQ: values for the matrix calibration curve.

atorvastatin metabolites. The reason might be the differences in C18 sorbents among individual producers, who consequently offer SPE sorbent of various properties (specific surface area, average pore diameter, surface pH, density of C18 ligand bonding, metal content etc.).

As MEPS and SPE extractions principles are similar the same washing and elution solvents were used [29]. Various elution and sample volumes were tested and finally the washing step was optimized in order to obtain clean extracts. Serum sample MEPS extraction procedure was optimized as following:

An internal standard (25 μl) was added to 225 μl of serum samples containing statins. 50 μl of this sample was drawn by means of syringe through the MEPS sorbent previously activated with 300 μl of acetonitrile and conditioned with 300 μl of 0.1 M ammonium acetate pH 4.5. MEPS sorbent with retained sample was washed with 200 μl of 0.1 M ammonium acetate pH 4.5 and 100 μl of mixture acetonitrile:0.01 M ammonium acetate pH 4.5 (15:85, v:v) and subsequently the analytes were eluted with 100 μl of mixture acetonitrile:0.1 M ammonium acetate pH 4.5 (95:5, v:v). Finally, the eluate was filtered via 0.2 μm PTFE filter and sample was injected onto UHPLC system.

During the development of off-line manual MEPS extraction procedure several problems arose. Although MEPS extraction should enable use of very small volume of elution solvents (until 20–50 μl) and samples (until 10 μl), this is suitable only for the automated MEPS set-up, where the MEPS syringe is connected on-line to an LC. Therefore the most of published articles presented a use of fully automated MEPS system connected on-line to an LC. However, this does not apply for the manual arrangement of MEPS. The main disadvantages of manual arrangement of MEPS are following: non-availability of vial inserts smaller than 100 μl, dependence of analyte recovery on the continuous speed of the movement of the plunger and inaccurate manual manipulation with volumes lower than 50 μl through the 100 μl syringe. In order to solve all above mentioned arising problems, the volumes of sample and solvents must be greater than 50 μl and continuous speed of the movement of plunger must be maintained during the extraction. Both features are very critical for off-line manual MEPS arrangement and are not in agreement with the on-line MEPS procedure.

3.2. Validation of UHPLC–MS/MS method using MEPS as sample preparation

A development of UHPLC–MS/MS method used for the determination of atorvastatin and its metabolites in biological samples was described previously [29], including method optimization, SST (system suitability test) measurements and method validation. Validation parameters including linearity, method recovery, accuracy, precision and limits of detection and quantification were evaluated newly because another sample preparation technique employing MEPS was introduced. The results could be seen in Table 1. Method linearity was measured in the calibration range 0.1–100 nM for standard calibration curves (2) and 0.5–100 nM for matrix calibration curves (1). An internal standard (atorvastatin-D5) was used for quantitation. The response was linear in tested concentration range for all analytes ($r^2 > 0.9990$), therefore the calibration curves could be used for quantitative purposes. Method accuracy expressed as recovery was established at the three concentration levels of calibration curve – at high (100 nM), medium (10 nM) and low (5 nN). Method accuracy ranged from 89 to 116%. Method precision was measured using spiked blank serum treated by MEPS at three different concentration levels in three replicates and finally RSD (%) was calculated. Intra-day precision for atorvastatin and atorvastatin lactone was lower than 5% and for hydroxy metabolites of atorvastatin it was lower than 10%. Interday precision values were also lower than 10% for each measured analyte (data not presented). The matrix effect was evaluated as the comparison of standard solution and spiked blank serum sample, which was first treated by MEPS and subsequently spiked by standard solution. Matrix effect values ranged from 93 to 105% therefore no significant matrix effects were observed and the method was found to be selective using UHPLC–MS/MS in connection with MEPS sample preparation step. Limits of detection were expressed as S/N = 3 and limit quantification as S/N = 10. LOD and LOQ for all analytes in real matrix could be seen in Table 1 and their values correspond to the values of LOD and LOQ in standard solution described in previously published article [29].

3.3. Application to real samples

Newly developed UHPLC–MS/MS method with MEPS as the sample preparation for the determination of atorvastatin and its metabolites was applied to the serum samples of patients with familiar hypercholesterolemia treated by atorvastatin and simultaneously by extracorporeal elimination procedures (LDL-apheresis, rheopheresis).

Both MEPS and SPE sample preparation methods were used for the treatment of the same real samples of serum, which were split before the procedure. The results of samples treated by MEPS and SPE procedures were compared by means of Student *t*-test (Table 2). The differences of the concentrations of measured

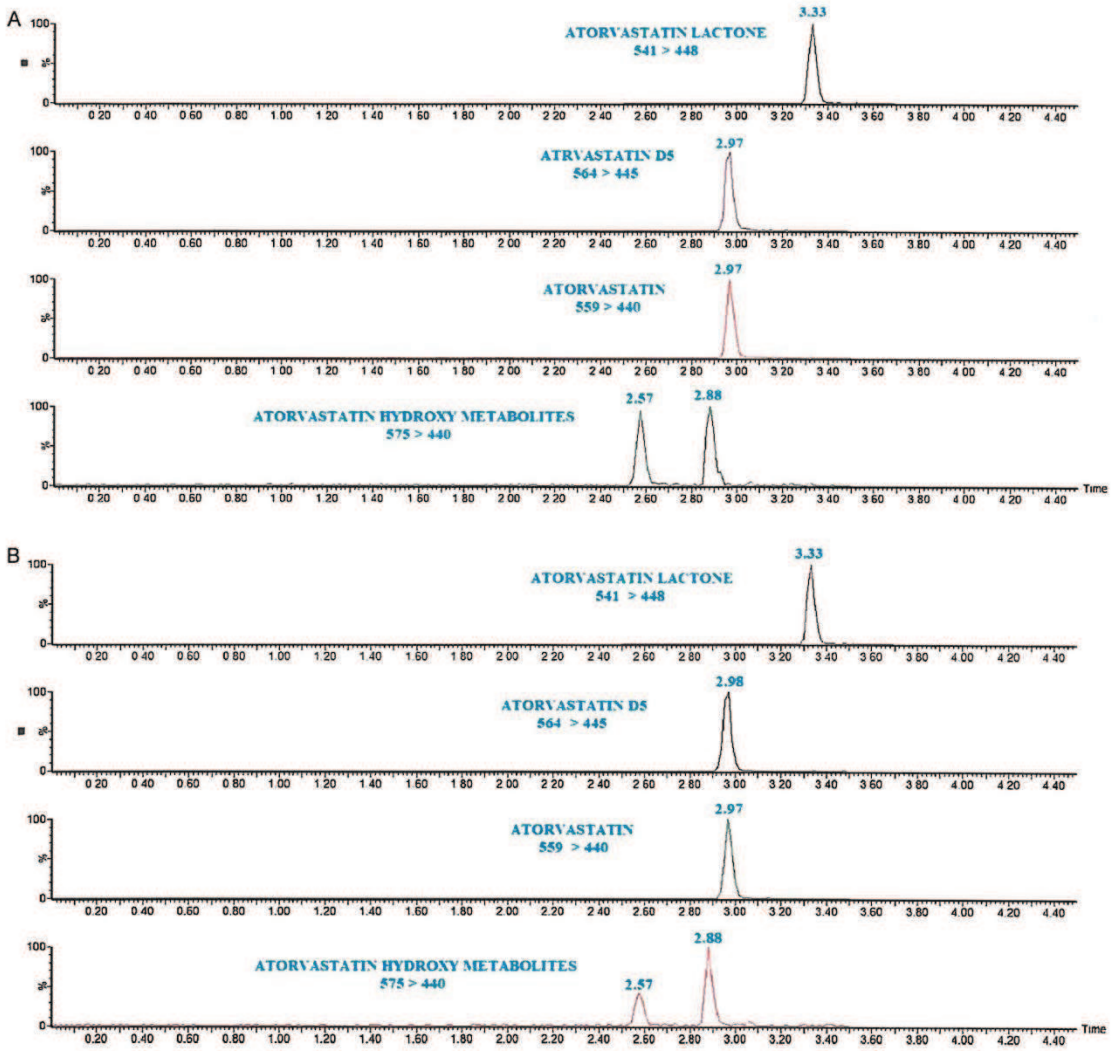

Fig. 3. UHPLC–MS/MS chromatogram of standard mixture of measured analytes (calibration level 5×10^{-7} M) (A) and of serum samples – patient treated by atorvastatin (B).

analytes obtained by MEPS and by SPE were statistically non-significant.

