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Review

The role of liquid chromatography–tandem mass spectrometry in the clinical laboratory☆

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ABSTRACT

Liquid chromatography coupled to mass spectrometry (LC–MS/MS) is increasingly used as a routine methodology in clinical laboratories for the analysis of low molecular weight molecules. The high specificity in combination with high sensitivity and multi-analyte potential makes it an attractive complementary method to traditional methodology used for routine applications. Its strength and weaknesses in this context will be discussed and examples of successful clinical applications will be given. For LC–MS/MS to truly fulfil its promise in clinical diagnosis, the prerequisite steps being sample pre-treatment, chromatographic separation and detection by selected reaction monitoring must become more integrated as they are in conventional clinical analysers. The availability of ready-to-use reagents kits, eliminating efforts needed for method development and extensive validation, are likely to contribute to a wider acceptance of LC–MS/MS in clinical laboratories. Growing applicability of LC–MS/MS in the clinical laboratory field is expected from quantitative protein analysis.

Contents

Abbreviations: LMW, low molecular weight; LC–MS/MS, liquid chromatography–tandem mass spectrometry; IAC, immunoaffinity chromatography; APEI, atmospheric pressure electrospray ionization; IS, internal standard; HILIC, hydrophilic interaction chromatography; LDTD, laser diode thermal desorption; APCI, atmospheric pressure chemical ionization; UHPLC, ultra-high performance liquid chromatography; SRM, selected reaction monitoring; SPE, solid phase extraction; GC, gas chromatography; IVD, in vitro diagnostics; PFP, pentafluorophenyl; ECL, electrochemiluminescence; hrMS, high-resolution mass spectrometry; LLOQ, lower limit of quantification; LOD, limit of detection; 3-MT, 3-methoxytyramine; NM, normetanephrine; M, metanephrine; MIP, molecularly imprinted polymer; SCX, strong cation exchange; MMA, methylmalonic acid; HVA, homovanillic acid; VMA, vanillylmandelic acid; WCX, weak cation exchange; RIA, radioimmunoassay; SA, succinic acid.

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1. Scope

This review focuses on the use of liquid chromatography coupled to mass spectrometry (LC–MS/MS) for analysis of low molecular weight (LMW) biomarkers for laboratory diagnostic purposes. It describes what LC–MS/MS can bring to a clinical laboratory, what are its pros and cons. It does, however, not pretend to give a comprehensive overview of all aspects related to this technique, as for each step (sample preparation, chromatography, matrix effects, automation, pitfalls etc.) extensive reviews have been published [1–4]. What we have tried here is to give our view on what is needed for LC–MS/MS to further become successful in clinical practice, show its strengths and weaknesses and some future perspectives.

2. Introduction

Once being a highly specialized analytical technique, LC–MS/MS is now increasingly becoming accepted as a routine diagnostic tool in clinical laboratories. Mass spectrometry has a relatively long history. Over 50 years ago gas chromatography coupled to MS (GC–MS) was introduced in the clinical diagnostic field. It required volatile compounds, and thus polar analytes had to be derivatized. Despite the facts that it required expert knowledge of mass spectrometry and necessitated laborious sample preparation, specialized diagnostic laboratories adopted this technique because of its enhanced sensitivity and specificity. With the introduction of innovative soft ionization techniques as electrospray ionization (ESI) at atmospheric pressure (API), HPLC coupling to mass spectrometry became accessible, allowing LMW molecules to be ionized in liquid phase [5].

Most clinical laboratories use triple quadrupole mass spectrometry in the selected reaction monitoring (SRM) mode for target analysis.

