Važni aspekti uspostave dvojne spektrometrije masa u uvjetima rutinskoga kliničkog laboratorija

Some important aspects of implementing tandem mass spectrometry in a routine clinical laboratory environment

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Sažetak

Tijekom posljednjih godina kombinacija tekućinske kromatografije visoke učinkovitosti (engl. *high performance liquid chromatography,* HPLC) i dvojne spektrometrije masa (engl. *tandem mass spectrometry*, MS/MS) (kombinacija koja je također poznata kao HPLC-MS/MS), postala je pouzdani analitički postupak. Njena primjena u terapijskom praćenju koncentracije lijeka (engl. *therapeutic drug monitoring*, TDM) pokazala se boljom od uobičajeno primjenjivanih imunokemijskih analiza. Ta je tehnika postala obveznim pomagalom u mnogim kliničkim laboratorijima, posebice u kontekstu praćenja koncentracije imunosupresijskih lijekova gdje se smatra “zlatnim” standardom. Međutim, postavljanje uređaja za HPLC-MS/MS je zahtjevno u smislu validacije analiza te robusnosti mjerenja. U ovom se pregledu daje gruba smjernica kao pomoć u procesu uspostave takvih uređaja u rutinskim kliničkim uvjetima.

Ključne riječi: dvojna spektrometrija masa, ciljana analiza, terapijsko praćenje koncentracije lijeka, rutinska analiza, tekućinska kromatografija visoke učinkovitosti, HPLC-MS/MS

Abstract

During past years, the combination of high performance liquid chromatography (HPLC) and tandem mass spectrometry (MS/MS), also known as HPLC-MS/MS, matured to reliable analytical instrumentation. Its application in therapeutic drug monitoring (TDM) has been shown to be superior to normally applied immunoassays. Especially in the context of immunosuppressant medication level surveillance, this technique has become an indispensable tool in many clinical laboratories and is considered the gold standard. However, setting up a HPLC-MS/MS platform is demanding in terms of assay validation and the robustness of testing. This review is to provide a rough guideline aiding the implementation process of such a platform into a routine clinical environment.

Key words: tandem mass spectrometry, targeted analysis, therapeutic drug monitoring, routine analysis, high performance liquid chromatography, HPLC-MS/MS

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Uvod

Tijekom posljednjih nekoliko godina dvojni maseni spektrometri (engl. *tandem mass spectrometers*, MS/MS, također poznati kao “trostruki kvadropolni” maseni spektrometri, postali su značajni i pouzdani rutinski analitički uređaji. Ti su analizatori, osobito ako su povezani tehnikama pripreme uzorka temeljene na tekućinskoj kromatografiji visoke učinkovitosti (HPLC), omogućili provedbu kvantitativne i kvalitativne analize malih organskih molekula u femtomolskom rasponu, - i to kako ksenobiotika, tako i endogenih metabolita. Zbog toga se njihova uporaba proširila u farmaceutskoj industriji, istraživačkim ustano-

Introduction

Within the last few years tandem mass spectrometers (MS/MS), also known as “triple quadrupole” mass spectrometers, matured significantly to reliable analytical routine instruments. With these detectors, especially if coupled to high performance liquid chromatography- (HPLC) based sample preparation techniques (HPLC-MS/MS), quantitative and qualitative analysis of small organic molecules – xenobiotics as well as endogenous metabolites – became feasible in the femtomole range. Hence they became widely applied in pharmaceutical industry, research-orientated institutions, and – within the last decade – in

vama, a tijekom posljednjeg desetljeća i u kliničkim laboratorijima. Terapijsko praćenje koncentracije lijeka (TDM) zasnovano na HPLC-MS/MS pojavilo se prije manje od dva desetljeća i trenutno je uglavnom usredotočeno na kontrolu koncentracija imunosupresijskih i antiretrovirusnih lijekova te psihofarmaka. Općenito, točno mjerenje analita koji se prate u TDM nužan je preduvjet za uspješno liječenje bolesnika. Većina analiza zasnovanih na reakciji antitijelo-antigen (imunokemijske analize) koje su danas u širokoj uporabi uspješno ne udovoljavaju tom mjerilu zbog nekoliko razloga. Križna reaktivnost komercijaliziranih antitijela s molekulama koje su strukturno slične endogenim metabolitima lijeka ili lijekovima iz iste skupine analita često dovodi do viših vrijednosti ciljanog lijeka. U slučaju praćenja koncentracije imunosupresiva takav nedostatak specifičnosti analize rezultira odstupanjem (razlikom, engl. *bias*) koje u prosjeku dostiže 20-40% (1-3). Nadalje, nepreciznost analize i osjetljivost često jedva da ispunjavaju kliničke potrebe, posebice ako su terapijski rasponi sniženi kod kombiniranih pristupa terapiji. Izvanredan primjer u tom kontekstu su komercijalno dostupni testovi za određivanje koncentracije takrolimusa koji su u međuvremenu postali dobro poznati po slaboj učinkovitosti u bolesnika s 35% nižom koncentracijom hematokrita zbog grubog precjenjivanja niskih koncentracija takrolimusa. Takve previsoke vrijednosti mogu dovesti i do lažno pozitivnih rezultata koji mogu doseći koncentracije unutar terapijskog raspona (4-9). Stoga je vrlo iznenađujuće da se taj test još uvijek uvelike koristi u rutinskim kliničkim laboratorijima, što je vidljivo iz najnovije statistike programa kontrole dijagnostičke učinkovitosti (10). Lani je prosječno 54% svih laboratorija (N = 353) koji su sudjelovali u spomenutom britanskom programu kontrole kvalitete provodilo tu analizu. U usporedbi s time, samo 16% laboratorija je sudjelovalo u skupini mjerenja metodom HPLC koja je uključivala tehnike HPLC-UV, HPLC-MS i HPLC-MS/MS.

Sve to jasno odražava trenutačan položaj HPLC-MS/MS u rutinskom kliničkom TDM. Iako je bolja učinkovitost analiza HPLC-MS/MS (poboljšana preciznost i točnost, bolja specifičnost i povećana osjetljivost koje rezultiraju manjim potrebnim količinama uzorka te nižim granicama kvantifikacije) jasno dokazana tijekom proteklih godina (ta se tehnika smatra zlatnim standardom za TDM imunosupresiva), još uvijek je primjena te tehnologije prisutna u relativno malo laboratorija. Razlozi su sasvim jasni: za spektrometriju masa je poznato da je zahtjevna u smislu potrebnih vještina operatera, a tu je i financijsko opterećenje zbog kupnje samog uređaja. Osim toga, ako se takva tehnika uvodi od početka, istraživača očekuje prilično duga eksperimentalna faza prije moguće provedbe temeljite konačne validacije pretrage. Konačno, za visokoautomatizirane imunokemijske pretrage govori se da imaju kraća vremena obrade uzorka od pretraga HPLC-MS/MS,

clinical laboratories. HPLC-MS/MS based therapeutic drug monitoring (TDM) emerged less than two decades ago and is currently mainly focused on controlling immunosuppressant drug levels, as well as anti-retroviral medications and psycho-pharmaceutics. Generally, accurate measurement of analytes to be monitored is in TDM a necessary prerequisite for successful patient care. At the present time, most of the widely applied antibody-antigen-based assays (immunoassays) do not satisfactorily meet this criterion due to several reasons. Frequently, cross reactivity of commercialized antibodies with molecules structurally similar to endogenous drug metabolites or drugs of the same analyte class leads to an overestimation of the targeted drug. In the case of immunosuppressant TDM, this lack of assay specificity results in a deviation (bias) easily reaching an average of 20 to 40% (1-3). Furthermore, assay imprecision and sensitivity often hardly meet clinical needs, especially if therapeutic ranges are lowered in combined therapy approaches. A remarkable example in this context is the one of commercially available tacrolimus assays which have in the meanwhile become well known to perform insufficiently in patients with a hematocrit level below 35% by grossly overestimating low tacrolimus levels. This can even lead to false positive results that may reach concentrations within the therapeutic range (4-9). It is highly surprising but this assay is still widely applied in routine clinical laboratories, as evident in the latest proficiency scheme statistics (10). Within the last year, an average of 54% of all laboratories (N = 353) participating in the mentioned UK-based testing scheme used this particular assay. In comparison, only 16% of laboratories took part in the HPLC testing group that included HPLC-UV, HPLC-MS and HPLC-MS/MS platforms.

This clearly reflects the current position of HPLC-MS/MS in routine clinical TDM. Although the superior performance of HPLC-MS/MS assays (improved precision and accuracy, higher specificity and increased sensitivity resulting in reduced sample volumes and lower limits of quantification) has been clearly demonstrated over the past years (this technique is considered the gold standard for immunosuppressant TDM), still not too many laboratories have switched to this technology. The reasons are quite clear – mass spectrometry is said to be demanding in terms of operator skills needed and the financial burden of hardware purchase. In addition, if implementing such a technique from the scratch, quite a prolonged experimental stage awaits the experimenter before the thorough final assay validation can be carried out. Finally, highly automated immunoassays are said to have shorter turnaround times than HPLC-MS/MS assays, which does not hold true for the latest generation of high throughput approaches.

In the following chapters, the authors will discuss how the successful transfer of such analytical platforms can be pursued to a routine clinical laboratory environment cha-

što zapravo nije točno za posljednju generaciju s visokim protokom.

Autori u sljedećim poglavljima raspravljaju o načinu kako uspješno provesti prijenos takvih analitičkih platformi u uvjete rutinskoga kliničkog laboratorija koje karakteriziraju visokoautomatizirani analizatori. Pregled se usredotočuje na postavljanje ciljane analize kakva se ostvaruje u TDM. Posebna se pozornost posvećuje TDM imunosupresiva (11-14) jer je to nova temeljna stručna vještina u laboratoriju autora. Glede pristupa probiranju koje se provodi u neonatalnoj dijagnostici, toksikologiji, sudskoj medicini, proteomici i metabolomici, čitatelji se upućuju na odgovarajuću literaturu (15-25).

Potrebe u kliničkim uvjetima

Uvjeti u rutinskome kliničkom laboratoriju razlikuju se od većine industrijskih ili istraživačkih struktura po: (i) mogućim visokim rizicima za bolesnike povezanima s bilo kakvom vrstom pogrešne kvalitativne ili kvantitativne analize; (ii) susretu s visoko složenim i nestandardiziranim matriksima uzoraka kao što su puna krv, plazma i mokraća od oboljelih osoba; (iii) visokim zahtjevima za robusnošću i postojanošću analiza; (iv) provedbi analiza od strane tehničkog osoblja; (v) zahtjevu za kratkim vremenom obrade (unutar pola dana u slučaju TDM). Tijekom posljednjeg desetljeća zakonodavstvo Europske zajednice je pokušalo riješiti tu izazovnu situaciju provedbom *In vitro dijagnostičke direktive* (IVDD) 98/79/EG (26,27) koja je posljednjih godina prenesena i u nacionalne zakone. Posljedica te direktive je da medicinski uređaj koji se koristi za dijagnostiku *in vitro*, uključujući bilo kakvu aparaturu, analizu ili sastavnicu analize (npr. kalibrator), mora biti podvrgnut studiji procjene učinkovitosti kako bi se dokazala prikladnost za navedenu svrhu uređaja ili analize. Cilj te validacije je deklaracija o usklađenosti (Dodatak 3 Direktive) od proizvođača u kojem je nedvosmisleno naznačeno da "uređaji moraju ispunjavati osnovne zahtjeve postavljene u Dodatku I koji se na njih odnosi, uzimajući u obzir planiranu svrhu uređaja koji su u pitanju" (članak 3 Direktive). Ako to vrijedi kao točno, svi "uređaji, osim uređaja za procjenu učinkovitosti, za koje se smatra da ispunjavaju osnovne zahtjeve naznačene u članku 3, moraju imati oznaku CE o usklađenosti kada se stavljaju na tržište" (članak 16 Direktive). Oznaka CE je obvezatan znak koji se koristi u Europskoj zajednici radi označavanja da je određeni proizvod ili skupina proizvoda usklađena sa zdravstvenim i sigurnosnim zahtjevima definiranima srodnim europskim direktivama. Tu oznaku ne treba zamijeniti za znanstvenu skraćenicu koja se koristi za kapilarnu elektroforezu, tj. također CE. U slučaju analitičkog ispitivanja, tj. analize, procjena učinkovitosti treba uključiti najmanje temeljitu validaciju analitičke platforme (npr. prema "Uputama za industriju; validacija bioanalitičke metode" (28) Američke uprave za

racterized by highly automated analyzers. This overview will concentrate on targeted analysis setups as realized in TDM. A special emphasis will be given to immunosuppressant TDM (11-14), since this is a novel core expertise of our laboratory. For screening approaches as realized in neonatal diagnostics, toxicology, forensics, proteomics, and metabolomics, the audience is referred to the literature (15-25).