Two blood samples (before and after extracorporeal elimination procedure) were obtained from one patient. In all samples of serum atorvastatin, atorvastatin lactone and its two metabolites were found (Fig. 3). Total losses of statins during the procedure were calculated. Such monitoring will enable individual adjustment of dosage scheme for each patient.

3.4. A comparison of SPE and MEPS extraction procedure

A traditional SPE was directly compared with newly developed MEPS procedure. The patient samples were split and were treated by both formerly developed SPE method [29] and newly developed MEPS. MEPS extraction procedure was found to be fast and simple method with good recovery using very small volume of sample, which is regardful to the patient and using a small volume of solvent, which is environmentally friendly approach. During MEPS extraction the evaporation of extract is not effected. Therefore it was found to be more suitable for the routine analysis of a large number of biological samples.

Moreover, it is possible to use MEPS cartridge repeatedly. We proved about 30 times re-use without any lose of extraction efficiency or sample carry-over (Fig. 4). On the other hand, one of the disadvantages of MEPS technique might be an unavailability of a great variability of sorbent chemistries, which are available for traditional SPE. Another great disadvantage of MEPS is a strong dependence of analyte recovery on continual movement of plunger and on rate of sample passing through the sorbent. This is a critical feature of manual manipulation, which requires skilled operators.

However, much higher speed of MEPS extraction procedure is overwhelming advantage in routine analyses in clinical laboratories. The comparison of data from MEPS and SPE extraction is displayed in Table 3 in detail.

Table 2
Atorvastatin and its metabolites in serum – comparison of results obtained using MEPS and SPE method.

Compounds [nM]	p-OH AT	o-OH AT	AT	ATL
Patient no. 1				
Before EEP				
MEPS	3.29	5.71	14.04	9.12
SPE	3.35	6.43	14.07	9.45
After EEP				
MEPS	1.23	2.32	4.30	3.09
SPE	1.38	1.91	4.63	4.03
Patient no. 2				
Before EEP				
MEPS	1.53	1.59	2.80	2.67
SPE	1.80	1.55	2.78	2.47
After EEP				
MEPS	1.25	0.93	1.71	1.85
SPE	1.46	1.17	1.67	1.70
For the differences of MEPS and SPE				
Average	0.1725	0.3375	0.0763	0.2288
Standard deviation	0.2704	0.5683	0.2911	0.4971
t-Values	0.1609	0.1069	0.4412	0.3627
Statistical significance	Non-significant	Non-significant	Non-significant	Non-significant

p-OH-AT: p-hydroxyatorvastatin, o-OH-AT: o-hydroxyatorvastatin, AT: atorvastatin, ATL: atorvastatin lactone, LCL: lower confidence limit, UCL: upper confidence limit, EEP: extracorporeal elimination procedure, MEPS: microextraction by packed sorbent, SPE: solid phase extraction.

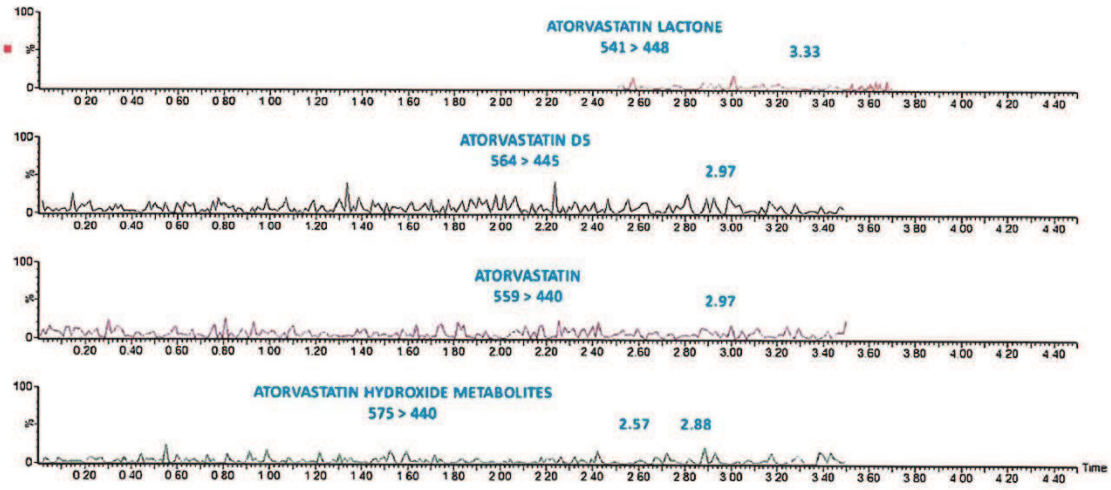

Fig. 4. Chromatogram of blank serum after the MEPS extraction: blank was prepared by MEPS procedure after the sample extractions. No carry-over effect was observed.

Table 3
Comparison of MEPS and SPE extraction.

	SPE	MEPS
Total time of extraction	25 min	7 min
Type of sorbent	Silica based C18 (100 mg)	Silica based C8 (1–2 mg)
Sample volume	500 μl	50 μl
Solvent volume – conditioning	2000 μl	600 μl
Solvent volume – washing	6000 μl	600 μl
Elution volume	1000 μl	100 μl
Reuse of cartridge	3–5×	30×
Evaporation	Yes	No

4. Conclusions

A new MEPS sample preparation method for the determination of atorvastatin and its metabolites was developed. MEPS extraction procedure is fast and simple sample preparation method using small volume of sample, washing and elution solvent therefore it is regardful to the patients and environmentally friendly. Because MEPS is less time-consuming, simpler and more regardful than formerly developed SPE method, this technique is predicted to be more suitable for the routine analyses of biological samples in clinical laboratories.

Using lower volume than 50 μl is not suitable for off-line arrangement of MEPS procedure compared to on-line MEPS arrangement which can use volumes even less than 10 μl. The effectiveness of MEPS extraction is highly dependent on the continuous speed of the movement of the plunger. From these reasons on-line connection of MEPS and chromatographic system is more convenient for routine analyses of large number of biological samples. However in case of precise manipulation with the syringe during sampling, using higher volume than 50 μl and the maintenance of continual plunger movement the manual MEPS method demonstrate good recovery, reproducibility within much shorter time needed for extraction (about 7 min). At these conditions manual MEPS method is further more suitable for the routine preparation of biological samples than SPE extraction.