Over the past decade, LC–MS/MS has been transformed into an accessible analytical technique, thereby becoming widely accepted also in the field of clinical chemistry. MS was first introduced as a routine technique in clinical laboratories in the areas of newborn screening, drug screening, and the diagnosis of organic metabolic disorders [6]. Early-day instruments were sensitive enough for detecting metabolites as amino acids and acyl-carnitines which appear in the micro- to millimolar range (Fig. 1). In order to become

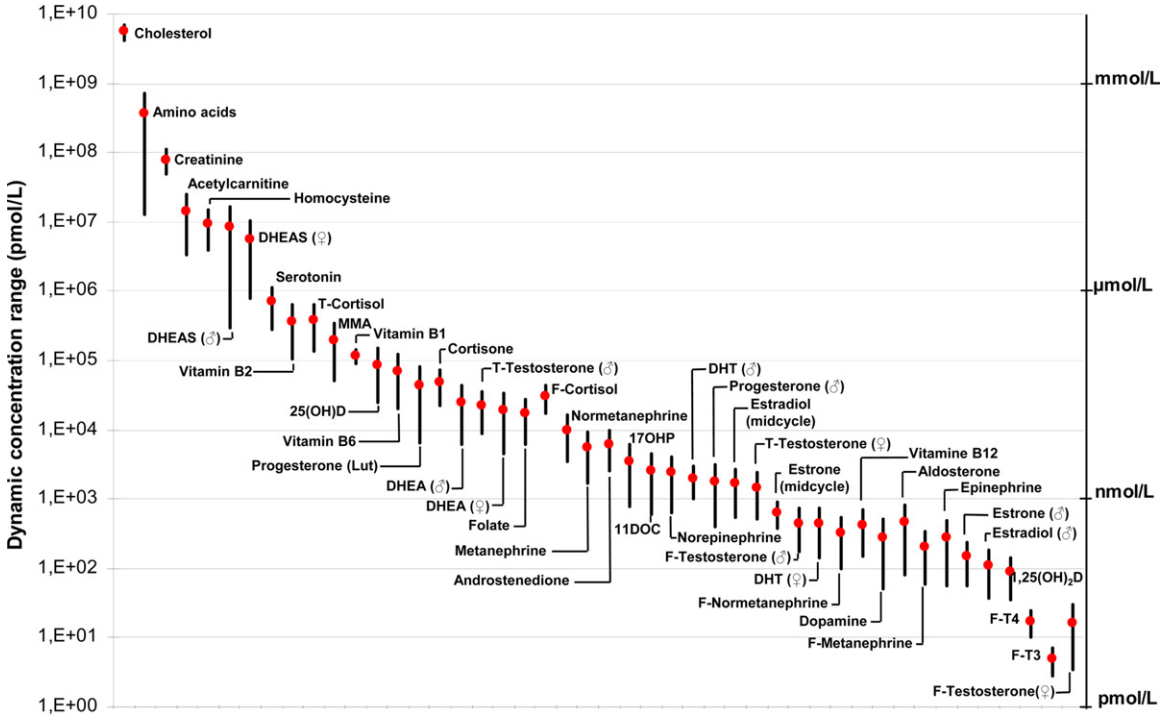

Fig. 1. Dynamic range of LMW biomarkers in human adult serum. Shown are mean and ranges. MMA, methylmalonic acid; DHEA(-S), dehydroepiandrosterone(-sulphate); 25(OH)D, 25-hydroxyvitamin D; 17OHP, 17-hydroxyprogesterone; 11-DOC, 11-deoxycortisol; DHT, dihydrotestosterone; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D; T4, thyroxine; T3, triiodothyronine; T, total; F, free; lut, luteal; ♂, male; ♀, female.
Adapted from Kushnir et al. [10].

applicable in the field of endocrinology, in which LMW molecules usually are present at much lower concentrations (Fig. 1), more sensitive equipment was required, additional to more sophisticated sample preparation and advanced chromatographic procedures. It is now accepted that LC–MS/MS is the method of choice for screening of inherited metabolic disorders [6], as well as for the measurement of certain steroid hormones, particularly for those which rely on accurate and specific measurement at low concentrations [7].