Needs of the clinical environment

A routine clinical laboratory environment differs from most industrial or research oriented setups by (i) possible high risks for patients associated with any kind of qualitative and quantitative analysis failure, (ii) encountering highly complex and non-standardized sample matrices like whole blood, plasma, and urine from non-healthy individuals, (iii) high demands to assay robustness and stability, (iv) assay execution by technical personnel, (v) demand for short turn around times (within half a day in the case of TDM). Within the last decade, EU legislation tried to meet this challenging situation by implementation of the In Vitro Diagnostic Directive (IVDD) 98/79/EG (26,27) which has been transferred into national legislation within the last years. As a consequence, a medicinal device used for in vitro diagnostics including any instrumentation, assay or assay component (i.e. a calibrator) has to undergo a performance evaluation study to prove the fitness for purpose of the device or assay. This validation aims toward a declaration of conformity (annex 3 of the directive) by the manufacturer indicating unequivocally that the "devices must meet the essential requirements set out in annex I which apply to them, taking account of the intended purpose of the devices concerned" (article 3 of the directive). If this holds true, any "devices, other than devices for performance evaluation, considered to meet the essential requirements referred to in article 3 must bear the CE marking of conformity when they are placed on the market" (article 16 of the directive). The CE marking is a mandatory sign used in the European Community to indicate that a certain product or product group has conformed to health and safety requirements defined by the related European directives. It should not be mistaken with the scientific abbreviation used for capillary electrophoresis. In the case of an analytical assay, a performance evaluation should at least include a thorough validation of the analytical platform (e.g. according to FDA „Guidance for Industry, Bioanalytical Method Validation" (28) and GHTF " Quality Management Systems – Process Validation" (29) guidelines) and risk analysis (e.g. failure modes and effects analysis as published by the GHTF in the "Implementation of Risk Management Principles and Activities Within a Quality Management System" guideline (30)). In summary, the aim of performance evaluation is to

hranu i lijekove (engl. *Food and Drug Administration*, FDA) te smjernicama GHTF (29) "Upravljanje sustavima kvalitete - validacija procesa") te analizu rizika (npr. načini rada kod zatajenja te analiza učinaka, kao što je objavila GHTF u smjernici (30) "Provedba načela upravljanja rizikom i aktivnosti unutar sustava upravljanja kvalitetom"). Ukratko, cilj procjene učinkovitosti je dokazati da uređaj osmišljen za IVD (npr. analitička platforma HPLC-MS/MS) ispunjava zahtjeve Dodatka I Direktive koji je osnova za deklaraciju o usklađenosti. U članku 1.5 Direktive spominje se "povlastica unutar ustanove": "ova se Direktiva ne primjenjuje na uređaje koji su izrađeni i koriste se isključivo unutar iste zdravstvene ustanove te u prostorijama gdje su izrađene ili u neposrednoj blizini i ne prenose se na drugu pravnu osobu. To ne utječe na pravo zemlje članice EZ da aktivnosti podvrgne zahtjevu za odgovorajućom zaštitom", i ona može vrijediti za analitičke platforme sa samo jednom jedinicom (npr. HPLC-MS/MS, analize PCR...) na kojima se analiziraju isključivo lokalni uzorci (npr. unutar jednog medicinskog centra). Međutim, tumačenje izraza "unutar ustanove" razlikuje se unutar EZ zbog nacionalnih zakonskih odredbi. Trenutačno se cijelo to pitanje čini neriješeno tako da se možemo nadati da će buduće rasprave dovesti do razjašnjenih odredbi u vezi s novim primjenama.

Uvod u uređaje za spektrometriju masa

Ionizacija analita

Spektrometrija masa se temelji na stvaranju, odabiru i prijenosu iona u vakuum uređaja za MS. Sljedeći odlomci služe kao kratak pregled kojime se daje uvid u postojeća tehnička rješenja za ionizaciju i odabir analita, kao i za povezivanje MS na kromatografske uređaje.

Tijekom posljednjih desetljeća razvijene su različite metodologije za stvaranje iona te ionski odabir analita, kao i raznolike strategije za kombiniranje tehnika razdvajanja analita sa spektrometrijom masa (Slika 1.). Kako su ionizirani samo ioni, najvažnija reakcija u MS mogla bi biti ona kojom se analiti od interesa, a bez naboja, pretvaraju u ione plinske faze. Najstarija, najčešće korištena i vjerojatno najrazumljivija ionizacijska tehnika je elektronska ionizacija (EI) u kojoj je ispareni analit podvrgnut udaru, tj. "bombardiranju" energijskim elektronima (tipično 70 eV). Kako se analiti u EI trebaju pojaviti kao ispareni, to je svakako ionizacija izbora za analizu GC-MS. Tijekom posljednjih desetljeća razvijeno je nekoliko ionizacijskih tehnika za analize nehlapljivih i termički nepostojanih spojeva, uz ionizaciju elektroraspršenjem (engl. *electrospray ionization*, ESI) i ionizaciju potpomognutu matriksom uz desorpciju laserskim zračenjem (engl. *matrix-assisted laser desorption ionization*, MALDI) kao primarnim metodama izbora. Kod ESI, ioni se stvaraju iz tekućine pri atmosferskom tlaku. Posta-

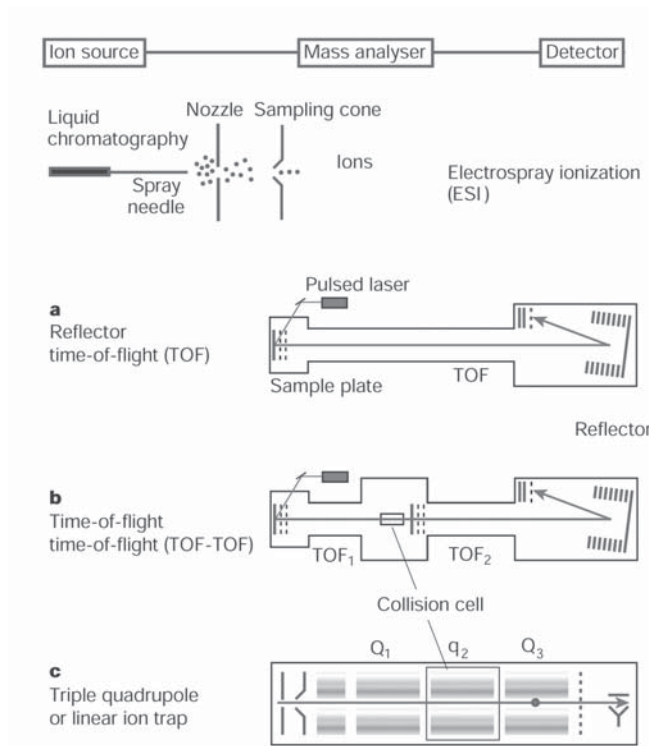
demonstrate that the designed IVD device (e.g. the HPLC-MS/MS platform) meets the requirements of Annex I of the Directive which is the basis for the declaration of conformity. The "in-house privilege" of the Directive, which is addressed in article 1.5 by stating "this Directive shall not apply to devices manufactured and used only within the same health institution and on the premises of their manufacture, or used on premises in the immediate vicinity without having been transferred to another legal entity. This does not affect the right of a Member State to subject activities to appropriate protection requirement", might be applicable to single-unit analytical platforms (e.g. HPLC-MS/MS hyphenations, PCR assays …) which operate solely with local (e.g. within one medical center) samples. However, due to national regulation the interpretation of the term "in-house" differs within the EU. Currently, the whole issue seems unresolved and further discussions will hopefully lead to clarified regulations for novel applications.

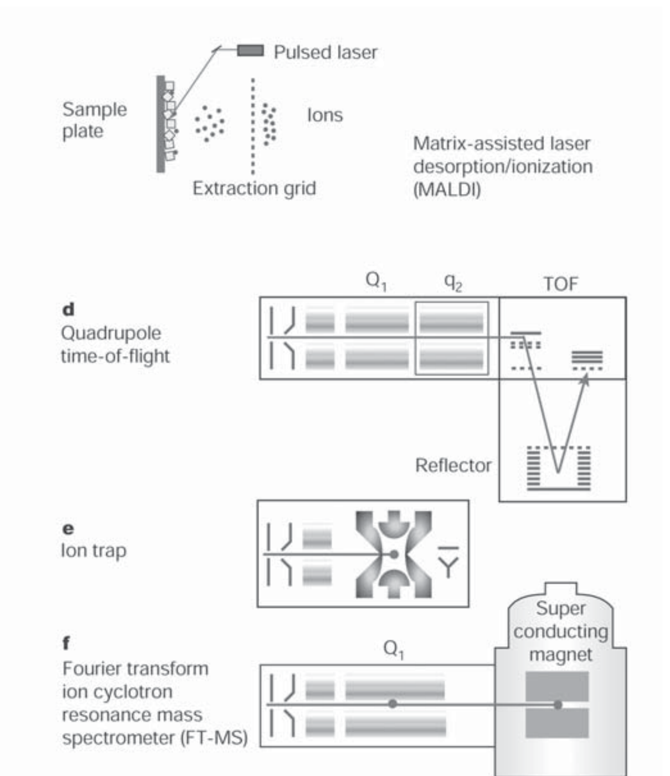
Introduction to mass spectrometry instrumentation

Analyte Ionization

Mass spectrometry is based on the formation, selection and transport of ions in the vacuum of a MS instrument. The following paragraphs shall serve as a short overview to provide an insight into current technical solutions of analyte ionization and selection, as well as of connecting a MS to chromatographic devices.

During the past decades, different methodologies have been developed for ion formation and ion selection of analytes and a variety of strategies have evolved to combine analyte separation techniques with mass spectrometry (Figure 1). Since only ions are analyzed, the most important reaction in MS might be the one that converts uncharged analytes of interest into gas–phase ions. The oldest, most used and probably best understood ionization technique is electron ionization (EI) in which analyte vapour is subjected to a bombardment by energetic electrons (typically 70 eV). Since analytes in EI have to be presented as vapours, it is the ionization of choice for GC–MS analysis. Over the past decades, a number of ionization techniques have been developed for analyses of non volatile and thermally labile compounds, with electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) emerging as primary methods of choice. In ESI, ions are generated from a liquid at atmospheric pressure. The electrospray setup is technically simple – a hollow needle through which the eluent flows (usually 1–1000 µl/min) is charged with a potential. The high field at the tip of the needle produces a cone-shaped liquid meniscus from which emerges a spray of highly charged





SLIKA 1. Shematski prikaz ionizacijskih tehnika i uređaja za odabir iona koji se koriste u bioanalitičkoj spektrometriji masa. Prikazane su različite konfiguracije instrumenata (**a**-**f**) s tipičnim ionizacijskim izvorima (lijeva kolona ESI, desna kolona MALDI). **a**, Uređaj-reflektor vremena proleta (engl. *time of flight*, TOF) koji se koristi kad se traže visoke točnosti mase. **b**, Instrument TOF-TOF koji omogućuje fragmentaciju analita između dvaju procesa odabira iona. **c**, Glede trostrukog kvadropola TOF-TOF MS (QqQ), taj uređaj kombinira dva procesa odabira iona i omogućuje, između ostalog, pokuse praćenja odabranih reakcija. Linearna ionska stupica (točka u Q3) može se koristiti za prikupljanje i očitanje iona nastalih u sudarnoj stanici. **d**. U kvadropolnom uređaju TOF (Q-TOF) (križani maseni spektrometar), prednji dio trostrukog kvadropolnog uređaja je kombiniran s TOF kao ionski selektor za očitavanje. **e**, U (trodimenzijskim) uređajima s ionskom stupicom, hvatanje iona, fragmentacija te očitavanje (obično pregledom) omogućava nastanak spektara MS/MS. **f**. Uređaj FT-ICR-MS hvata ione pomoću jakoga magnetskog polja. Može se kombinirati s linearnom ionskom stupicom (kvadropol s točkom) radi djelotvornog izdvajanja, fragmentacije i odabira fragmenta prije odjeljka FT-MS. Pretiskano uz dozvolu Macmillan Publishers Ltd: Nature (24), autorska prava 2003.

FIGURE 1 Schematic overview of ionization techniques and ion selection devices used in bio-analytical mass spectrometry. Different instrumental configurations (**a**-**f**) are shown with their typical ionization sources (left column ESI, right column MALDI). **a**, Reflector time of flight (TOF) instrument used if high mass accuracies are desired. **b**, TOF-TOF instrument allowing analyte fragmentation between two ion selection processes. **c**, As for the TOF-TOF MS a triple quadrupole (QqQ) instrument combines two ion selection processes allowing, among others, selected reaction monitoring (SRM) experiments. A linear ion trap (dot in Q3) can be used to collect and read out ions generated in the collision cell. **d**, In a quadrupole TOF (Q-TOF) instrument (hybrid mass spectrometer), the front part of a triple quadruple instrument is combined with a TOF as a readout ion selector. **e**, In (three-dimensional) ion trap instruments ion capture, fragmentation and ion read out (usually by a scan) allows to generate MS/MS^n spectra. **f**, The FT-ICR-MS instrument traps ions by the aid of a strong magnetic field. It can be combined with a linear ion trap (quadrupole with dot) for efficient isolation, fragmentation and fragment selection prior to the FT-MS section. Reprinted by permission from Macmillan Publishers Ltd: Nature (24), copyright (2003)

va ionizacije elektroraspršenjem je tehnički jednostavna: šuplja igla kroz koju protječe izlazna tekućina (obično 1-1000 µL/min) je nabijena električnim potencijalom. Visoko polje na vrhu igle stvara stožasti tekući "polumjesec" iz kojega nastaju raspršene, visoko nabijene kapljice. Daljnje isparavanje kapljica rezultira stvaranjem iona. ESI ima neke vrlo dojmljive prednosti koje joj omogućuju primjenu kod vrlo različitih bioloških problema. Stvaranje višestru-

droplets. Subsequent evaporation of the droplets results in ion formation. ESI has some very impressive attributes that allow its application to a wide variety of biological problems. The production of multiply charged species extends the dynamic range of the mass spectrometer to the range of 10^6 u allowing analysis of macromolecules. Furthermore, it can be considered a very "soft" ionization technique which does not destroy non-covalently bound

ko nabijenih čestica proširuje dinamički raspon masenog spektrometra do 10^6 u i omogućuje analizu makromolekula. Nadalje, ESI se može smatrati vrlo "blagom" ionizacijskom tehnikom koja ne razara čak ni nekovalentno vezane biomolekularne komplekse kao što su proteini (31) i čak čitavi virusi (32). To je najvjerojatnije najkorišteniji ionizacijski izvor koji se kombinira s različitim sustavima analizatora HPLC-MS i CE-MS (33,34). Priličan nedostatak ESI, osobito kod pokusa s izravnom infuzijom (engl. *direct infusion experiments*, DIESI) je njena osjetljivost na supresijske učinke matriksa i iona, npr. kod koncentracija s mnogo soli u biološkim uzorcima ili visokokoncentriranim analitima koji mogu spriječiti ili ukloniti ionizaciju analita (35-37). Alternativa ESI, koja se često kombinira unutar istog izvora, je kemijska ionizacija uz atmosferski tlak (engl. *atmospheric pressure chemical ionization*, APCI).