A new MEPS method was validated with good results of linearity precision and accuracy. Analytes could be quantified at nM concen-

trations with typical LOQ 0.08–0.66 nM. MEPS method was used as the sample preparation method for the determination of atorvastatin and its metabolites in serum of two patients with familiar hypercholesterolemia treated by atorvastatin together with extracorporeal elimination procedure and it is planned to be applied to a large number of samples in routine clinical laboratory. MEPS is fast and simple method enabling determination of more serum samples within the same period of time compared to SPE or LLE and using less organic solvent and also less amount of sample thereby lower stress for the patients. For these reasons formerly developed SPE sample preparation method for determination of atorvastatin and its metabolites was replaced by MEPS sample preparation method.

Acknowledgements

The authors gratefully acknowledge the financial support of research projects MSM0021620822 and SVV/2011/263002.

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PŘÍLOHA V

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Determination of pravastatin and pravastatin lactone in rat plasma and urine using UHPLC- MS/MS and microextraction by packed sorbent as the sample preparation step

Talanta, podáno k publikaci.

DETERMINATION OF PRAVASTATIN AND PRAVASTATIN LACTONE IN RAT PLASMA AND URINE USING UHPLC-MS/MS AND MICROEXTRACTION BY PACKED SORBENT

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Abstract

A simple and reproducible method for the determination of pravastatin and pravastatin lactone in rat plasma and urine by means ultra high performance liquid chromatography- tandem mass spectrometry (UHPLC- MS/MS) using deuterium labeled internal standards for quantification is reported. Separation of analytes was performed on BEH C_{18} analytical column (50 x 2.1 mm, 1.7 μm), using gradient elution by mobile phase consisting of acetonitrile and 1mM ammonium acetate at pH 4.0. Run time was 2 minutes. Quantification of analytes was performed using the SRM (selective reaction monitoring) experiment in ESI negative ion mode for pravastatin and in ESI positive ion mode for pravastatin lactone. Sample treatment consisted of a protein precipitation by ACN and microextraction by packed sorbent (MEPS) for rat plasma. Simple MEPS procedure was sufficient for rat urine. MEPS was implemented using the C8 sorbent inserted into a microvolume syringe, eVol hand-held automated analytical syringe and a small volume of sample (50μl). The analytes were eluted by 100 μl of the mixture of acetonitrile: 0.01 M ammonium acetate pH 4.5 (90:10, v:v). The method was validated and demonstrated good linearity in range 5- 500 nmol/l ($r^2 > 0.9990$) for plasma and urine samples. Method recovery was ranged within 97 - 109 % for plasma samples and 92- 101% for the urine samples. Intra-day precision expressed as the % of RSD was lower than 7% for the plasma samples and lower than 8% for the urine samples. The method was validated with sensitivity reaching LOD 1.5 nmol/l and LOQ 5 nmol/l in plasma and urine samples. The method was applied for the measurement of pharmacokinetic plots of pravastatin and pravastatin lactone in rat plasma and urine samples.

Keywords: pravastatin, pravastatin lactone, UHPLC- MS/MS; MEPS; rat plasma and urine

1. Introduction

Statins represent the most efficient drugs for the treatment of the severe forms of hypercholesterolemia and reduce the morbidity and mortality associated with cardiovascular diseases [1] [2]. Pravastatin is a comparative hydrophilic liver-specific inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG–CoA) reductase, a key enzyme of biosynthesis of cholesterol [3][4][5]. This agent lowers the total plasma cholesterol and low-density lipoprotein concentrations. Pravastatin has a pleiotropic and antioxidant effect too [2]. Pravastatin as well as the other statines exists in two forms, lactone and open-ring hydroxy acid form [6]. Hydroxy acid form is the active drug with cholesterol-lowering effect while the lactone form is inactive (prodrug). Pravastatin is administered as the sodium salt of the active hydroxy acid form [7]. It is absorbed from gastrointestinal tract and undergoes extensive first- pass metabolism in liver [8]. Approximately 50% of pravastatin are bound to plasma protein and extracted mainly in the feces via bile and in a smaller portion in urine [9].

A number of statins have been introduced into clinical practice. The first substance was lovastatin followed by atorvastatin and simvastatin. Many pharmacokinetic studies comparing different HMG-CoA reductase inhibitors have been performed. Pravastatin was characterized as one of the most efficient for its greater hydrophilicity [4] and the unique pharmacokinetic properties compared the other HMG-CoA reductase inhibitors. The pharmacokinetics of pravastatin is characterized by low absorption and bioavailability (about 20% of the total doses after oral administration), a fast absorption rate limiting elimination and a relatively low protein binding [10]. The peak plasma concentration of pravastatin after the oral administration is attained within 1- 1.5 h. Plasma elimination half- life ranges from 1.3- 2.6 h [11]. Several high performance liquid chromatography methods with UV or MS/MS detection have been developed for the determination of pravastatin in human plasma or serum [3] [4][5] [8] [12][13] [14] [15]. The determination of pravastatin in urine has not been described so far. Only one method using UV, which is not selective and sensitive enough towards biological samples, was published [12]. Most of LC-MS/MS assays currently available focus only on pravastatin, which substantially decreases method value. Pravastatin is one of the drugs which is subjected to interconversion between lactone and open-ring hydroxy acid. For this reason the analysis of lactone and acid forms and their chromatographic separation is the key for their accurate quantification [6]. The important step for the minimized interconversion is also maintaining of pH between 4 and 5 [1]. Just two reported methods enabled simultaneous quantification of pravastatin and pravastatin lactone in biological fluids. Both methods used the electrospray ionization in positive mode and analytical column with particle size > 4µm. For the quatification of analytes they employed internal standard method bud only one of them using the deuterium labeled standard. Because the separation of pravastatin and pravastatin lactone is necessary, using internal standard for each analytes individually is preferable for elimination of matrix effects and accuracy quantification using LC- MS [5] [10].

All reported LC-MS/MS methods used protein precipitation or solid phase extraction (SPE) on the reverse phase sorbent as sample preparation step. An overview of analytical methods for determination of pravastatin and its related substances in biological samples is shown in Table 1. In clinical laboratories the main requirements for sample preparation are rapidity, simplicity and miniaturization, especially when using small volume of samples and organic solvents, while maintaining sufficient selectivity, precision and accuracy. MEPS as miniaturized SPE is logical extension of SPE for the analysis of biological fluids [16]. It can handle small sample volumes (10 µl of plasma, urine or water) as well as relatively large volumes (1000 µl). Small sample volumes allow for analysis of not only human but also the animal samples for example rat plasma whose volume availability is very limited [17][18]. Sample preparation takes place on the packed bed. Many sorbent material for example silica- based (C2, C8, C18), strong cation exchanger (SCX), restricted access material (RAM), HILIC, molecularly imprinted polymers (MIPs) are available [17]. MEPS enable on- line connection to some of GC, HPLC or UHPLC system [16][17][19]. The disadvantages of manual approach of MEPS include irrepeatable the speed of plunger movement and non-accurate manual injection of very small volumes of sample (< 50µl) [1]. A compromise between manual and automatic approaches is usage of the eVol hand- held automated analytical syringe which removes the influence of operator on the above mentioned disadvantages of

manual attitude. Unlike SPE sorbent MEPS can be reused several times, more than 100 extractions without any loss in its performance for water, urine and centrifuged plasma samples and 30- 40 times for non-centrifuged plasma [16] [19]. MEPS technique has been used to extract a wide range of analytes from biological samples such as urine, plasma and serum [1][20][21][22].