3. Positioning LC–MS/MS among other diagnostic methodologies

Analysis of LMW compounds can be performed by either manual, semi- or fully automated immunoassays or by chromatography-based techniques such as HPLC, GC(–MS) and more recently LC–MS/MS. Over the years many laboratories have abandoned manual radio-immunoassays, sometimes including extensive purification, and embraced automated analysis of steroids and other LMW compounds for reasons of convenience, throughput and more at the expense of reduced quality for certain analytes, as exemplified by cross-reactivity and diminished sensitivity [8–10]. Additional problems are the lack of concordance between assays, e.g. due to differences in antibodies used, specificity problems due to the nature of interaction of antibodies with small molecules, presence of interfering auto-antibodies (e.g. thyroglobulin, insulin), heterophilic antibodies and hook effects due to limited dynamic range [11–13]. Also, noteworthy is the little progress that has been made in improvement of instrument sensitivity over the last ten years. Measurement of certain steroids, circulating in the picomolar range (e.g. aldosterone, 1,25-dihydroxyvitamin D), still require radioimmunoassay (RIA) for detection.

LC–MS/MS has several advantages over immunoassays for the measurement of steroids, including superior specificity and the ability to quantify entire steroid profiles in a single run. Nevertheless, challenges remain for LC–MS/MS as well in the detection of steroids circulating at low (picomolar) concentrations, e.g. estrogens in postmenopausal women, men and children, androgens in females, children, and males suffering from hypogonadism, and in disease states where specific enzymatic blocks in steroid metabolism are present [10]. Also specificity remains a critical issue in LC–MS/MS, especially in steroid analysis where many fragments used for SRM are shared among the multiple steroids that are present in biological fluids. For an extensive description on steroid analysis by LC–MS/MS readers are referred to a dedicated review by Kushnir et al. [10].

In a comparison of LC–MS/MS to other analytical techniques commonly used for esoteric testing in the diagnostic laboratory, such as HPLC with various detectors and GC(–MS), it is obvious that LC–MS/MS generally has clear advantages over these traditional chromatography-based analysers due to its higher sensitivity, specificity with reduced sample preparation and analysis time.

With the arrival of more sensitive LC–MS/MS instruments, combined with sophisticated sample preparation, certain analytes can now accurately be measured in body fluids that were not suited before due to their low circulating concentrations. Alternatives for the laborious 24 h urine collections (e.g. plasma, saliva) may show improved diagnostic accuracy, easier sample collection, and reduced efforts for both patient and laboratory personnel (e.g. plasma metanephrines [14,15], methylmalonic acid (MMA) [16–18], salivary cortisol [19]. In (neuro)endocrine oncology, rapid testing for exclusion of pheochromocytoma is mandatory before a decision on surgical intervention can be made. In this situation analysis of plasma metanephrines with superior specificity provides added value as and no longer requires time consuming and laborious 24 h urine collection.

Still, the costs and requirements to develop and validate an LC–MS/MS assay in-house may in part have been responsible for the limited application of LC–MS/MS in routine laboratories, especially when compared with readily available immunoassays. Despite this, the move towards greater use of LC–MS/MS in the clinical laboratory seems obvious in the goal of achieving better patient care. Table 1 shows LC–MS/MS performance characteristics in terms of

Table 1
Analysis of LC–MS/MS strength, weakness, opportunities, and threats (SWOT[a]) in clinical diagnostics.

Strengths	Weaknesses
High sensitivity	High instrument costs
High specificity	Serial (batch-wise), non random-access operation
High speed of development at low costs of new assays when compared to immunoassays by IVD companies	Need for highly skilled personnel for method development, validation, operation and troubleshooting
Low costs per sample in terms of reagents	Lack of clearly defined quality regulations
Possibility to measure multiple analytes in the same sample simultaneously	Limited sample throughput in conventional set-up
Versatility	Absence or limited availability of CE/IVD approved reagent-kits
Near reference methodology in routine setting	Limited experience of IVD requirements from MS vendors
Matrix independency (saliva, CSF, urine etc.)	
Compatible with automated sample handling configurations	
Opportunities	**Threats**
Progress towards more user-friendly instruments (with integration of all components into a single system)	Speed of development of new instruments > hard to keep up with (e.g. development of commercially available kits) and requires regular adaptation of routine practice.
Adoption of MS technology by major IVD companies	Growing difficulty finding (skilled) technicians (and experience at an academic level)
Broader availability of CE/IVD approved kits for LC–MS/MS analysis	Lack of commitment from major IVD companies
Quantitative measurement of peptides and proteins	Regulatory bodies applying restrictions on using home-brew assays for diagnostic purposes
Profiling of metabolically related metabolites (context)	Competition from innovations in immunoassays or from the introduction of new technologies

[a] SWOT analysis is a tool for auditing an organization and its environment. It is the first stage of planning and helps marketers to focus on key issues. Strengths and weaknesses are internal factors. Opportunities and threats are external factors.

its strengths (S), weaknesses (W) as internal factors and opportunities (O) and threats (T) as external factors, according to the SWOT model of analysis.