Ionizacija započinje kružnim pražnjenjem iz igle koje stvara finu plazmu reagencijskih iona koje potječu iz hlapljivih tvari u izvoru (pomoćni plinovi i isparena otapala). Složeni uzastopni niz reakcija dovodi do najdjelotvornije ionizacije analita. APCI je poznat po osjetljivosti, robusnosti i pouzdanosti, te mnogo nižoj sklonosti za kemijske interferencije nego ESI (33).

Daljnja nova alternativa ESI je fotoionizacija uz atmosferski tlak (engl. *atmospheric pressure photo ionization,* APPI) koja se temelji na apsorpciji visokoenergijskog fotona proizvedenog u svijetlećem tijelu koje koristi električni luk (engl. *discharge lamp*) (38, 39). Premda sve ionizacijske tehnike s atmosferskim tlakom, tj. ESI, APCI i APPI, imaju značajne prednosti, treba uvijek imati na umu da sve tri pokazuju osjetljivosti koje su specifične za određene vrste spojeva: vrlo nizak broj dobivenih iona ne može se isključiti u pojedinim slučajevima.

Vidljivo različit način uvođenja analita u analizator masa čini osnovu ionizacije MALDI (40). Analit se istodobno kristalizira s matriksom, a ioni se stvaraju laserskim zračenjima. Izuzetna osobina MALDI je pulsna priroda lasera čije stvaranje "paketa" iona predstavlja odvojene događaje visoke osjetljivosti. Zbog kratkog trajanja tih pulseva broj iskoristivih masenih analizatora je ograničen jer su potrebne visoke brzine pregleda. MALDI je metoda izbora za analizu visokog protoka jer se mogu koristiti ciljane ploče sa stotinama uzoraka. Njena relativno visoka podnošljivost soli i pufera čini je metodom izbora za analizu složenih biogenih matriksa. Glavni nedostatak MALDI nalazi se u prirodi korištenih matriksa, osobito ako se moraju analizirati spojevi niske molekularne težine. Općenito, dok ionizacijske tehnike s atmosferskim tlakom (API) kao što su ESI, APCI i APPI pripadaju u "blage" ionizacijske tehnike koje uglavnom stvaraju spektre sa slabom fragmentacijom, višefazni ionski analizatori kao što su QqQ, Qq-TOF ili ionske stupice (vidjeti nastavak teksta) moraju se koristiti da bi se dobili fragmentirani ioni koji omogućuju identifikaciju analita usporedbom s referentnim materijalom.

bio-molecular complexes as proteins (31) and even whole viruses (32). ESI is probably the most utilized ionization source combined with any sort of analyzer for HPLC–MS and CE–MS analysis. (33,34). A considerable shortcoming of ESI, particularly in direct infusion experiments (DIESI), is its susceptibility to matrix and ion suppression effects, e.g. by high salt concentrations in biological samples or by high concentrated analytes which might hinder or deplete ionization of analytes (35-37). An alternative to ESI, often combined within one source housing, is atmospheric pressure chemical ionization (APCI). The ionization is initiated by a corona discharge from a needle, producing a fine plasma of reagent ions stemming from volatiles in the source (auxiliary gases and vaporized solvents). A complex reaction cascade leads to a most efficient ionization of analytes. APCI is known for its sensitivity, robustness, ruggedness and reliability, and much lower susceptibility to chemical interferences than ESI (33). A further emerging alternative to ESI is atmospheric pressure photo ionization (APPI), based on absorption of a high energy photon which is produced in a discharge lamp (38,39). Although all atmospheric pressure ionization techniques, i.e. ESI, APCI, and APPI have impressive attributes, one has always to bear in mind that all the three of them show compound class specific sensitivities – very low ion yields cannot be excluded in certain cases.

A distinctly different concept of introducing analytes into a mass analyzer forms the basis of MALDI (40). The analyte is co-crystallized with a matrix and ions are formed by laser irradiations. An outstanding feature of MALDI is the pulsed nature of the laser which produces ion packages in discrete events of high sensitivity. Due to the short duration of these pulses, the number of utilizable mass analyzers is limited as high scanning rates are required. MALDI is the method of choice for high throughout analysis since target plates loaded with hundreds of samples can be used. Its relatively high tolerance to salts and buffers makes it the method of choice for analyzing complex biogenic matrices. The major drawback of MALDI lies in the nature of utilized matrices, especially if low-molecular weight compounds have to be analyzed. Generally, as atmospheric pressure ionization (API) techniques as ESI, APCI, and APPI are "soft" ionization technique producing mainly spectra with hardly any fragmentation, multistage ion analyzers as QqQ, Qq–TOF or ion–traps (see below) have to be used to obtain fragment ions that aid analyte identification by comparison with reference material.

Ion selection

Transmission quadrupoles are used as mass filters by applying precisely controlled voltages to opposing sets of poles. By ramping the voltages on each side of the poles, a complete range of masses can be passed to the detector in a time dependent manner. Quadrupoles were the

Odabir iona

Prijenosni kvadropolovi koriste se kao maseni filtri primjenom precizno kontroliranih napona na suprotnim serijama polova. Promjenama napona na krajevima polova omogućuje propuštanje čitavog raspona masa na detektor u zadanom vremenu. Analizatori s kvadropolom su u prošlosti bili najčešće korišteni, osobito u kombinaciji s ionskim izvorom koji stvara strukturno značajne fragmente, kao npr. EI u platformama GC-MS. Iako kvadropolovi mogu mjeriti mase > 4000 u, zapravo su najprikladniji za primjenu masa < 1000 u. Značajan nedostatak kvadropolnih masenih analizatora je relativno mala brzina pregleda (< 4000 u/s). Zbog toga se u načinu rada koji uključuje potpuni pregled koristi samo mali udio raspoloživih iona, što takve analizatore čini nepogodnima za suvremena visokobrzinska izdvajanja s osjetno nižim širinama vršnih vrijednosti (engl. *peak widths*). Oni se također ne mogu koristiti kao selektori jako velikih masa u kombinaciji s MALDI. Spomenuta se situacija, međutim, doima potpuno drugačijom kad se tri kvadropola poredaju tako da je središnji nerazdvojivi kvadropol omeđen dvama prijenosnim kvadropolovima koji čine dvojni spektrometar masa - također poznat kao trostruki kvadropolni (QqQ) maseni spektrometar. Takav razmještaj omogućuje kontroliranu fragmentaciju iona u drugom kvadropolu koji se koristi kao sudarna stanica. Dobivena sekundarna raspodjela iona može poslužiti kao skup strukturnih parametara u identifikaciji analita. Nadalje, struktura QqQ omogućuje kombiniranje odabira iona, ionsku reakciju, te procese pregleda iona u novim pokusima, npr. visokospecifični ionski produkt, prethodnički ion, ili način potpunog snimanja bez gubitaka (engl. *neutral loss scan mode*). Praćenje odabrane reakcije (engl. *selected reaction monitoring,* SRM) kao ciljanog analitičkog pokusa najviše osjetljivosti i točnosti je metodološka osnova primjene spektrometrije masa u sudskoj medicini, farmaceutskoj industriji i kliničkoj kemiji, o čemu će se raspravljati u ovom pregledu (33,34,41).

Alternative prijenosnim kvadropolovima su Paulove ionske stupice koje se tehnički mogu smatrati varijacijama općeg dizajna kvadropolnoga masenog filtra. Ioni se zatvaraju ("hvataju u stupicu") u tim uređajima elektrodinamičnim fokusiranjem. Za razliku od kvadropola gdje ioni imaju postojanu putanju do detektora, uhvaćeni ioni su prisiljeni napustiti stupicu prijelazom u nepostojane orbite povećanjem rf-napona. Sposobnost ionskih stupica za provedbu ponovljenih spektrometrijskih pregleda (MS^n s N teorijski do 10) zasnovanih na sudaru analita unutar stanice, njihova lagana uporaba te relativno niska cijena učinila ih je čvrstim radnim pomagalom u mnogim laboratorijima. Iznimno povećana kvantitativna učinkovitost i dinamični raspon postignuti su razvojem linearnim ionskih stupica - dvodimenzionalnih Paulovih ionskih stupica koje zatvaraju ione u osnu dimenziju pomoću električnog polja na krajevima stupice. Kod takvih su stupica prednos-

most common analyzers in the past, particularly in combination with an ion source which produces structurally significant fragments as EI in GC-MS platforms. Although quadrupoles can operate up to masses > 4000 u, they are best suited for applications < 1000 u. One notable disadvantage of a quadrupole mass analyzer is a relatively slow scanning rate (< 4000 u/s). Thus, in full scan mode only a small fraction of available ions is used, a fact which makes them unsuitable for modern high speed separations with appreciably smaller peak widths. Also, they cannot be used as final mass selectors in combination with MALDI. However, the situation appears totally different when three quadrupoles are arranged in such a way that a central non-resolving quadrupole is flanked by two transmission quadrupoles forming a tandem mass spectrometer – also known as a triple quadrupole (QqQ) mass spectrometer. This setup allows controlled ion fragmentation in the second quadrupole which is used as a collision cell. The obtained secondary ion distribution might serve as a set of structural parameters in analyte identification. Furthermore, the QqQ setup allows combining ion selection, ion reaction, and ion scan processes to novel experiments, e.g. a highly specific product ion, precursor ion, or neutral loss scan modes. The selected reaction monitoring (SRM), a targeted analysis experiment of highest sensitivity and accuracy, is the methodological backbone of the application of mass spectrometry in forensics, pharmaceutical industry and clinical chemistry, which is to be discussed in this overview (33,34,41).

Alternatives to transmission quadrupoles are Paul ion traps which can be technically considered as variations of the general quadrupole mass filter design. Ions are confined ("trapped") in these devices by electrodynamic focusing. In contrast to a quadrupole, where ions have a stable trajectory on their way to the detector, trapped ions are forced to leave the trap by putting them in unstable orbits through increasing the rf–voltage. The capability of ion traps to perform analyte collision-based repeated MS/MS experiments (MS^n with n theoretically up to 10) within the cell, their ease in use, and their relatively low cost have made them rugged workhorses in many laboratories. Notably increased quantitative performance and dynamic range have been gained by the development of linear ion traps – two-dimensional Paul ion traps confining ions in axial dimension by means of an electric field at the ends of the trap. With such traps, benefits are mainly associated with the optimized volume of the mass analyzer (42). TOF mass analyzers, in principle some of the simplest devices in mass spectrometry, separate ions based on their velocity which is mass dependent. All ions are formed at once in the ion source and subsequently accelerated through a fixed potential. Hence, small mass ions arrive in the detector earlier than large mass ions. A common variation of TOF incorporates an electrostatic mirror, a reflectron,

ti uglavnom povezane s optimiranjem sadržine u masenom analizatoru (42). TOF-maseni analizatori, koji su u načelu jedan od najjednostavnijih uređaja u spektrometriji masa, odvajaju ione na temelju njihove brzine koja je ovisna o masi. Svi se ioni odjednom stvaraju u ionskom izvoru i zatim se ubrzavaju kroz fiksni potencijal. Zbog toga ioni male mase pristižu u detektor prije od iona velike mase. Uobičajena varijacija TOF uključuje elektrostatično ogledalo, tj. reflektron, u području bez polja kako bi se nadoknadili učinci raspodjele kinetičke energije početnih iona. Analizator TOF je postao vrlo popularan u ranim danima MS no zbog toga što zahtijeva puls iona nije bio spojiv s ionizacijskim metodama dostupnima u to doba. Razvoj novih tehnika pulsne ionizacije kao što je MALDI doveo je do renesanse TOF-detektora tijekom 1990-ih. Danas se uglavnom koristi kao maseni analizator za "očitavanje" te je kombiniran s drugim TOF, ionskom stupicom, ili pak - najčešće, - s jednim ili dva kvadropola (Q-TOF ili Qq-TOF) ispred njega. Takvi se križani maseni spektrometri ističu neograničenim rasponom masa, izuzetno visokim brzinama pregleda (do 10^6 u/s), te visokom snagom razlučivosti koja rezultira točnošću mase u rasponu ppm. Stoga ti spektrometri predstavljaju tehniku izbora kod primjena koje zahtijevaju visok protok uz najvišu djelotvornost, kao što je slučaj u proteomici i metabolomici (24,25,34,43-45).

Spajanje na sustave kromatografskog razdvajanja

Spajanje HPLC i MS omogućuje analizu najrazličitijih spojeva, uključujući termički nepostojane analite i analite s visokom molekularnom težinom gdje je analiza pomoću GC-MS nemoguća. Komercijalno je dostupan široki raspon stacionarnih faza HPLC-a kako u smislu dimenzija kolone, tako i modifikacija površine, a to omogućuje optimiranje kromatografskog razdvajanja na medij koji se ispituje. Najnoviji razvoj ovog područja uključuje također nedavno uvođenje strojne podrške "ultra"-učinkovitosti koje bi moglo dovesti do značajnog smanjenja vremena analize. Spajanje tekućinske kromatografije i spektrometrije masa ograničava otapala u HPLC-u na hlapljive pufere i organske aditive radi izbjegavanja taloženja soli u masenom spektrometru. Zbog toga su natrijevi/kalijevi fosfatni puferi, koji su bili često korišteni kao otapala u HPLC-UV, morali biti zamijenjeni amonijevim formijatom ili acetatima.