The aim of this work was to develop and validate a fast and simple UHPLC-MS/MS method for the determination of pravastatin and pravastatin lactone in rat plasma and urine using MEPS and PP as sample preparation methods. The combination of two sample preparation techniques enabled use of small sample volumes, reuse of MEPS sorbent more than 100 times for the precipitated plasma and urine samples. Simultaneously it provided sufficiently clean extracts with good recovery of analytes. The suitability of the method for the detection of the drug and its lactone metabolite in samples with limited volume availability was verified using rat plasma and urine obtained after intravenous administration of the drug. The concentration-time profiles were consequently measured. Novelty of method consists in simultaneous quantification of pravastatin and pravastatin lactone using two deuterium labeled standards for both analytes individually. This UHPLC-MS/MS method was applied not only to the real rat plasma but also to urine samples. MEPS was employed as fast and effective sample preparation technique use of small sample volumes.

2. Experimental

2.1 Chemicals and reagents

Working standards of pravastatin, pravastatin lactone, pravastatin deuterium labeled (D3) and pravastatin lactone deuterium labeled (D3) were purchased from Toronto Research Chemicals (Ontario, Canada).

The acetic acid, LC- MS grade (>99%), the ammonium hydroxide, LC- MS grade (>25%) and the acetonitrile, LC-MS grade, were purchased from Sigma Aldrich. HPLC grade water was prepared by Milli-Q reverse osmosis Millipore (Bedford, MA, USA) and it meets European Pharmacopoeia requirements.

2.2 *Chromatography and Mass Spectrometry*

UHPLC system Acquity UPLC (Waters, Prague, Czech Republic) was used for the purpose of this study. It consisted of ACQ-binary solvent manager and ACQ-sample manager. All UHPLC-MS/MS analyses were performed on BEH C_{18} analytical column (50 x 2.1 mm, 1.7 μm, Waters, Prague, Czech Republic) based on Bridged Ethyl Hybrid (BEH) particles. Mobile phase was composed of acetonitrile and 1 mM ammonium acetate pH 4.0 using gradient elution with initial mobile phase composition acetonitrile: 1mM ammonium acetate pH 4.0 (05:95). Thereafter the concentration was changed within 1.0 minutes to 70% of acetonitrile and subsequently to 95% of acetonitrile within 2.00 minutes. Mobile phase flow rate was 0.2 ml/min. The analytical column was kept at 35°C by column oven. The solutions were stored in the autosampler at 4 °C.

The MS/MS triple quadrupole system was used in this study. Quattro Micro (Micromass, Manchester, GB) was equipped with a multi- mode ionization source (ESCI). Ion source was set-up in ESI polarity-switching mode as follows: capillary voltage: 3000 V, ion source temperature: 130 °C, extractor: 3.0 V, RF lens: 1.0 V. The desolvation gas was nitrogen at flow 550 l/h and at the temperature 450 °C. Nitrogen was used also as a cone gas (50 l/h). Cone voltage (CV) was set up individually for each analyte (Table 2). Quantification of all analytes was performed using SRM (selected reaction monitoring) experiment. Product ions were chosen according to the fragmentation pathways in Product ion scan mode (Fig. 2). Argon was used as a collision gas and collision energy (CE) was optimized for each analyte individually (Table 2). The MassLynx 4.1 Data System was used for MS system control and data gathering. QuanLynx software was used for data processing and quantitation - regression analysis of standard, matrix calibration curves and calculation of concentrations.

2.3 Preparation of standard solutions

The stock solutions of standards were prepared by dissolving of the amount corresponding to 1.0 mmol/l of appropriate working standard into 1.0 ml of dissolution media, due to significant differences in solubility. The stock solutions of pravastatin lactone and pravastatin lactone D3 were prepared in pure acetonitrile. The stock solutions of pravastatin and pravastatin D3 were prepared in mixture acetonitrile: 1mM ammonium acetate pH 4.0 (90:10, v:v). Working solutions of all analytes were further diluted by mixture of ACN: 1 mM ammonium acetate pH 4.0, 05:95 (initial mobile phase) to achieve individual points of calibration curve in the range 5 - 500 nmol/l, using five calibration points (500, 100, 50, 10, 5.0 nmol/l). Working solution of ISs at concentration of 200 nmol/l was prepared by diluting with 0.5 M ammonium acetate pH 3.0 for the stability reason (see section 2.4). Stock solutions of all tested compounds in mixture of ACN: 1 mM ammonium acetate pH 4.0, (90:10) pH 4.0 and solutions of analytes in 0.5 M ammonium acetate pH 3.0 were tested for stability reason at 20, 4 and -18 °C Fresh stock solutions were prepared.

2.4 Sample preparation

Protein precipitation (PP) and MEPS were used as the sample preparation techniques. MEPS was utilized for the cleaning up of urine samples and the combination of PP and MEPS for the plasma samples. The eVol hand-held automated analytical syringe was used for MEPS extraction. 25 µl of the working solution of ISs and 25 µl of 0.5 M ammonium acetate pH 3.0 were added to 50 µl of plasma or

urine samples containing the analytes. Addition of this buffer was crucial for the stability reason, to adjust and keep pH of sample between 4.0– 5.0. Such pre-treated sample solutions were applied to the PP or MEPS extraction. The adjustment of pH is crutial, because sample without addition of buffer has pH about 7, which facilitates the conversion of lactone to acid form.

Plasma and urine samples were stored at -80 °C and after the thaw cycle they were processed immediately by MEPS procedure and analyzed by UHPLC-MS/MS.

2.4.1. Sample preparation for rat plasma

The first step was PP with ACN as the deproteinization agent. 100 µl of ACN was added to 100 µl pre-treated sample solution. This mixture was shaken and incubated for 15 minutes. The precipitated sample was centrifuged at 4200 RPM for 10 minutes. The supernatant was withdrawn and diluted by 1.80 ml of 0.01 M ammonium acetate buffer pH 4.5. The C8 MEPS sorbents packed in bin, which is inserted into a needle assembly connected to 500 µl syringe (SGE Analytical science, Germany) was chosen. The whole volume of supernatant was aspirated through MEPS previously activated three times with 250 µl of acetonitrile and conditioned three times with 250 µl of 0.01M ammonium acetate buffer pH 4.5. The sorbent was washed two times with 250 µl of 0.01M ammonium acetate buffer pH 4.5 and 250 µl of mixture acetonitrile: 0.01M ammonium acetate buffer pH 4.5 (05:95, v:v). Analytes were eluted with 100 µl of mixture of acetonitrile: 0.01M ammonium acetate buffer pH 4.5 (90:10, v: v). The eluate was filtrated via PTFE microfilter (4mm x 0.20 µm) and the sample was injected onto UHPLC system.

2.4.2. Sample preparation for rat urine

Only MEPS without PP was used for the cleaning up of urine samples. The type of sorbent and solvents and the whole procedure of MEPS was identical as in 2.4.1.

2.5 Method validation

SST (system suitability test) is an important part of method validation, details of which are usually given Pharmacopoeias. The SST was performed under the optimized chromatographic conditions. In LC- MS only repeatability of retention times and peak areas are checked.

Newly developed MEPS-UHPLC-MS/MS was validated in terms of linearity, selectivity, sensitivity (limits of detection and quantitation), method accuracy, precision and matrix effects according to the requirements of ICH (International Conference on Harmonization) [23]. For the determination of linearity, two calibration curves of all analytes were prepared:(1) matrix calibration curve using blank rat plasma or urine samples, that were spiked and then treated by MEPS procedure in the concentration range 5 – 500 nmol/l and (1) standard calibration curve, where stock standard solutions were diluted by mobile phase in the concentration range 1 – 500 nmol/l.

For method precision, spiked blank rat plasma and urine samples treated by MEPS at three different concentration levels were measured in three replicates in order to calculate % of RSD, which describes the closeness of agreement between series of measurements.

Method accuracy was described as the recovery experiment. Recovery was determined via a comparison of the response of plasma and urine samples spiked prior to MEPS extraction with the response of rat blank plasma and urine samples that were first treated by MEPS procedure and then spiked with the analytes. It was complemented at three concentration levels in three replicates to establish the closeness of agreement between the true and measured value as it corresponds to ICH requirements [23]. QC samples were prepared at the same concentrations as were the concentration levels prepared for precision and accuracy experiments. Matrix effects were evaluated using blank rat plasma and urine samples, which were first treated by MEPS procedure and then spiked by standard solution at three concentration levels within the calibration range. The results were compared with the measurement of standard calibration curves and matrix effects were calculated.

Limits of detection and quantification were established based on signal-to-noise (S/N) ratio approach. Limit of detection was expressed as S/N=3, limit of quantification was expressed as S/N = 10.

The stability of samples in standard stock solutions was evaluated at 20, 4 and -18 °C in a short-term and long-term period.

3. Results and discussion

3.1 Ultra high performance liquid chromatography tandem mass spectrometry detection

UHPLC was used for separation of pravastatin and pravastatin lactone. Incorporation of chromatographic separation of two compounds is nessesary because of the potential in-source interconversion between a lactone and its corresponding hydroxy acid form [6]. A selection of analytical column Acquity BEH C18 (50 x 2.1mm, 1.7 µm) and development of method was carried out with the regard to physical- chemical properties, the stability of the analytes and mass spectrometry detection, which is limited in terms of solvents that could be used. The crucial aspect was maintaining the pH in the range 4 to 5 [1]. Ammonium acetate and ammonium formate at different concentration and pH were tested in the study. In compromise of response of mass spectrometer, analysis time and resolution, acetonitrile in combination with 1mM ammonium acetate pH 4.0 was finally chosen as the mobile phase. Flow rate was 0.20 ml/min. The gradient elution was applied. Firstly the composition of the mobile phase was optimized on the standard solution. The initial ratio of mobile phase was 70:30 (1mM AmAc pH 4.0: ACN). When these conditions were applied to the matrix samples, very intensive matrix enhancement was observed because analytes were not enough separated from matrix components. AT this point, the profile of gradient elution with different initial conditions was tested. 95% of 1mM ammonium acetate pH 4.0 was selected as a suitable initial composition of mobile phase. Time of equilibration have been prolonged therefore the total time increased from two to six minutes.

A MS/MS triple quadrupole system with electrospray ionization was used for quantification. Both positive and negative ionization modes were examined. The precursor and product ion spectra of pravastatin and pravastatin lactone could be seen in Fig. 1. In full scan spectra pravastatin provided a precursor ion $[M-H]^-$ in negative ion mode and $[M+H]^+$ in positive ion mode. In positive ion mode pravastatin lactone offered the protonated molecule $[M+H]^+$ and an ammonium adduct $[M+NH_4]^+$ together with the other adducts inconvenient for quantification, such as $[M+Na]^+$ and $[M+K]^+$. In negative ion mode pravastatin lactone provided acetate adduct $[M+CH_3COO]^-$. The best response was obtained in ESI negative ion mode for the pravastatin $[M-H]^-$ and in ESI positive ion mode for of pravastatin lactone $[M+H]^+$, which offered more reproducible results during quantification using SRM experiment. The ammonium adduct of pravastatin lactone $[M+NH_4]^+$offered the better S/N ratio than protonated molecule, however provided no repeatability of peak area and linear response. Therefore only a precursor ion $[M+H]^+$ for pravastatin lactone and $[M-H]^-$ for pravastatin were further used for the quantification and fine tuning of all parameters of mass spectometer (see section 2.2, Table 2).

Two specific transitions were optimized for each molecule to increase selectivity and reliability of the method. The first transition was used for the quantification of analytes, the second one was confirmatory.

3.2 Sample preparation

MEPS was selected as a sample preparation method because it is fast and simple miniaturized technique. The main reason was the need for very small volume of sample. Accessible volume of real sample was less than 200 µl. The pH of sample without buffer was about 7, therefore a selection of suitable buffer was the key step for keeping of pH between 4 to 5. To ensure this 50 µl of 0.5 M ammonium acetate pH 3.0 was added to 50 µl of sample. While urine is relatively simple matrix, plasma is more complex containing many contaminants and a protein together. To prolong the life-time of MEPS sorbent PP was used before MEPS extraction of plasma. PP was used mainly to remove proteins from plasma and MEPS to eliminate large amounts of contaminant and salts.

Several types of deproteinization agents and their volume were tested. Using the acids for precipitation was not possible because shifted pH towards low values caused interconversion of pravastatin to pravastatin lactone. Therefore ACN was finally the most convenient deproteinization

agent. The volume of ACN was chosen with regard to compatibility of MEPS extraction and sufficient precipitation. Large volume of ACN in sample caused low recovery after the MEPS extraction, because analytes were not captured into the sorbent. For the deproteinization of 50 µl of plasma sample, 100 µl ACN was chosen as the lowest volume providing sufficient precipitation and good recovery. After the centrifugation the removed supernatant was diluted by1.8 ml of 0.01 M ammonium acetate buffer pH 4.5, thus the volume of sample for aspiration through the MEPS sorbent increased considerably. Nevertheless the growth of whole volume of sample solution was much smaller than for the SPE extraction. Lower volume of sample and solvents are one of the main advantages of MEPS extraction. Selection of suitable buffer was the other key step. 0.01 M ammonium acetate pH 4.5 was used for the dilution of supernatant after PP for the elimination of interconversion.

Off-line semiautomatic MEPS technique using the eVol hand-held automated analytical syringe was employed. Two different MEPS cartridges – C18 and C8 were tested during the optimization of sample preparation procedure. Good results of recovery and precision during the validation of method were obtained using of C8 sorbent. C18 sorbent showed very different recovery values for pravastatin and pravastatin lactone. Adsorption on sorbent and the choice of elution and wash solvents was problematic probably due to greatly different physical- chemical properties. For the pravastatin lactone 100% of ACN was the most suitable as an elution solvent, while the reduction of the % of ACN in elution solvent decreased recovery of pravastatin lactone and increased that of pravastatin. A compromise between recovery of pravastatin and pravastatin lactone was elected and 90% of ACN was used. The same problem concerned the wash solvent, only 5% of ACN could have been used to prevent washing out of pravastatin from sorbent during this cleaning step.

The critical point of MEPS procedure and whole step of sample preparation was interconversion of pravastatin and pravastatin lactone each other. The maintanance of pH between 4 and 5 in all sample solutions, supernatants and extracts was absolutely essentially for prevention of interconversion. Plasma and serum samples were finally prepared according to the procedure described in section 2.4.

3.3 Method validation

The SST and validation parameters included the repeatability of reference standard solution injection, linearity, method recovery, accuracy, precision, matrix effects and limits of detection and quantification. The excellent repeatability of injection was obtained for the retention time (RSD < 0.5%) and for peak area (RSD< 6%). The results of SST and validation could be seen in Table 3.