Postavljanje uređaja HPLC-MS/MS

Maseni spektrometri su izuzetno složeni i skupi analitički uređaji. Unatoč tome, osiguranje uvjeta za analitički specifičnu spektrometriju masa predstavlja samo manji dio uspješne uspostave kvantitativnog rutinske analitičke primjene HPLC-MS/MS, s time da MS služi isključivo kao detektor. Zbog svojstava te aparature, osobito osjetljivosti na kontaminacije i bilo kakve nestalnosti okoline, uspješna primjena uvelike ovisi o prethodnoj obradi uzorka i kontroliranim uvjetima u okolini. Klimatizacija je kod

into the field-free region to compensate for effects of kinetic energy distribution of initial ions. The TOF analyzer gained great popularity in the early days of MS, but, as it needs a pulse of ions, was not compatible with available ionization methods at that point of time. The development of new pulsed-ionization techniques as MALDI led to a TOF detector renaissance in the 1990s. Nowadays it is mainly used as "readout" mass analyzer, combined with either another TOF, an ion trap, or – most frequently – with one or two quadrupoles (Q–TOF or Qq–TOF) preceding it. These hybrid mass spectrometers stand out through an unlimited mass range, exceptionally high scan rates up to 10^6 u/s, and high resolving power resulting in mass accuracy in the ppm range. Therefore, they are the technique of choice for applications demanding high throughput at utmost efficiency, as it is the case in proteomics and metabolomics (24,25,34,43-45).

Hyphenation to chromatographic separation systems

The hyphenation of HPLC and MS allows the analysis of a wide variety of compounds including thermo-labile and high molecular weight analytes which cannot be addressed by GC-MS. A broad range of HPLC stationary phases, both in terms of column dimensions and surface modifications, are commercially available allowing optimization of chromatographic separation to the matrix under investigation. The latest developments in this field also include the recent introduction of "ultra–performance" hardware, which may lead to a significant reduction in analysis time. Coupling liquid chromatography to mass spectrometry limits HPLC solvents to volatile buffers and organic additives in order to avoid salt precipitation in the mass spectrometer. Hence, sodium/potassium phosphate buffers, frequently used as eluents in HPLC-UV, had to be replaced by ammonium formiate or acetate systems.

HPLC-MS/MS Instrumentation setup

Mass spectrometers are impressively complex and expensive analytical devices. Nevertheless, setting up analyte-specific mass spectrometry conditions is only a minor part of the successful establishment of a quantitative analytical routine HPLC-MS/MS assay with the MS serving solely as a detector. Due to its nature, especially its sensibility to contaminations and environmental fluctuations of any kind, a successful application highly depends on sample pre-treatment and controlled environmental conditions. Air-conditioning of the mass spectrometry laboratory is mandatory, especially if a small room is chosen. Gas and pressurized air supplies have to meet the requirements of manufacturers, which might exceed the qualities commonly used in a routine laboratory environment. Complications in the analysis of complex "real world" samples might arise from chromatographic interferences, matrix effects, adduct and cluster formations, and ion suppression

spektrometrije masa obvezatna, posebice ako je izabrana mala prostorija. Dovodi plina i zraka pod pritiskom moraju udovoljiti zahtjevima proizvođača koji mogu premašivati kvalitete uobičajene u rutinskoj laboratorijskoj okolini. Komplikacije u analizi složenih uzoraka "iz stvarnog svijeta" mogu nastati zbog kromatografskih interferencija, učinaka medija, tvorbi adukata i nakupina, te potiskivanja iona zbog konkurentnih metabolita ili drugih sastojaka matriksa. Zbog toga je temeljito validiran protokol za pripremu uzorka, zasnovan na uzorku bolesnika, od izuzetne važnosti za održanje željene dugotrajne učinkovitosti mjerenja.

Dosta je često moguće optimirati eksperimentalne parametre na temelju prihvatljivih polaznih uvjeta koji se nalaze u stručnoj literaturi. Tu su uključeni parametri HPLC kao što su svojstva mobilne (otapala, aditivi za otapala, koncentracije pufera, pH...) te stacionarne faze, postavke za ionski izvor kojima se određuje koji ion izabrati kao matični ion, te koju ionsku reakciju pratiti (SRM). Nužni pokusi ručnim prilagođavanjem danas su obično podržani i vođeni programskom podrškom masenog spektrometra. Usto, svaki će od većih dobavljača masenih spektrometara vrlo rado pomoći stručnim znanjem svojih specijalista za primjenu ukoliko je potrebno razviti nove pretrage.

Osmišljenje analitičke platforme, osobito odabir sastavnica strojne podrške, ima snažan utjecaj na ukupan uspjeh uvođenja analize. Radni tok od primarnog neobrađenog uzorka do konačne otopine uzorka u bočici za HPLC mora se planirati isto toliko pažljivo kao i postavljanje kromatografskih uređaja koje predstavlja konačne korake pročišćivanja uzorka. Na temelju osjetljivosti dostupnoga masenog spektrometra, zatim odabranog pokusa (npr. SRM), te poznavanja ciljne koncentracije analita koja se prati u uzorku potrebno je provesti ili predkoncentraciju uzorka ili razrjeđenje uzorka kao primarni korak u pripremi uzorka. Stupanj automatizacije te veličina serije koju treba obraditi ovisiti će o potrebama laboratorija kao i raspoloživoj aparaturi. Visoki povrat analita iz matriksa mora se postići kako bi se osigurala robusnost i preciznost analiza. Uporaba internih standarda s najvećom mogućom fizikalno-kemijskom sličnošću ciljanim metabolitima je daljnja obveza u razvoju analiza HPLC-MS/MS. Konačno, nužno je uspostaviti komunikaciju između programske podrške uređaja i laboratorijskog informacijskog sustava, ako je to moguće. Dijagram toka u kojem je sažet čitav analitički proces od uzorka bolesnika do kvantitativnog rezultata LC-MS/MS prikazan je na slici 2.

Priprema uzorka

Endogeni metaboliti, kao i ksenobiotici, često se ili vežu na proteine u plazmi ili se integriraju u stanične membrane (npr. eritrocit) . Stoga je kvantitativno otpuštanje analita iz tih sastojaka matriksa preduvjet za uspješnu analizu. Kidanje staničnih membrana može biti prouzročeno bilo

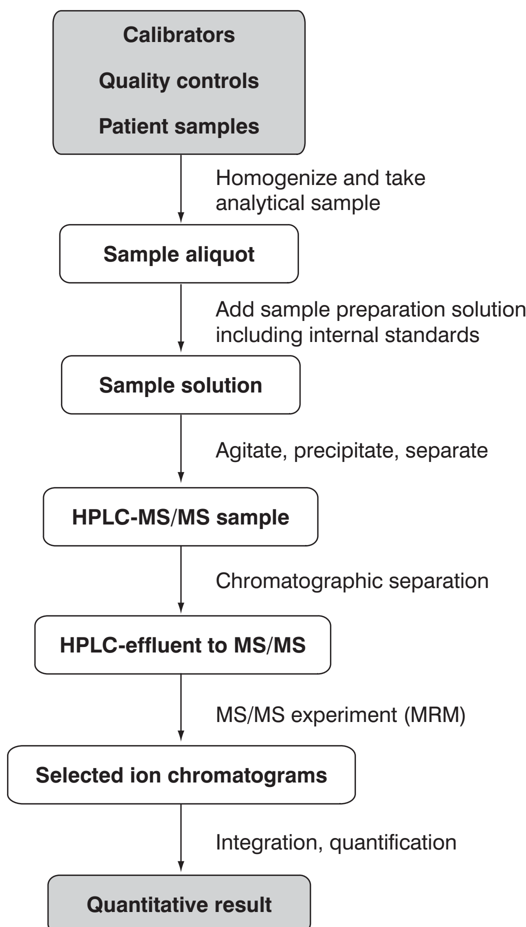
by competing metabolites or other matrix constituents. Hence, a thoroughly validated sample preparation protocol based on patient specimen is of outmost importance to maintain the desired long-term assay performance.

Quite often experimental parameters can be optimized from reasonable good starting conditions found in literature. This includes HPLC parameters as mobile phase (solvents, solvent additives, buffer concentrations, pH …) and stationary phase (column packing material, column dimensions, separation temperature …) properties, ion source settings including which ion to choose as a parent ion, and which ion reaction to monitor (selected reaction monitoring – SRM). The necessary manual tuning experiments are nowadays usually supported and guided by the mass spectrometer software. In addition, any of the major mass spectrometer providers is eager to help with the expert knowledge of their application specialists if novel assays have to be developed.

The design of the platform – especially the selection of hardware components has a strong influence on the overall success of assay introduction. The workflow from the primary crude sample to the final sample solution in the HPLC vial has to be planned as carefully as the setup of the chromatographic devices representing the final sample purification steps. Based on the sensitivity of the available mass spectrometer, the chosen experiment (e.g., a SRM), and the knowledge of the analyte target concentration to be monitored in the specimen, either sample preconcentration or sample dilution has to be performed as a primary sample preparation step. The degree of automation and the batch size to be processed will depend on the needs of the laboratory as well as on available instrumentation. High analyte recoveries from matrix have to be achieved to ensure assay robustness and precision. The use of internal standards with the highest possible physico-chemical similarity to the targeted metabolites is another must in HPLC-MS/MS assay development. Finally, the communication of instrument software to the laboratory information system (LIS) has to be established, if possible. A flowchart summarizing the complete analytical process from the patient sample to the quantitative LC-MS/MS result is given in Figure 2.

Sample preparation

Endogenous metabolites, as well as xenobiotics, are often either bound to plasma proteins or incorporated into cell (i.e. erythrocyte) membranes. Hence, quantitative release of analytes from these matrix constituents is a prerequisite for successful analysis. Disruption of cell membranes can be supported either by an osmotic shock (addition of distilled water) or by the addition of divalent heavy metal cations like zinc or copper. Protein unfolding and subsequent precipitation is usually carried out by the addition of organic solvents and is aided by the abovemen-



Slika 2. Sažetak procesa analitičke platforme LC-MS/MS kojom se dobivaju rezultati kvantitativne analize.

Figure 2 Process summary of an analytical LC-MS/MS platform producing quantitative assay results.

osmotskim šokom (dodatak destilirane vode) ili dodatkom divalentnih kationa teških metala kao što su cink ili bakar. Denaturacija proteina i daljnje taloženje obično se provodi dodatkom organskih otapala i potpomognuto je navedenim kationima (46). U većini slučajeva koristi se acetonitril ili metanol u konačnim koncentracijama > 50% (v/v). Za uzorke pune krvi, koji se susreću u slučaju TDM imunosupresiva (osim za mikofenolnu kiselinu) kombiniraju se oba pristupa. Najčešće se koristi umjereno (obično 1 + 2) razrjeđenje uzorka (tj. kalibrator, kontrola te alikvoti uzorka bolesnika) sa smjesom cinkovog sulfata/metanola (47-50). Alternativni protokol u dvije faze u kojem se koristi acetonitril u drugoj fazi razrjeđenja (konačno razrjeđenje 1 + 14) uspješno su proveli Keevil i suradnici (51,52) primjenjujući 10 µL alikvota uzorka i koristeći mikrotitarsku ploču. U međuvremenu se ta metoda proširila u struci te je uspješno primijenjena uz različite modifikacije (53,54). Drugi značajan protokol su predstavili Annesley i Clayton koji su dodali alikvot vode uzorku prije taloženja

tioned cations (46). In most cases, acetonitrile or methanol in final concentrations > 50% (v/v) is employed. For whole blood specimens, as encountered in the case of immunosuppressant TDM (except for mycophenolic acid), both approaches are combined. A moderate (usually 1 + 2) dilution of the specimen (i.e. calibrator, control and patient sample aliquots) with a zinc sulfate/methanol mixture is most often employed (47-50). An alternative two step protocol using acetonitrile in the second dilution step (final dilution 1 + 14) was successfully implemented by Keevil and coworkers (51,52) by using only a 10 µL sample aliquot and operating in a microtiter plate. In the meanwhile, this method has spread in the community and has been successfully applied in different modifications (53,54). Another noteworthy protocol was presented by Annesley and Clayton who added an aliquot of water to the sample prior to protein precipitation to prevent sample agglutination (55). During work with plasma/serum specimens and organic, solvent-based samples, protein

proteina radi sprječavanja sljepljivanja uzorka (55). Tijekom rada s uzorcima plazme/seruma i uzorcima s organskim otapalom, utvrđeno je da su protokoli za taloženje proteina dovoljni u većini slučajeva. Općenito, potrebno je postići da uzorak postane potpuno homogeniziran tijekom faze taloženja snažnim protresanjem; bilo kakvo stvaranje grudica značajno šteti kvaliteti analiza i treba ga izbjeći. Uklanjanje ostataka stanica i denaturiranih makromolekula provodi se centrifugiranjem, a postojan i čvrst talog je poželjniji radi omogućavanja jednostavnog odlijevanja supernatanta u bočicu za HPLC koja služi kao krajnji spremnik za uzorak. Otopina uzorka mora biti jasna i bez vidljivih kontaminacija. Kako je proteinski talog obrnuto povezan s temperaturama, uporaba centrifuga s hlađenjem (tj. 4 °C) rezultira poboljšanom kvalitetom uzorka.

Jedna od najizrazitijih razlika između analiza zasnovanih na kromatografiji i imunokemijskih analiza je uporaba internih standarda koji se moraju dodati u fazi pripreme uzorka. Ako se ispravno dodaju precipitacijskom otapalu, ti standardi nadoknađuju gubitke koji su tijekom postupka pripreme uzorka prouzročeni isparavanjem organskog otapala, pogreškama u rukovanju, ili opadajućom kromatografskom učinkovitošću ako su obuhvaćene i faze ekstrakcije. Usto, oni nadoknađuju nepreciznost svojstvenu sustavu uvođenja uzorka, prolazne nestalnosti procesa nastanka iona i - što je najvažnije, - drugih učinaka matriksa kao što je potiskivanje iona ako se istodobno ispiru s analitima. Vršni omjeri analita/internog standarda moraju se dobro uravnotežiti kako bi se izbjegle bilo kakve razlike u analizama zbog učinaka potiskivanja iona uzrokovanih internim standardom. Kako bi se ispunili kriteriji što veće kemijske sličnosti analitima, ti su spojevi ili postojani izotopno označeni derivati ili strukturni homolozi analita. Označavanje postojanih izotopa provodi se namjernim umetanjem izotopa ^{2}H ili ^{13}C u molekularnu strukturu kvantitativnom zamjenom određenih atoma ^{1}H ili ^{12}C (56). Na taj se način uvodi pomak molekularne mase i razlučuje interni standard od analita u masenom spektrometru. Ako označavanje postojanih izotopa nije odabrani postupak zbog prirode analita (tj. fungalni metabolit nastao fermentacijom u slučaju većine imunosupresiva i antibiotika), može se koristiti strukturni homolog. U slučaju TDM imunosupresiva, askomicin najčešće služi kao interni standard za takrolimus, a ciklosporin D za ciklosporin A. U oba slučaja interni standardi imaju istu veličinu i geometriju prstenastog sustava. Oni pokazuju samo fine razlike u jednom postranom lancu: metilnoj skupini u ciklosporinu A/D i metilenskoj skupini u slučaju takrolimusa/askomicina.