Method linearity and sensitivity – two types of calibration curves using stable isotopically labeled internal standards were measured, matrix and standard calibration curves. The response was linear in the calibration range 5 – 500 nmol/l for both analytes ($r^2 > 0.9990$), therefore the calibration curves could be used for quantitative purposes. Inter-day reproducibility of calibration curve was estimated as % RSD and was lower than 5%. Limits of detection and quantification for all analytes in real matrix were LOD = 1.5 nmol/l and LOQ = 5 nmol/l respectively.

Method accuracy and precision were established at three concentration levels of calibration curve, at high (500 nmol/l), medium (50 nmol/l) and low (5 nmol/l). Method accuracy expressed as recovery was within the range of 97-109% for the plasma samples and of 92-101% for urine samples. Method precision was measured as intra- and inter-day variability of three different levels expressed as % RSD (see Table 3). Intra-day precision for pravastatin and pravastatin lactone in plasma samples was lower than 8 % and in urine samples it was lower than 7 %. Inter-day precision was lower than 15% for both analytes in plasma and urine samples.

The matrix effects were evaluated as the comparison of standard solution and spiked blank plasma samples, which was first treated by MEPS and subsequently spiked by standard solution. Matrix effect values ranged from 82 - 118% for plasma samples and 84 - 110% for urine samples – (Table 3). No significant matrix effects (<18%) were observed and the method was found to be selectiveenough using UHPLC-MS/MS in connection with MEPS sample preparation step.

Short-term stability of analytes was assessed at pH 3, and at pH 4 using 0.5 M ammonium acetate buffer (dilution media). Long-term stability was assessed in stock solvent (mixture of ACN and 1mM ammonium acetate pH 4.0 (90:10) at 20, 4 and -18°C. After short term storage at 4°C, at pH 3 and pH 4 pravastatin and pravastatin lactone were stable for at least 13 h with concentration deviation lower than 5%. During long-term stability both analytes were stable in stock solution at 20, 4 and -18°C at least 4 weeks with concentration deviation lower than 5%. The results demonstrated that pravastatin had better stability during time at pH 4 than pravastatin lactone which is more stabile at pH 3. These results demonstrated that suitable storage condition in stock solvent o appropriate pH and temperature can significantly increase the stability.

3.4 Application to real samples

Newly developed UHPLC-MS/MS method with MEPS as the sample preparation for the determination of pravastatin and pravastatin lactone was applied to the plasma and urine samples of rats administered intravenously by pravastatin. Sequential blood samples during pharmacokinetic study allowed only minimal volume to be taken. Usage of the microextraction method was therefore essential step for the drug analysis because only about 50 µl of plasma was required, which allowed repeated experiments.

Seven plasma and urine samples (samples at regular intervals) were obtained from one rat. The concentration of pravastatin and pravastatin lactone were determined. This approach enabled detailed characterization of pravastatin and its lactone metabolite concentration-time profiles during initial periods after drug administration including kinetics of their renal excretion (Fig. 3).

Conclusions

A fast, sensitive and selective method was developed for the determination of pravastatin and pravastatin lactone by UHPLC-MS/MS in rat urine and plasma samples. MS/MS detection utilized two SRM transition for each compound to ensure high selectivity and reliability of the method. Deuterium labeled internal standards pravastatin d3 and pravastatin lactone d3 were used for precise and accurate quantification.

A sample pretreatment by means of MEPS was applied for these analytes. Maintaining of pH between 4 and 5 was important to prevent the interconversion of analytes. Therefore ammonium acetate was used for the stabilization of samples during sample preparation and as the part of the mobile phase. The analyses were carried out using small sample volume (50µl) and in a short time period. The MEPS cartridges could be used more than 100 times, with urine and precipitated plasma sample. Using the eVol hand-held automated analytical syringe removed the influence of operator on the speed of plunger movement and manual injection of very small volumes of samples (< 50µl) which is very critical for the repeatability and recovery of analytes. MEPS methods enable fast and simple sample preparation using small volume of sample, washing and elution solvent therefore it is regardful, environmentally friendly and suitable for the samples with limited volume availability such as plasma from small laboratory animals.

The MEPS-UHPLC-MS/MS method for the determination of pravastatin and pravastatin lactone was validated according to the requirements of ICH with good results of linearity, precision, accuracy and matrix effect. Thus the proposed method could be used for the determination of analytes in the rat urine and plasma samples and to evaluate pharmacokinetic profiles.

Acknowledgement

The authors gratefully acknowledge the financial support of research projects ***MSM0021620822*** and ***SVV/2011/263002***.

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Figures:

Fig 1: Precursor (1) and product (2) ion spectra of pravastatin (A) and pravastatin lactone (B)

Fig. 2: UHPLC-MS/MS chromatogram of standard mixture of measured analytes (calibration level 5*10-7 mol/l) (A) and chromatogram of plasma samples (B) –rat treated by pravastatin

Fig. 3: Pharmacokinetic profile of rat pravastatin measured in rat plasma samples (A), urine samples (B)

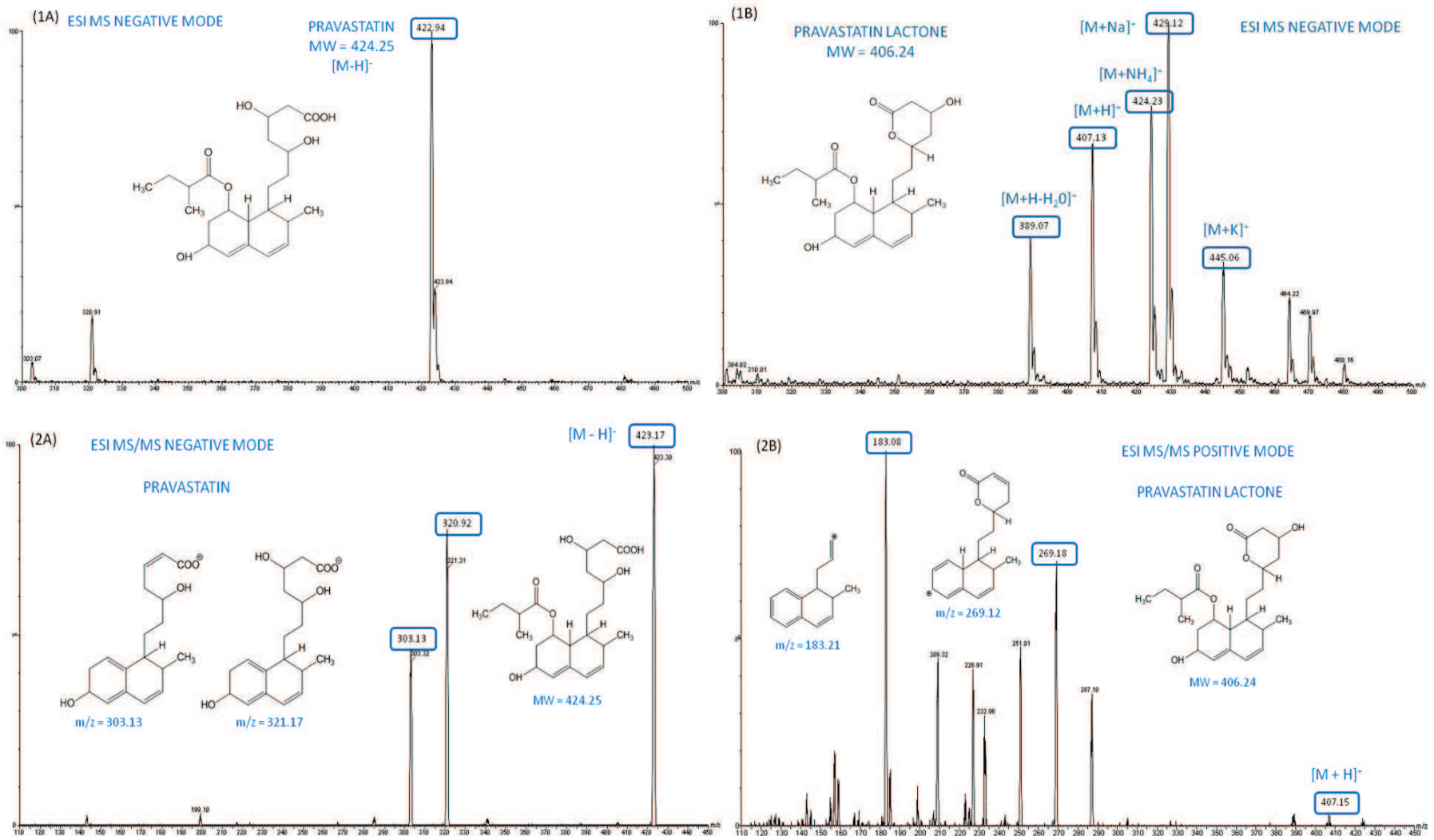
Tables:

Table 1: An overview of LC- MS/MS methods used for the determination of pravastatin in biological samples

Table 2: Optimization of specific transitions for all analytes

Table 3: The results of SST and validation: linearity, method accuracy, precision and matrix effects for urine and plasma samples, linearity and sensitivity test

Fig. 1:


(1A) ESI MS NEGATIVE MODE
PRAVASTATIN
MW = 424.25
[M-H]⁻
422.94
(1B)
PRAVASTATIN LACTONE
MW = 406.24
ESI MS NEGATIVE MODE
[M+Na]⁺
429.12
[M+NH4]⁺
[M+H]⁺
424.23
407.13
[M+H-H2O]⁺
389.07
[M+K]⁺
445.06
(2A)
ESI MS/MS NEGATIVE MODE
PRAVASTATIN
[M - H]⁻
423.17
320.92
303.13
m/z = 303.13
m/z = 321.17
MW = 424.25
(2B)
183.08
ESI MS/MS POSITIVE MODE
PRAVASTATIN LACTONE
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m/z = 183.21
m/z = 269.12
MW = 406.24
[M + H]⁺
407.15

Fig. 2:

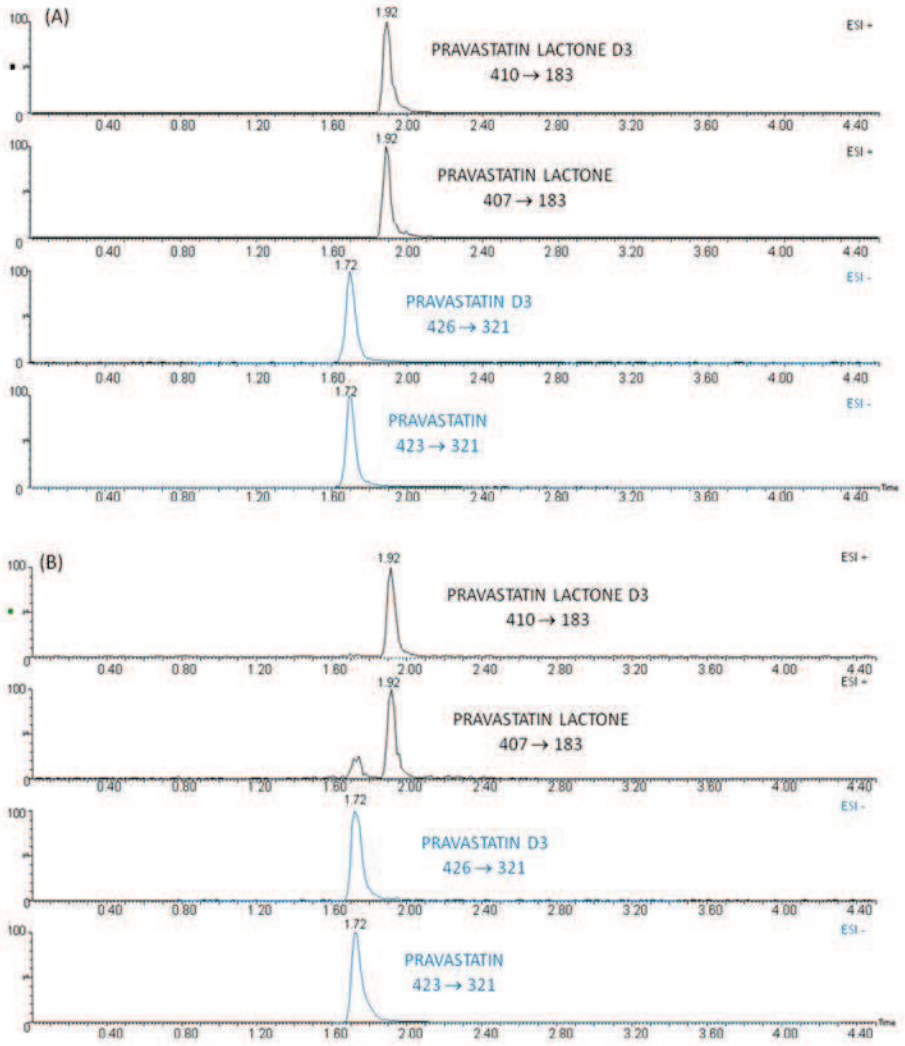
(A)
PRAVASTATIN LACTONE D3
410 → 183
PRAVASTATIN LACTONE
407 → 183
PRAVASTATIN D3
426 → 321
PRAVASTATIN
423 → 321
(B)
PRAVASTATIN LACTONE D3
410 → 183
PRAVASTATIN LACTONE
407 → 183
PRAVASTATIN D3
426 → 321
PRAVASTATIN
423 → 321

Fig. 3:

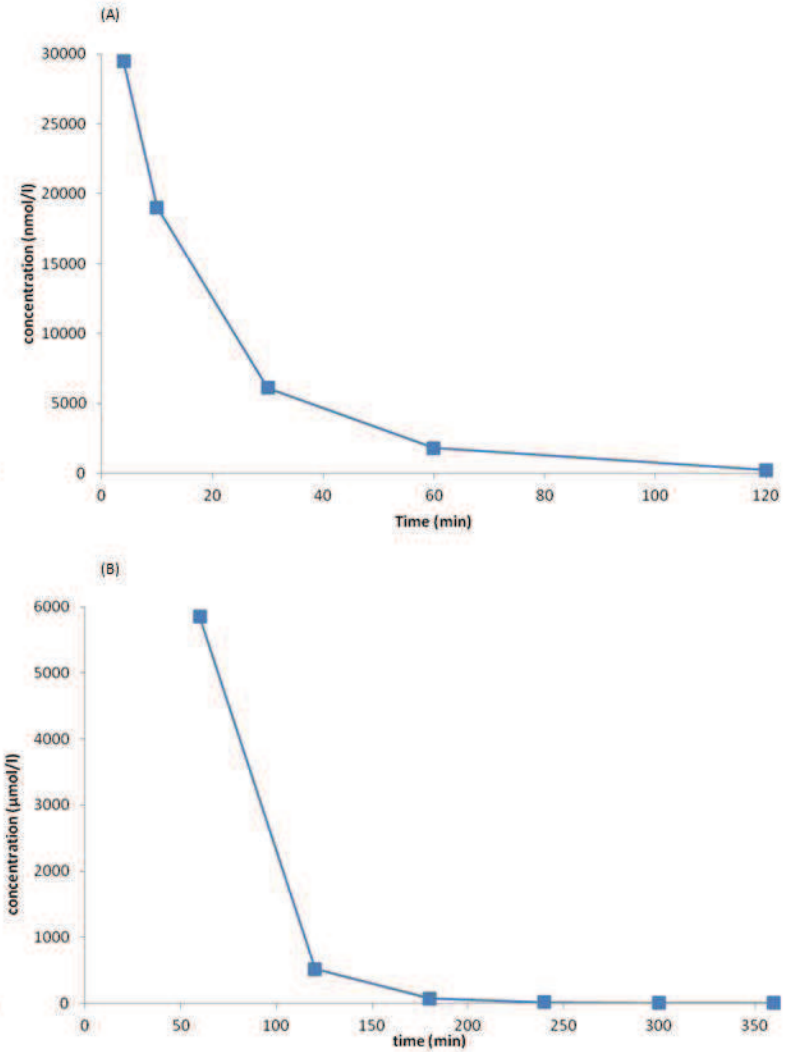
(A)
concentration (nmol/l)
Time (min)
(B)
concentration (µmol/l)
time (min)