Priprema uzorka provodi se u serijama, s uzorcima za kontrolu kvalitete u svakoj seriji. Veličina serije ovisi o dostupnim uređajima; ograničenje može, primjerice, predstavljati broj mjesta za bočice u rotoru centrifuge. Radi omogućavanja nedvosmislene identifikacije uzorka kroz čitavu prijeanalitičku fazu, uporaba pojedinačnih reakcijskih posudica i bočica za HPLC može biti vrlo korisna. Ako se

precipitation protocols have been found to be sufficient in most cases. Generally, care must be taken that the sample becomes completely homogenized during precipitation step by vigorous agitation; any formation of clumps impairs assay quality significantly and must be avoided. Removal of cell debris and denaturated macromolecules is performed by centrifugation, a stable and hard precipitate is preferred to allow simple decanting of supernatant to the HPLC vial serving as a final sample container. The sample solution has to be clear and without visible contaminations. As protein precipitation is invertely correlated with temperatures, employing cooled centrifuges (i.e. 4 °C) yields improved sample quality.

One of the most striking differences between chromatography- based assays and immunoassays is the use of internal standards which have to be added in the sample preparation step. If added correctly with the precipitation solvent, they compensate for losses during sample preparation procedure caused by evaporation of organic solvent, handling errors, or declining chromatographic performance if extraction steps are involved. Additionally, they compensate for the inherent imprecision of the sample introduction system, temporary fluctuations of the ion formation process and – most importantly – other matrix effects as ion suppression if co-eluting with analytes. Analyte/internal standard peak ratios have to be well balanced to avoid any assay bias due to ion suppression effects caused by internal standard. To meet the criteria of outmost physico-chemical likeness to analytes, these compounds are either stable isotope-labelled derivatives or structural homologues of analytes. Stable isotope labelling is performed by deliberately introducing ^{2}H or ^{13}C isotopes into molecular scaffold by quantitatively replacing certain ^{1}H or ^{12}C atoms (56). By doing so, a shift of the molecular mass is introduced, discriminating the internal standard from the analyte in mass spectrometer. If stable isotope labeling is not a choice due to the nature of the analyte (i.e. a fungal metabolite produced by fermentation as in the case of most immunosuppressants and antibiotics), a structural homologue can be used. In the case of immunosuppressant TDM, ascomycin most often serves as an internal standard for tacrolimus, and cyclosporine D for cyclosporine A. In both cases, internal standards have an identical ring system size and geometry. They feature only subtle differences in one side chain – a methyl group in cyclosporine A/D and a methylene group in the tacrolimus/ascomycin case.

Sample preparation is performed in batches with quality control samples running in each batch. Batch size depends on available instrumentation; a limitation, for example, can be the number of vial places in the centrifuge rotor. To allow unequivocal sample identification throughout the whole pre-analytical phase, the use of individual reaction cups and HPLC vials can be beneficial. If sample mix-

zamjena uzorka može isključiti drugim mjerama opreza (tj. dvodimenzijskim crtičnim kodovima), postupci obrade mogu se značajno pojednostaviti uporabom mikrotitarskih pločica s 96 jažica (51,52,57,58). Alikvoti uzorka mogu se staviti ili u jažice ručno ili pomoću robota za uzorke (59-61). Temeljem njihove široke uporabe u farmaceutskoj industriji, na raspolaganju su različite vrste pločica s jažicama i odgovarajućih tresilica, naprava za zatvaranje, te centrifugalnih rotora za pločice s jažicama. Suvremeni automatski uređaji za uzorkovanje omogućuju uzimanje alikvota izravno s mikrotitarskih ploča, čak i uz prisutnost taloga. Zbog toga je moguće izbjeći vremenski zahtjevan ručni prijenos uzoraka iz precipitacijske epruvete u bočicu u HPLC.

Pročišćavanje uzorka

Nakon taloženja proteina i lize stanica matriks za uzorke ostaje toliko nečist da ga je nemoguće izravno umetnuti u vakuum masenog spektrometra. Ona se sastoji od sastojaka kako male, tako i velike molekularne težine koji obuhvaćaju široki raspon polariteta: hidrofilni (tj. aminokiseline, organske kiseline, peptidi, derivati šećera...), amfolitički (tj. fosfolipidi...) i hidrofobni (kolesterol, trigliceridi, lipidi...) metaboliti su prisutni bilo u otopini, bilo kao vezikule. Stoga se i za lipofilne (npr. lijekovi, endogeni steroidni hormoni) i hidrofilnije analite (npr. organske kiseline, kateholamini) najčešće primjenjuje dvodijelni protokol pročišćavanja uzorka u kojem se kombinira ekstrakcija na krutoj fazi (SPE) i analitički RP-HPLC kako bi se osigurali ponovljivi odgovori detektora.
Primjena neintegrirane SPE za pročišćavanje uzorka ili ugušćivanje analita je dobro uhodana tehnika koja se razvijala tijekom prošlih desetljeća kao alternativa protokolima ekstrakcije tekućina-tekućina. Danas je dostupno mnogo različitih materijala za kolone, od klasičnih materijala reverzne faze (RP-18) i lipofilnih polimera do faza izmjene aniona (SAX) i kationa (SCX). Ta se tehnika primjenjuje u većini analitičkih laboratorija i korištena je za gotovo bilo koji matriks (62,63). U kliničkom se laboratoriju često koristi kao tehnika ugušćivanja uzorka u kontekstu HPLC-analiza s UV ili fluorescentnom detekcijom. Može se koristiti u različitim formatima, od jednostrukih kolona SPE do ploča s 96 jažica (64). Nakon prethodnog kondicioniranja materijala za SPE dodaje se alikvot uzorka. Daljnjim se fazama ispiranja uklanjaju neželjeni sastojci matriksa. Na kraju se analit od interesa ispire iz kolone koja se obično odbacuje nakon jednokratne uporabe. Protok otapala kroz stacionarnu fazu SPE obično je potpomognut primjenom blagog vakuuma ili centrifugalnim silama. Radi se, međutim, o ručnoj metodi sa svim svojim nedostatcima: troši puno vremena i zahtjevna je s obzirom na uključenost osoblja. Ukoliko se traži veći protok uzoraka neintegrirani SPE treba zamijeniti integriranim metodama. To znači da metoda treba biti integrirana u postavu HPLC prije analitičke kolone korištenjem osmišljene izmjene kolona. Zbog

up can be excluded by other precautions (i.e. by two-dimensional barcodes), the use of 96-well microtiter plates simplifies workup procedures significantly (51,52,57,58). Specimen aliquots can be either placed into the wells by hand or by the aid of a sample robot (59-61). Due to their broad application in pharmaceutical industry, different types of well plates, sealing devices, well plate shakers, and well plate centrifugation rotors are available. Modern auto samplers allow drawing sample aliquots directly from microtiter plates, even if a precipitate is present. Hence, the time consuming manual transfer of a sample from the precipitation tube to the HPLC vial can be avoided.

Sample purification

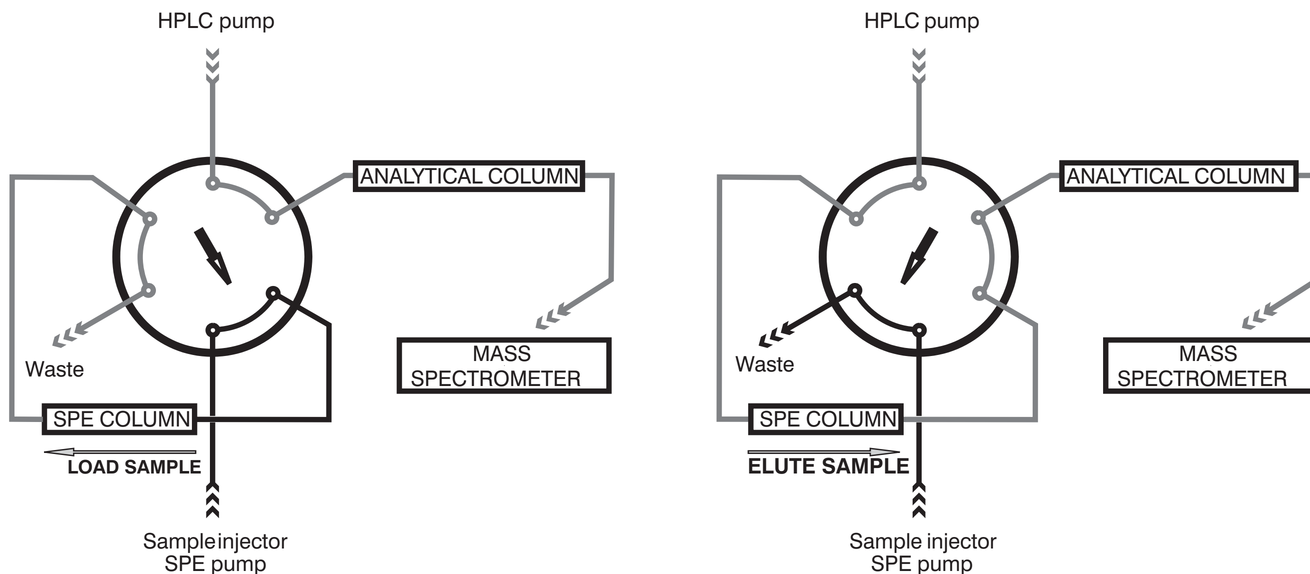
Protein precipitation and cell lysis leave a sample matrix much too dirty to be directly introduced into the vacuum of a mass spectrometer. It consists of both low and high molecular weight constituents covering a broad range of polarities; hypdrophilic (i.e. amino acids, organic acids, peptides, sugar derivatives …), ampholytic (i.e phospholipids …), and hydrophobic (cholesterol, triglycerides, lipid rafts ….) metabolites are present either in solution or as vesicles. Hence, both for lipophilic (e.g. drugs, endogenous steroidal hormones) and more hydrophilic analytes (e.g. organic acids, catecholamines), a two step sample purification protocol combining solid phase extraction (SPE) and analytical RP-HPLC is most often applied to provide reproducible detector responses.
The use of offline SPE for sample purification or analyte enrichment is a well established technique evolving over the past decades as alternative to liquid-liquid extraction protocols. A lot of different column materials are available, ranging from classical reversed phase materials (RP-18) and lipophilic polymers to anion (SAX) and cation exchange (SCX) phases. This technique found its application in most analytical laboratories and has been applied for nearly any matrix (62,63). In the clinical laboratory, it is often used as sample enrichment technique in the context of HPLC analyses with UV of fluorescence detection. It can be applied in different formats, ranging from single SPE columns to 96 well plates (64). After the preconditioning of SPE material, a sample aliquot is added. Subsequent washing steps remove unwanted matrix constituents. Finally, the analyte of interest is eluted from the cartridge, which is usually discharged after single use. Solvent flow through the SPE stationary phase bed is usually supported by applying a gentle vacuum or by the aid of centrifugal forces. However, it is a manual method with all its drawbacks – it is time consuming and demanding in terms of personnel involvement. If a higher sample throughput is desired, offline SPE should be replaced by online methods. This implies that it has to be integrated into the HPLC setup prior to the analytical column by using a column switching design. As a consequence, SPE columns

toga je, pak, obvezno koristiti kolone SPE veće trajnosti i tlačne postojanosti, a potrebna je i uspostava protoka otapala koja omogućuje kontrolirani prijenos svih analita od prvoga do drugog elementa kolone. Najznačajnija kromatografska razlika prema neintegriranom SPE jest da se, kod rada na visokim brzinama protoka (< 4 mL/min), kod nekih od komercijalno dostupnih integriranih kolona za SPE (npr. proizvodi zasnovani na materijalu s ograničenim pristupom, engl. *restricted access material*, RAM) mogu pojaviti adsorpcijski učinci te učinci isključenja prema veličini (65,66). Stoga se sastojci makromolekularnog matriksa lagano uklanjaju, čak i kad pokazuju afinitet prema površini materijala za SPE u konvencionalnoj postavi SPE, kao što to čine denaturirani proteini.

Primjena integriranih kolona za pripremu u tekućinsku kromatografiju uvedena je prije više od 40 godina (67). Tijekom dva desetljeća takva je kolona našla primjenu od industrije do kliničke analize, i to počevši od TDM imunosupresiva (68,69), osim ostalih primjena (70,71). Njena široka uporaba u TDM imunosupresiva koje se temelji na spektrometriji masa započela je prije manje od 10 godina kada je nekoliko skupina istraživača skoro istodobno predstavilo svoje prve integrirane analize SPE-HPLC-MS i SPE-HPLC-MS/MS (48,50,72-74). Mogući plan postave integrirane SPE-HPLC-MS/MS, kao što je realiziran u našem laboratoriju, prikazan je na slici 3. Obje stacionarne faze, i integrirana SPE-kolona i analitička HPLC-kolona, pune se odvojenom HPLC-sisaljkom i time se omogućava izmjena među mobilnim fazama ili stvaranje gradijenta mobilne faze u obje kromatografske dimenzije. Prijenos s jedne na drugu kolonu omogućen je uključenjem šestosmjernog ventila. Kako se dovođenje i pročišćavanje uzorka na SPE obično obavlja s istom mobilnom fazom, drugi kanal za otapalo (ako se koristi dvojna sisaljka) HPLC-sisaljke koja opslužuje kolonu za SPE može se koristiti za čišćenje/obnovu stacionarne faze SPE nakon svake analize (tj. ispiranjem s metanolom). Analiti zadržani na SPE prenose se u drugu kromatografsku fazu pomoću otapala kojega prosljeđuje HPLC-sisaljka; obrnuti protok (reverzno ispiranje, engl. *back flush mode*) dovodi do izoštrenog profila ispiranja sa SPE. Zbog dostupnosti dvaju (dvojna sisaljka) ili više kanala za otapala na HPLC-sisaljci moguće je proizvesti bilo koji željeni gradijent mobilne faze. To je od izuzetnog značaja ako je potrebno odvojiti analite pod istim tlakom ili ako treba odvojiti metabolite lijeka podložnog fragmentaciji ionskog izvora od matičnog lijeka koji se ispituje (48,75-77). Vrijeme ciklusa takve postave ovisi o fazi SPE (oko 1 minute), vremenu potrebnome za temeljito odvajanje analita u drugoj kromatografskoj dimenziji ako je ono potrebno (tj. ako treba odvajati analite pod istim tlakom), te vremenu za obnovu stacionarnih faza. U postavi našeg laboratorija koja se koristi za TDM imunosupresiva postignuto je ukupno vrijeme kromatografije od 2,6 minuta, uz vršno ispiranje analita između 1,2 i 1,5 minuta (Slika 4.).

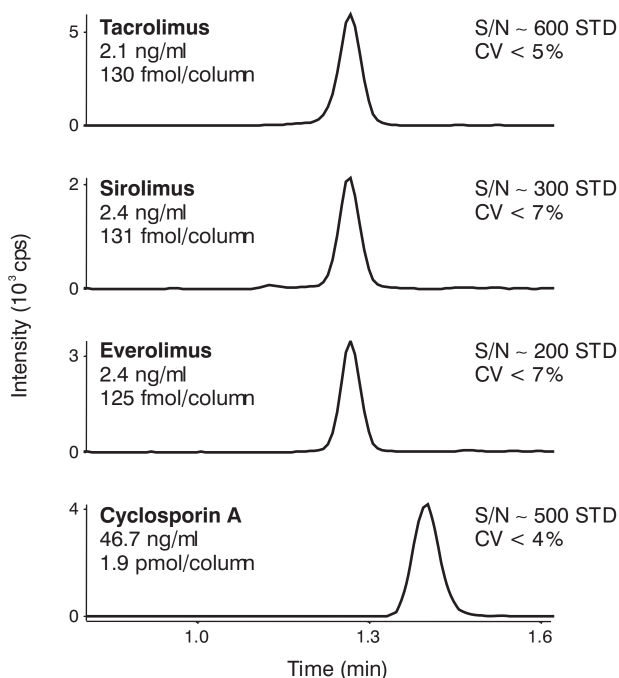
of increased durability and pressure stability have to be used and a solvent flow setup allowing the controlled transfer of all analytes from the first to the second column element is needed. The most significant chromatographic difference to offline SPE is that, if operated at high flow rates (< 4 ml/min), some of the commercially available online SPE columns (e.g. the RAM – restricted access material – based products) can show both size exclusion and adsorptive effects (65,66). Hence, macromolecular matrix constituents are easily removed, even if they show an affinity to the surface of the SPE material in a conventional SPE setup, as denaturated proteins can do.

Online column switching was introduced to liquid chromatography more than forty years ago (67). Within two decades it found its way from industry to clinical analysis starting with immunosuppressant TDM (68,69), besides other applications (70,71). Its broad application in mass spectrometry-based TDM of immunosuppressants started less than ten years ago, when several research groups presented more or less simultaneously their first online-SPE-HPLC-MS and online-SPE-HPLC-MS/MS assays (48,50,72-74). A possible layout of an online SPE-HPLC-MS/MS setup – as realized in our laboratory – is given in Figure 3. Both stationary phases, the online SPE column and the analytical HPLC column, are fed by a separate HPLC pump, allowing mobile phase switching or mobile phase gradient formation in both chromatographic dimensions. The transfer from one to the next column is aided by switching a six-way valve. Since sample introduction and sample purification on the SPE is usually performed with the same mobile phase, the second solvent channel (if a binary pump is used) of the HPLC pump serving the SPE column can be used to clean/regenerate the SPE stationary phase after each analysis (i.e. by flushing it with methanol). The analytes retained on the SPE are transferred to the second chromatographic step with the aid of the solvent delivered by the HPLC pump; the reversion of flow direction ("back flush mode") leads to a sharpened elution profile from the SPE. Due to the availability of two (binary pump) or more solvent channels on the HPLC pump, any desired mobile phase gradient can be formed. This is of special importance if isobaric analytes have to be separated or if drug metabolites susceptible for ion source fragmentation have to be separated from the parent drug under investigation (48,75-77). The cycle time of this setup depends on the SPE step (about 1 min), the time needed for proper analyte separation in the second chromatographic dimension if needed (i.e. if isobaric analytes have to be separated), and the time to regenerate stationary phases. In our laboratory setup used for immunosuppressant TDM, a total chromatography time of 2.6 min has been realized, with the analyte peaks eluting between 1.2 and 1.5 min (Figure 4).



SLIKA 3. Shematski prikaz tehnike zamjene uzorka koja se često primjenjuje kod postava HPLC-MS/MS. Najprije se injicirani alikvot uzorka dovodi do kolone za SPE pomoću mobilne faze SPE-sisaljke. Analiti su zadržani na koloni za SPE dok se sastojci matriksa ispiru kao otpad. Ventil sa 6 ulaza kao prekidač dovodi do povratnog ispiranja analita iz kolone za SPE na analitičku kolonu za HPLC pomoću smjese mobilne faze HPLC-sisaljke. Tu su analiti odvojeni od glavnih izvora potiskivanja iona; prednji dio otapala i sastojci lipofilnog matriksa istodobno se ispiru iz kolone za SPE.

FIGURE 3 Schematic overview of the column switching technique frequently applied in HPLC-MS/MS setups. First the injected sample aliquot is carried to the SPE column by the aid of the SPE pump mobile phase. Analytes are retained on the SPE column whereas matrix constituents are flushed into waste. Switching the 6 port valve leads to the back flush elution of the analytes from the SPE column onto the analytical HPLC column by the aid of the HPLC pump mobile phase mixture. Here, analytes are separated from the main sources of ion suppression - the solvent front and lipophilic matrix constituents co-eluting from the SPE column.



SLIKA 4. Kromatogram dobiven pomoću HPLC-MS/MS niskostupanjskog, višekomponentnog kalibratora TDM imunosupresiva. I CV ponovljenih mjerenja (N = 6) i brojevi S/N pokazuju da je ova razina analita prilično iznad granice kvantifikacije pojedinačne analize koja je ustanovljena u našem laboratoriju.

FIGURE 4 HPLC-MS/MS derived chromatogram of a low level multicomponent immunosuppressant TDM calibrator. Both the CV of repeated measurements (N = 6) and the S/N numbers show that this analyte level is well above the limit of quantification of this particular assay established at our laboratory.

Alternativno takvoj dvodimenzijskoj postavi HPLC, koja osigurava maksimalno pročišćavanje uzorka prije ulaza u maseni spektrometar, često se realizira i jednodimenzijski HPLC. Takva, pak, postava omogućava kraća vremena ciklusa, uz česta izvješća o vremenima zadržavanja ispod jedne minute za TDM imunosupresiva (51,52,54,60,61,78). Takva vrsta postave HPLC je znatno jeftinija i njome se rukuje lakše nego analizom koja uključuje tehnologije za izmjenu kolona. Stoga i nije iznenađujuće da ona predstavlja tehnološku osnovu trenutno na tržištu jedine analize za TDM koja se zasniva na HPLC-MS/MS i ima ovjeru CE te funkcionira bez prethodnog pročišćavanja uzorka na neintegriranoj SPE. Nedostatci takve postave mogu se vidjeti u nepotpunom pročišćavanju uzorka koje dovodi do neželjenih učinaka potiskivanja iona i povećanog zagađenja masenog spektrometra. Za izbjegavanje takvih problema korisno je primijeniti protokole za pročišćavanje uzorka utemeljene na neintegriranom SPE ili visoke omjere razrjeđenja uzorka u fazi taloženja proteina. Nadalje, zbog povećane kontaminacije kolone za HPLC preostalim sastojcima matriksa postiže se isključivo kratak vijek trajanja kolona - obično ispod 1000 injiciranja. Uz gore opisanu postavu integrirane SPE-HPLC-MS/MS ostvarenu u našem laboratoriju, kolone za SPE izdrže 2000 injiciranja matriksa, a kolone za HPLC se mogu koristiti za više od 6000 uzoraka. Time su troškovi za potrošni HPLC materijal (uključujući i bočice za HPLC) blizu 0,5 EUR po uzorku. To ujedno čini spektrometriju masa, unatoč visokim troškovima analitičke strojne podrške i laboratorijskih prilagodbi, čak i u financijskom smislu jakim konkurentom rutinskoj analitici zasnovanoj na imunokemijskim analizama, uz već dobro poznate kvalitativne prednosti kao što su poboljšana točnost, smanjena nepreciznost, te smanjeni učinci matriksa.

Danas predvidljivo kretanje u optimiranju analitičkih platformi za HPLC-MS/MS prema sve većem protoku uzoraka uključuje uporabu kolona s manjim unutarnjim promjerima napunjenu materijalima sa smanjenom veličinom čestica (1,8 µm) na koje se djeluje većim brzinama protoka i višim temperaturama. Međutim, zbog očekivanih viših povratnih tlakova, nužno je poboljšati i stupanj strojne opreme kako bi ispunila te povećane zahtjeve. Drugi trend uključuje spajanje dviju (ili više ako je to primjenljivo) neovisnih HPLC-jedinica u jedan maseni spektrometar, što se uobičajeno naziva "umnogostručenje". Pri tome se HPLC-jedinica spaja na MS kroz ventil kao prekidač isključivo tijekom vremena ispiranja analita iz analitičke kolone. Tijekom uvođenja uzorka kao faze integrirane SPE te ponovljenog uravnoteženja SPE, takva se jedinica odvaja od MS omogućujući pripajanje drugog HPLC na MS. Radi toga radna programska podrška treba biti u stanju potpuno kontrolirati istodobno dva ili više sustava za HPLC (istovjetne preklapajuće metode; sisaljke moraju biti pod punim nadzorom čak i ako nisu integrirane u maseni spektrometar). Kontrolna programska podrška masenog spek-

Alternatively to this two-dimensional HPLC setup, which ensures maximum purification of a sample prior to its entering the mass spectrometer, one dimensional HPLC setup is often realized. Such setup makes possible lower cycling times, while retention times below one minute were frequently reported for immunosuppressant TDM (51,52,54,60,61,78). This type of HPLC setup is significantly cheaper and easier to handle than an assay involving column switching technologies. Hence it is not surprising that it is the technological backbone of the currently only CE certified HPLC-MS/MS-based TDM assay on the market operating without offline SPE sample pre-purification. Drawbacks of this setup can be seen in an incomplete sample purification leading both to unwanted ion suppression effects and an increased pollution of the mass spectrometer. To circumvent these problems, the application of offline SPE-based sample purification protocols or high sample dilution ratios in the protein precipitation step can be beneficial. Furthermore, due to the increased contamination of the HPLC column by residual matrix components, only short column life times – usually below 1000 injections – are achieved. With the above described online-SPE-HPLC-MS/MS setup realized in our laboratory, SPE columns last about 2000 matrix injections and HPLC columns can be used for more than 6000 samples. This brings the HPLC consumable costs (including HPLC vials) close to 0.5 € per sample. This makes mass spectrometry, despite high costs for analytical hardware and laboratory adaptations, even in financial terms a strong competitor to immunoassay-based routine analytics, besides the well known qualitative advantages as improved accuracy, reduced imprecision, and reduced matrix effects.

A currently foreseeable trend in optimizing HPLC-MS/MS platforms toward higher throughput is the use of a column of smaller inner diameters packed with materials of decreased particle size (1.8 µm) to be operated at higher flow rates and increased temperatures. However, due to expectable higher backpressures, the hardware equipment has to be upgraded to meet these requirements. A second trend involves the coupling of two (or more if applicable) independent HPLC units to one mass spectrometer, commonly known as "multiplexing". Herein, a HPLC unit is connected to the MS through a switching valve only in the time window of analyte elution from the analytical column. During sample injection, the online SPE step, and the SPE re-equilibration, this unit is separated from the MS allowing the other HPLC to be connected to the MS. Hence, the operating software has to be able to completely control two or more HPLC systems simultaneously (identical overlapping methods; the pumps have to be under full control even if being offline from the MS device). These requirements are usually not fulfilled by the controlling software of the MS. Currently such a setup

Seger C., Griesmacher A.	Tandem mass spectrometry in the clinical laboratory

trometra obično ne ispunjava te zahtjeve. Trenutačno je takvu postavu ostvario barem jedan neovisan prodavač opreme za HPLC.