Table 1:

Analytes	Internal standard	Sample volume	Sample preparation			Liquid chromatography and mass spectrometry detection				
			Extraction technique	SPE column	Elution solvent	Analytical column	Mobile phase and flow rate	Ionisation mode and analyzer	Matrix	Ref.
Pravastatin	Hydrochlor-thiazide	0.5 ml	SPE	HLB	MeOH	Betabasic C8 (100x 4.6 mm, 5µm)	0.1% Ammonium: ACN, (20:80) 0.5 ml/ min, isocratic elution	ESI negative QQQ	human plasma	**[3]**
Pravastatin	hydroxy-lovastatin	1ml	SPE	C_{18} Bond Elut	-	Zorbax XDB C8 (50x 2.1 mm, 5µm)	ACN: 1 mM AmF pH 3.3 (2:1) 0,25ml/min, isocratic elution	ESI Negative QQQ	human plasma	**[4]**
Pravastatin Pravastatin lactone	Pravastatin- d_5 SQ- 1906- d_5	0.50 ml	SPE	Isolute C8 cartridges	ACN: H_2O (70: 30)	Keystone Betasil ODS (100x 2.1 mm, 5µm)	ACN: MeOH: 5mM AmAc pH 4.5, (30: 30: 40), 0.2 ml/min	ESI Positive QQQ	human serum	**[5]**
Pravastatin 3α- isopravastatin 6α –epipravastatin	Pravastatin- d_3	0.020ml	PP (ACN)	-	-	Atlantis dC18 (2.1x 150 mm, 3 µm)	ACN: 0.1% HCOOC in water, (29: 71), 0.5ml/min isocratic elution	ESI Negative QQQ	mouse plasma tissue homogenate	**[10]**
¨Pravastatin Pravastatin lactone 3-OH metabolites Fenofibric acid	triamcinolone	1.4 ml	Automatic SPE	DECs	MeOH	Synergi Max-RP (150× 2.0mm, 4 µm.)	ACN: MeOH: 5mM AmAc pH 4.5, (30: 30: 40), 0.2 ml/min	ESI Positive QQQ	human plasma	**[11]**
Pravastatin Pitavastatin	Fluvastatin	0.30 ml	SPE	Waters C18 Sep-Pak	MeOH	Luna C18 (50x 2 mm, 3 µm)	ACN: 0,1% HCOOC (90: 10), 0.2 ml/min, isocratic elution	ESI Negative QTrap	human plasma	**[12]**
Pravastatin R-416	R- 122798 (pravastatin analog)	1 ml	SPE	C8vBond Elut	ACN	Inertsil ODS-3 C18 (150 mm×4.6 mm, 5µm)	ACN: H_2O: AmAc: HCOOC: TEA (400: 600: 0.77: 0.2: 0.6, v/v/g/v/v), 1 ml/min	APCI Negative QQQ	human plasma	**[14]**
Pravastatin SQ- 31906	Pravastatin- d3	50 µl	-	-	-	Symmetry C18 (50x3.9mm, 5µm)	ACN: 1 mM FAc (100- 0% HCOOH) 0.5 ml/ min gradient elution	ESI Negative QQQ	rat plasma rat serum	**[15]**

Abbreviations: SPE - solid phase extraction, HLB - Hydrophilic- lipophilic balance, QQQ - triple quadrupole analyzer, DECs - disposable extraction cartridges, AmAc - ammonium acetate, ES I- electrospray ionization, TEA - triethylamin, APC I- atmospheric pressure chemical ionization, AmF - ammonium formate, PP - protein precipitation

Table 2:

	compounds	precursor	precursor type	fragment	cone V	collision E	dwell time	t_R
1	**PV**	423.5	$[M-H]^-$	321.5	35	15	0.1	1.72
				303.5	35	15		
2	**PV-d3**	426.5	$[M-H]^-$	321.5	35	15	0.1	1.72
				303.5	35	15		
3	**PVL**	407.5	$[M+H]^+$	183.5	20	15	0.1	1.92
				269.5	20	10		
4	**PVL-d3**	410.5	$[M+H]^+$	183.5	20	15	0.1	1.92
				269.5	20	10		

PV = pravastatin, PV-d3 = pravastatin deuterium labeled, PVL= pravastatin lactone, PVL-d3 = pravastatin lactone deuterium labeled

Table 3:

		PV	PV d3	PVL	PVL d3
t_R		0.72	0.72	0.92	0.92
LINEARITY (r^2) - standard calibration curve		0.9994	0.9996	0.9998	0.9995
Repeatability of calibration curve (%RSD) – inter- day		4.6	-	2.6	-
Repeatability t_R (%RSD) – intra- day		0.3	0.3	0.4	0.3
Repeatability A (%RSD) – intra- day		3.4	4.1	5.7	4.2
		PLASMA SAMPLES		URINE SAMPLES	
METHOD VALIDATION		PV	PVL	PV	PVL
LINEARITY (r^2) - matrix calibration curve		0.9999	0.9992	0.9994	0.9990
METHOD ACCURACY [%]	L1	103.4	96.7	99.4	99.5
	L2	102.7	109.3	98.2	101.1
	L3	98.3	102.6	92.4	97.1
METHOD PRECISION [RSD %] – intra- day	L1	0.8	4.8	0.7	2.0
	L2	2.4	7.3	1.6	3.6
	L3	6.5	6.6	6.9	4.1
METHOD PRECISION [RSD %] – inter- day	L1	6.6	3.7	0.7	6.3
	L2	5.0	6.0	4.1	12.4
	L3	2.8	15.0	8.9	15.9
MATRIX EFFECT [%]	L1	102.7	108.0	107.0	100,9
	L2	107.6	82.2	109.9	86.3
	L3	118.3	103.5	97.5	83.9
LOD [nmol/l]		1.5	1.5	1.5	1.5
LOQ [nmol/l]		5	5	5	5

PV = pravastatin, , PV-d3 = pravastatin deuterium labeled, PVL= pravastatin lactone, PVL- d3 = pravastatin lactone deuterium labeled, L1, L2, L3 = concentration 500, 50 and 5 nmol/l, LOD, LOQ = values for the matrix calibration curve, L1, L2, L3 = concentration level 1, 2, 3 (500, 50 and 5nmol/l)