Detekcija analita

U rutinskoj dvojnoj spektrometriji masa, primjena praćenja odabrane reakcije (SRM), koje je također poznato kao praćenje višestrukih reakcija (MRM), jest najčešće mjerenje kod MS/MS. Izlazna tekućina iz HPLC koji se sastoji od analita, sastojaka matriksa i mobilne faze dovodi se do izvora iona kroz čeličnu kapilaru koja služi kao elektroda. Tu svi sastojci matriksa koje je moguće ionizirati dobivaju naboj, dok veći dio otapala ostaje bez naboja i treba se ukloniti. Dodatci mobilnoj fazi, kao što su hlapljive organske kiseline i soli (npr. trikloroctena kiselina, mravlja kiselina, octena kiselina i njihove amonijeve soli) olakšavaju proces ionizacije prinosom iona. Odabir analita započinje već u ionskom izvoru jer su parametri strojne podrške (tj. temperatura izvora, potencijal za razdvajanje) usklađeni tako da je maksimalno povećana relativna količina iona željenog analita (tj. ion amonijeve skupine u slučaju imunosupresivnih lijekova). Nadalje se taj primarni ion odabire unutar prvog kvadropola. Zbog prirode tog procesa svi ionizirani sastojci medija pod istim tlakom s omjerom mase i naboja (m/z) koji je istovjetan analitu također se odabiru, dok su ioni s različitim vrijednostima m/z izbačeni iz ionske putanje. Reakcijom plinske faze u sudarnoj stanici primarni ion analita se razgrađuje u postupni niz produkata reakcije. Taj se proces može optimirati kako bi se povećao prinos odabranog ionskog fragmenta analita te se strogo kontrolira postavkama MS. Nadalje, očitava se omjer m/z samo sekundarnog iona u trećem kvadropolu te se prenosi na ploču detektora. Takva kombinacija tvorbe primarnog iona (izvora), odabira iona (kvadropol 1), tvorbe ionskog fragmenta (sudarna stanica) i odabira ionskog fragmenta (kvadropol 2) čini osnovu visoke selektivnosti dvojne spektrometrije masa. Tradicionalno se za analit bilježe dva SRM; jedan služi kao kvantifikator, a drugi (obično onaj manje osjetljivosti) kao dodatna kontrola kvalitete kojom se dokazuje identitet analita, pa se stoga naziva kvalifikator.

S obzirom da se u određenoj vremenskoj točci može zabilježiti samo jedan SRM (ne postoji istodobno mjerenje!), programska podrška masenog spektrometra mora kružiti kroz sve željene reakcije MS/MS u vremenskom okviru jedne milisekunde. Trajanje prikupljanja podataka za svaku odabranu ionsku reakciju, tj. period prekida, jest važan parametar za optimiranje analize koja mora pratiti niz analita. S jedne strane, osjetljivost masenog spektrometra opada s kraćim periodima prekida zbog relativnog porasta buke. Periodi prekida ispod 50 ms često se ne mogu primijeniti, premda ih je moguće lako ostvariti pomoću suvremenih uređaja. S druge strane, kromatografske vršne

has been realized by at least one independent vendor of HPLC equipment.

Analyte detection

In routine tandem mass spectrometry, the use of selected reaction monitoring (SRM), also known as multiple reaction monitoring (MRM), is the predominant MS/MS experiment. The HPLC effluent consisting of analytes, matrix constituents, and mobile phase is introduced to the ion source via a steel capillary serving as an electrode. Thus all ionizable matrix constituents become charged whereas the bulk solvent remains uncharged and has to be removed. Mobile phase additives, like volatile organic acids and salts (e.g. trichloroacetic acid, formic acid, acetic acid and their ammonium salts) facilitate the ionization process by providing ions. Analyte selection starts already in the ion source, since hardware parameters (i.e. the source temperature, declustering potential) are tuned in such a way that the relative amount of the desired analyte ion (i.e. the ammonium cluster ion in the case of immunosuppressant drugs) is maximized. Subsequently, this primary ion is selected within the first quadrupole. Due to the nature of this process, all ionized isobaric matrix constituents with the mass to charge (*m/z*) ratio identical to the analyte are also selected, whereas ions of different *m/z* values are ejected from the ion trajectory. By gas phase reaction in the collision cell, the primary analyte ion is degraded to a cascade of reaction products. This process can be optimized to increase the yield of the chosen fragment ion of the analyte and is tightly controlled by the MS settings. Subsequently, only the secondary ion *m/z* ratio is read out by the third quadrupole and transferred to a detector plate. This combination of primary ion formation (source) – ion selection (quadrupole 1) – fragment ion formation (collision cell) and fragment ion selection (quadrupole 2) is the basis of the high selectivity of tandem mass spectrometry. Traditionally two SRMs are recorded for an analyte, one serving as a quantifier and the other (usually the less sensitive one) being used as the additional quality control proving the identity of the analyte and hence named qualifier.

Given that only one SRM can be recorded at a certain time point (no simultaneous measurement!), the mass spectrometer software has to cycle through all desired MS/MS reactions on a millisecond timescale. The duration of data collection for each selected ion reaction, the dwell time, is an important parameter for optimizing an assay which has to monitor a series of analytes. One the one hand, the sensitivity of a mass spectrometer decreases with shorter dwell times due to a relative increase in noise. The dwell times below 50 ms are often not applicable although easily realizable with modern-day instrumentation. On the other hand, chromatographic peaks elute within seconds

vrijednosti se ispiru tijekom sekundi u brzim analizama i širine tih vrijednosti < 10 s su većinom normalne. Kako je u kromatografiji dobro poznato da se vršna vrijednost analita treba karakterizirati s barem 10-15 točaka da bi se omogućila precizna integracija, povezana vremena ciklusa SRM trebala bi biti prilično ispod 1 s. Stoga - kao primjer izračuna s periodima prekida od 50 ms kod svih prijelaza, - nije moguće pratiti koeluirajuće vršne vrijednosti kod više od desetak SRM. To pak iznosi pet analita i jedan interni standard, ako se prate kvantifikatori i kvalifikatori.

Kako je pristup sa SRM usredotočen na pojedinačne analite, nije moguće promatrati istodobno isprane sastojke matriksa kao što su endogene komponente (tj. fosfolipid, kolesterol itd.) ili ksenobiotici (tj. lijekovi bilo koje vrste) koji mogu kvalitativno i kvantitativno varirati od bolesnika do bolesnika. Kako bilo koji element matriksa može uzrokovati neželjene gubitke signala zbog potiskivanja iona (35-37), takva pitanja moraju podrobno obraditi protokoli za validaciju metode HPLC-MS/MS. Kod uzoraka bolesnika sa svojstvima za koje je poznato da pokazuju analitičke interferencije u drugim rutinskim analizama (povišene koncentracije hemoglobina, bilirubina, triglicerida, kolesterola itd.) obvezatno treba provjeriti učinke potiskivanja iona, kao i kod lijekova koji se često koriste u bolesničkoj skrbi (tj. antivirusni ili antifungalni lijekovi u slučaju transplantiranih bolesnika). Pokusi s dodavanjima u kojima se koriste problematični uzorci bolesnika (tj. visoko iktèrični ili lipemični uzorci) pojačani s poznatim koncentracijama ispitivanih analita omogućuju mjerenje razlika u ponovnom dobivanju signala analita u usporedbi s uzorcima zdravih ispitanika pod istovjetnim tretmanom, u što su uključeni i učinci potiskivanja iona te interferencije matriksa u fazi ekstrakcije. Pokusi koji se temelje na uštrcnoj crpki, a u kojima se uvodi postojani protok ispitivanih analita u izlaznu tekućinu u HPLC između kolone za HPLC i masenog spektrometra korištenjem t-komadnog priključka, predstavljaju drugu dobro procijenjenu mogućnost za ispitivanje učinaka potiskivanja iona (37). Uzorcima zdravih ispitanika, koji su bez analita koji se ispituju, dodaju se ksenobiotici u klinički relevantnoj koncentraciji. Primjenjuje se uobičajena priprema uzorka koja se koristi za rutinske analitičke uzorke te se otopine uzorka uvode u sustav HPLC-MS/MS. Kako bi se nastali kromatogrami trebali pojaviti kao ravne crte zbog stalnog protoka analita iz uštrcne crpke, bilo kakvo odstupanje od te idealne situacije (koja se može oponašati ispitivanjem injiciranja uzorka otapala) istovjetno je poremećaju signala zasnovanog na matriksu. Dodatne vršne vrijednosti, koje je moguće prosuditi kao križne reakcije, jedva da se zapažaju. Češće se zapaža iznenadan gubitak signala analita - potiskivanje iona. Ako se to događa sustavno, tj. u bilo kojem uzorku čak i bez dodatka ksenobiotika, potrebno je istražiti alternativne strategije pripreme uzorka. Učinci potiskivanja iona u prisutnosti lijeka koji se često daje zajedno s ispitivanim

in fast assays, and peak widths of < 10 s are rather normal. Since it is well known in chromatography that an analyte peak has to be characterized by at least 10-15 data points to allow precise integration, associated SRM cycle times should be well below 1 s. Hence – as a calculation example using 50 ms dwell times on all transitions – not more than about a dozen SRMs can be monitored for co-eluting peaks. This translates to five analytes and one internal standard, if quantifiers and qualifiers are monitored.

Since the SRM approach focuses on single analytes, coeluting matrix constituents as endogenous components (i.e. phospholipids, cholesterol, etc.) or xenobiotics (i.e. drugs of any kind), which may vary qualitatively and quantitatively from patient to patient, cannot be observed. As any matrix element can give rise to unwanted signal losses due to ion suppression (35-37), HPLC-MS/MS method validation protocols must address such issues in detail. Patient samples with the properties known to show analytical interferences in other routine assays (increased levels of hemoglobin, bilirubin, triglycerides, cholesterol, etc.) have to be checked for ion suppressive effects, as well as medications commonly used for patient care (i.e. antiviral or antifungal drugs in the case of transplant patients). Spiking experiments using problematic patient samples (i.e. highly icteric or lipemic specimens) enforced with the known levels of the analyte under investigation allow the testing of differences in analyte signal recovery compared to healthy subject samples treated identically, covering both ion suppression effects and matrix interferences in the extraction step. Syringe pump-based experiments introducing a steady flow of investigated analytes into the HPCL effluent between the HPLC column and the mass spectrometer by using a t-piece connector are another well evaluated possibility to investigate ion suppression effects (37). Samples from healthy subjects which are free from the analytes under investigation are spiked with xenobiotics to a clinically relevant concentration. Conventional sample preparation, as used for routine analytical samples, is applied and sample solutions are introduced to the HPLC-MS/MS system. Since the resulting chromatograms should appear as flat lines due to constant analyte flow from the syringe pump, any deviation from this ideal situation (which can be mimicked by investigating the injection of a solvent sample), equals a matrix-based signal disturbance. Additional peaks, which can be judged as cross reactions, are hardly observed. More often a sudden loss of analyte signal – the ion suppression – can be observed. If this takes place systematically, i.e. in any sample even if no xenobiotics have been added, alternative sample preparation strategies should be sought. Ion suppression effects in the presence of a drug frequently co-administered with the analyte under investigation are more critical. If chromatographical co-elution with the analyte occurs, irreproducible quantitative results of the

analitom su pogibeljniji. Ako se kromatografsko ispiranje odvija zajedno s analitom, ne mogu se isključiti neponovljivi kvantitativni rezultati analize TDM. U takvom slučaju treba provesti dodatna mjerenja kao provjeru bilo primjenom promijenjenih uvjeta kromatografije ili dodatnom provedbom SRM moguće interferirajuće tvari u pokusu.

Analiza podataka

Analiza baznih podataka kromatograma utemeljenih na HPLC-MS/MS obično uključuje fazu "poravnanja" podataka prije potpuno automatizirane identifikacije vršnih vrijednosti. Integracijski algoritmi se dostavljaju uz svaki maseni spektrometar i obično se cijele serije (npr. sve serije u istom danu) mogu obraditi unutar nekoliko minuta. Ipak, provjera cjelovitosti vršnih vrijednosti je nužna kao dio procesa tehničke validacije jer se pogoršanje procesa stvaranja iona, koje je obično rezultat kontaminacije ionskog izvora ili istrošenih prijenosnih kapilara, brzo ogleda u gubitku uzorka vršnih vrijednosti. Sve se vršne vrijednosti analita uspoređuju s odgovarajućim internim standardom, a omjeri područja vršnih vrijednosti čine osnovu za kvantifikaciju analita. Kadgod je to moguće, treba koristiti komercijalno dostupne kalibracijske i kontrolne materijale s ovjerom CE; kalibratori s više razina su obvezatni kod validacije analiza (barem ako se primjenjuje smjernica FDA (28) o validaciji bioanalitičkih metoda).

Kontrola kvalitete i održavanje

Osim provjera preciznosti i točnosti analize praćenjem kontrole kvalitete i podataka kalibratora kroz određeni period (Slika 5.), potrebno je provoditi i niz provjera strojne podrške kako bi se osigurala dugotrajna postojanost postave uređaja. Obvezatan je i nadzor tlaka u kromatografskom sustavu jer je porast povratnog tlaka obično rani znak začepljenja kolona za HPLC zbog taloženja medija. Kolone za HPLC mogu se blokirati nakon dulje uporabe tako da preventivna zamjena može smanjiti isključenost uređaja iz rada. Kućište ionskog izvora treba često provjeravati radi vidljivih kontaminacija, te redovito čistiti pokretne dijelove strojne podrške koji štite MS radi izbjegavanja kontaminacija kvadropolova. Kvaliteta vakuuma ovisi o sustavu isisavanja; stoga se savjetuje česta provjera kvalitete ulja u prvom stupnju sisaljke. Glede postojanosti masenog spektrometra, djelotvornost prijenosa iona i razlučivosti mase trebali bi biti stalni kroz duga razdoblja u rutinskim analitičkim uvjetima. Preporučljivo je, ipak, provjeriti učinkovitost MS/MS provedbom pokusa usklađivanja prema uputama prodavača. Zadnje, ali ne i najmanje važno jest da se obvezatno treba procijeniti postojanost svake mobilne faze i materijala za pripremu uzoraka, uključujući i matične otopine internog standarda, a pravila za pohranu uzoraka treba razviti ili primijeniti iz literaturnih podataka. Stalna izobrazba osoblja te dobro napisani protokoli za rad upotpunjuju skup mjera za kontrolu

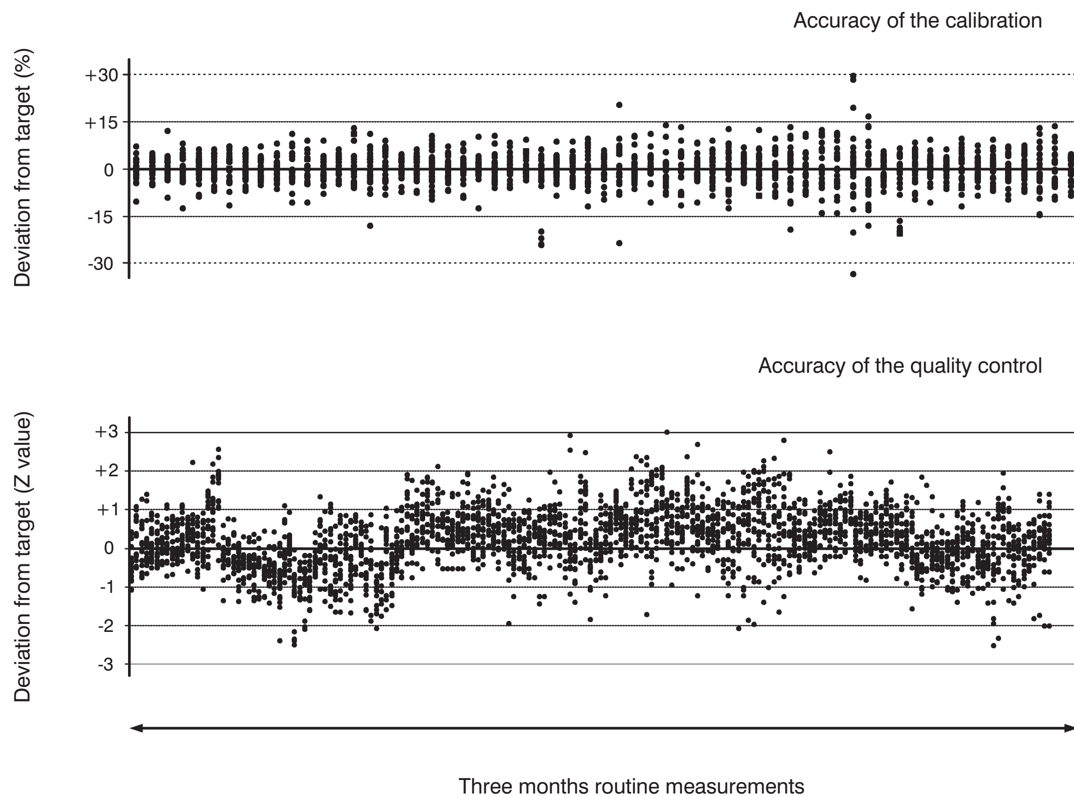
TDM assay cannot be ruled out. In such case additional verification measurements should be carried out either by using changed chromatographic conditions or by adding a SRM of the possibly interfering substance to the experiment.

Data analysis

Raw data analysis of HPLC-MS/MS-based chromatograms usually includes a data smoothing step prior to fully automated peak identification. Integration algorithms are supplied with any mass spectrometers, usually whole batches (e.g. all runs of one day) can be processed within minutes. Nevertheless, peak integration should be checked as part of the technical validation process since deterioration of ion formation process, usually due to ion source contaminations or worn out transfer capillaries, quickly mirrors the loss of peak shapes. All analyte peaks are referred to the respective internal standard, and peak area ratios are the basis of analyte quantification. Whenever possible, commercially available CE certified calibration and control materials should be used; multilevel calibrators are a must for assay validation (at least if the FDA guideline on bio-analytical method validation (28) is applied).

Quality Control and Maintenance

Besides checking the precision and accuracy of an assay by monitoring quality control and calibrator data over time (Figure 5), a series of hardware checks should be performed to ensure the long term stability of the setup. Pressure profiles of chromatographic runs have to be surveyed since the build up of back-pressure is usually an early sign of congestions of HPLC columns due to the precipitation of sample matrix. HPLC columns might block after prolonged use, and a preventive change can reduce the instrument down time. The ion source housing has to be frequently checked for visible contaminations, removable hardware pieces shielding the MS should be cleaned on a regular basis to avoid contaminations of quadrupoles. The quality of the vacuum relies on the pumping system; hence a frequent check on the oil quality of the first stage pumps is advisable. Regarding the stability of a mass spectrometer, ion transfer efficiency and mass resolution should be stable over quite long time spans under routine analysis conditions. Nevertheless, it might be advisable to check the MS/MS performance by running tuning experiments as suggested by the vendor's guidelines. Last but not least, the stability of any mobile phases and sample preparation materials including the internal standard stock solutions has to be assessed, and the rules for sample storage have to be developed or derived from literature data. Recurrent personnel training sessions and well written operation protocols complete the package of quality control measures toward a stable and well working HPLC-MS/MS platform.



SLIKA 5. Dugotrajna postojanost analize HPLC-MS/MS za TDM imunosupresiva u Innsbrucku. Standardizirani grafovi odstupanja komercijalnog, s CE ovjerenog višeanalitnog kalibratora i materijala za kontrolu kvalitete koji sadrži ciklosporin A, takrolimus, sirolimus i everolimus, u kojima je sažet tromjesečni period mjerenja. Podatci (64 vremenskih točaka s 24 podatkovnih točaka za svaku) o višestupanjskom (N = 6) kalibratoru predstavljeni su kao postotno odstupanje od dodijeljene ciljane vrijednosti. Za uspješnu kalibraciju, 4 od 6 podatkovnih točaka moraju biti unutar granice od 15%. Višestupanjski (N = 4) podatci o kontroli kvalitete (181 vremenska točka unutar 24 podatkovne točke za svaku) prikazani su kao odstupanje vrijednosti z (korišteni su rasponi 2S određeni od proizvođača) od dodijeljene ciljane vrijednosti.

Figure 5 Long-time stability of the HPLC-MS/MS immunosuppressant TDM assay at Innsbruck. Standardized deviation plots of commercially CE certified multi-analyte calibrator and quality control materials containing cyclosporine A, tacrolimus, sirolimus, and everolimus summarizing a three-month measurement period. The multilevel (n = 6) calibrator data (64 time points with 24 data points at each) is presented as percentage deviation from the assigned target value. For successful calibration, 4 out of 6 data points have to be within the 15% margin. The multilevel (N = 4) quality control data (181 time points within 24 data points at each) is given as z-value deviation (2S ranges assigned by the manufacturer were used) from the assigned target value.

kvalitete kojima je cilj postojanost i dobro funkcioniranje uređaja za HPLC-MS/MS.

Usporedivost s imunokemijskim analizama

Posebne razlike zbog metode zapažene su između metoda HPLC-MS/MS i imunokemijskih analiza između različitih proizvođača za svaki praćeni imunosupresijski lijek. Čak i različite provedbe imunokemijskih analiza od strane iste tvrtke mogu pokazati značajne razlike u kvantitativnim rezultatima ako se međusobno usporede (najvjerojatnije zbog različitih korištenih antitijela i razlika u metodama detekcije), te prema HPLC-MS/MS. Te razlike ne samo da su bile opisane u literaturi (47,48,49,52) nego se ogledaju i u rezultatima programa testiranja dijagnostičke učinkovitosti u Europi i Sjevernoj Americi.

Comparability to immunoassays

For any of the immunosuppressant drugs monitored, a distinct method bias has been observed between HPLC-MS/MS methods and immunoassays of various producers. Even different immunoassay realizations provided by the same company can show significant differences in quantitative results if compared among each other (most likely due to different antibodies used and due to differences in detection methods) and to HPLC-MS/MS. These differences were not only described in the literature (47,48,49, 52), but are also mirrored by proficiency testing scheme results in Europe and Northern America.

Since assay deviations to the HPLC-MS/MS are mainly based on the cross-reactivity of immunoassay antibodies with drug metabolites (11,12,13,79,80), an observed bias

Kako se odstupanja prema HPLC-MS/MS uglavnom temelje na križnoj reaktivnosti antitijela u imunokemijskoj analizi s metabolitima lijeka (11,12,13,79,80), zapažena razlika predstavlja individualan parametar koji snažno ovisi o brzini metaboliziranja osnovnog lijeka. Odstupanja od bolesnika do bolesnika moraju se očekivati, kao i nestalnost razlika kod pojedinačnih bolesnika ako se prate kroz vrijeme zbog, primjerice, promjenljivosti općega zdravstvenog stanja ili promjena u koktelu istodobno uzetih lijekova. Kao posljedica toga srednje vrijednosti razlike pokazuju visoki koeficijent varijacije, tipično u rasponu od 20 do 30%. To čini "razliku zbog metode", o kojoj je izvještavano u literaturi, nedjelotvornom u vođenju kliničara kroz promjenu metode kad su u pitanju imunokemijske analize. Stoga jedva da je korisno procijeniti utjecaj promjene analitičke platforme na korištene terapijske raspone. Prema našem osobnom uvjerenju i temeljem naših vlastitih zapažanja u laboratoriju, samo dulje usporedno mjerenje podataka iz TDM (potrebno je nekoliko tisuća podatkovnih točaka) na obje analitičke platforme, tj. "nove" i one koja se zamjenjuje - uključujući i stvaranje longitudinalnih profila bolesnika, - može uključenim kliničarima pružiti pouzdanje koje je potrebno za uspješno uvođenje nove analitičke metode kao što je HPLC-MS/MS za TDM imunosupresiva.

Zaključak

Maseni spektrometri su među najskupljim analitičkim uređajima primjenjivima za rutinsku analizu u kliničkim laboratorijima. Ipak, oni su konkurencija imunokemijskim analizama kako s obzirom na kvalitetu analize, tako i po troškovima po analizi. Analize koje se zasnivaju na dvojnoj spektrometriji masa visoko su selektivne i osjetljive; precizno mjerenje ciljanog analita bez križnih reakcija s bilo kojim drugim strukturno srodnim ili nesrodnim analitima je standardno. Rutinska analiza metodom HPLC-MS/MS u femtomolskom rasponu je provediva i time čini analize zasnovane na spektrometriji masa čak i osjetljivijima od mnogih danas dostupnih imunokemijskih analiza. U slučaju TDM imunosupresiva granica kvantifikacije može se lako sniziti za čimbenik 5-10 u usporedbi s bilo kojim dostupnim imunokemijskim analizama. Uporabom strategije obrade uzorka zasnovane na integriranim SPE-HPLC, troškovi po analizi mogu se smanjiti do ispod 1 EUR, a uz kraći vijek kolone, kao što je to slučaj u jednodimenzijskim postavama HPLC, moguće je dostići prag od 2 EUR po uzorku. To nas ostavlja s razlikovnim čimbenikom 5-10 u odnosu na komercijalne imunokemijske analize čime je, ako je protok uzoraka dovoljno velik, omogućena brza amortizacija početnih troškova za strojnu podršku od 250.000 - 300.000 EUR. Uzevši sve to u obzir, ciljana analiza zasnovana na HPLC-MS/MS zasigurno predstavlja nadu u budućnost rutinskih kliničkih analiza koja se već danas

is an individual parameter, strongly depending on the metabolization rate of the administered parent drug. Deviations from a patient to patient have to be expected, as well as bias fluctuations of individual patients if monitored over time due to, e.g., variations of the overall health state or due to changes in the cocktail of co-administered drugs. As a consequence, mean bias values show a high coefficient of variation, typically in the range of 20–30%. This makes "the method bias" as reported in the literature inefficient to escort clinicians through a method change if immunoassays are involved. Consequently, it is hardly useful to evaluate the impact of an analytical platform change on the used therapeutic ranges. To our personal conviction and from our own observations at our laboratory, only a prolonged parallel measurement of TDM data (several thousand data points are needed) on both analytical platforms – the "new one" and the one to be replaced, including the generation of longitudinal patient profiles, can give the involved clinicians the confidence needed for successful introduction of a novel analytical method like HPLC-MS/MS for immunosuppressant TDM.

Conclusion

Mass spectrometers are among the most expensive analytical devices usable for routine analysis in clinical laboratories. Nevertheless, they are competitive to immunoassays both in terms of assay quality and costs per analysis. Tandem mass spectrometry-based assays are highly selective and sensitive; the precise measurement of a targeted analyte without cross-reactions to any other structurally related or unrelated analytes is standard. Routine HPLC-MS/MS analysis in the femtomole range is feasible, making mass spectrometry-based assays even more sensitive than many currently available immunoassays. In the case of immunosuppressant TDM, the quantification limit can be easily lowered by a factor of 5–10 compared to any available immunoassays. Using an online-SPE-HPLC- based sample workup strategy, costs per analysis can be cut below 1 € and, with shorter column life times as in one-dimensional HPLC setups, a 2 € per sample threshold can be reached. This leaves still a gapping factor of 5-10 to commercial immunoassays, allowing a swift amortization of the initial hardware costs of about 250.000–300.000 € if the sample throughput is high enough. Taken together, HPLC-MS/MS-based target analysis is certainly a promise for the future of routine clinical analyses currently starting to become real. It can be expected that all major vendors will within the forthcoming years become more and more aware of this market. Hence the essential improvements in machine handling and software will become real and mass spectrometer packages will develop from research-oriented instruments to routine equipment. It can be expected that sooner or later several CE-certified

počinje ostvarivati. Može se očekivati da će svi glavni prodavači tijekom nadolazećih godina postajati sve svjesniji tog tržišta. Stoga će osnovna poboljšanja u strojnom rukovanju i programskoj podršci postati stvarna i paketi spektrometrije masa razviti će se iz instrumenata usmjerenih na istraživanja u rutinsku opremu. Prije ili kasnije može se očekivati da će biti dostupno nekoliko analiza s ovjerom CE, kao što je danas slučaj za analize zasnovane na HPLC-UV i HPLC-FLD, a možda ih čak i zamijeniti.

assays will be available as they are nowadays for HPLC-UV and HPLC-FLD-based analyses, maybe even replacing them.

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