4. Considerations when entering the LC–MS arena

For clinical laboratories considering to start performing LC–MS/MS analyses, several aspects should be taken into account. These include investment costs and return on investment (ROI) in terms of a positive business case, as well as the significant investments in technicians and academically skilled persons to get them experienced in method development, validation, daily operation and troubleshooting. A "minimal scenario" is when LC–MS/MS is used for dedicated analysis of a single high volume test (e.g. immunosuppressants), either using commercially available kits or by use of an instrument that has been fully installed and programmed for this particular activity by the MS vendor. In either way, the hurdle for development and extensive validation is eliminated. Limited training may suffice to cope with troubleshooting activities, with a major role for the MS vendor for keeping the instrument up and running. A second MS instrument often is needed for back-up purposes, since MS vendors cannot (yet) deliver full 24 h service, or only at very high costs. MS vendors as well as other smaller companies have begun making complete kits for LC–MS to facilitate clinical laboratories who, for various reasons, cannot, or do not want to develop their own in-house tests.

4.1. Cost aspects

If LC–MS/MS wants to be an attractive alternative to other methodologies, such as immunoassays, it not only needs to compete on quality grounds, but also on test price.

LC–MS/MS instruments are expensive analytical tools, with investments ranging from €200,000 to €400,000. Expected lifetime is 7–10 years, although a 5 year depreciation scenario would be more realistic given the rapid developments of new generation analysers. Additional infrastructural costs for installation, configuration of the working place, liquid nitrogen supply and costs for training of dedicated technicians and people with academic background, all have to be taken into account. Maintenance costs are approximately 10% of the primary instrument costs but may vary depending on the type of contract, with highest costs for a clinical contract service which guarantees 24 h service including all parts, versus a minimal contract for fixed yearly maintenance only. LC–MS assays make up to <1% of all tests in most hospital laboratories, with exception of some academic settings, commercial and reference laboratories. Nevertheless, these represent the more expensive tests, and cost savings when moving from HPLC, GC or immunoassays to LC–MS may pay off. When coming from manual RIA and HPLC applications, savings can be made both in reduced time for sample preparation as well as in lower reagent costs. We experienced a 33% staff reduction from transfer of HPLC-based assays to LC–MS/MS, aside the better quality tests that were obtained. In order to compete on test price with automated immunoassays, one needs to reach high test volume on LC–MS/MS. Cost-calculations based on maximum throughput scenarios with instruments running 22 h a day, 350 days a year with few minutes runtime per test may be real scenarios for some large commercial or reference laboratories in the United States, where hundreds of thousands of tests are run on a yearly basis and instruments are dedicated for testing a single high volume test. However, most hospital laboratories, at least in Europe, use a single LC–MS/MS instrument for a variety of tests, which make annual throughputs of 50,000 tests per instrument a more realistic scenario. The argument can be made that 20,000 tests per instrument is the lower level for cost justification, but the actual number may vary depending on the test repertoire and on the cost of sending tests out for mass spectrometric analysis. We realize that there are still quite a number of LC–MS/MS laboratories having difficulties reaching these numbers of tests with concomitant problems in operating LC–MS/MS in a cost-effective manner. Instead of pursuing central LC–MS/MS facilities, the tendency, at least in the Netherlands, is that more and more tertiary laboratories acquire their own LC–MS instruments for performing these tests themselves. The downside of such a scenario is that, due to working at sub-maximal instrument capacity, these laboratories will not be operating in a cost-effective manner, aside the investments in training and getting familiar with all aspects of performance of LC–MS/MS. Sometimes LC–MS/MS instruments are shared with adjacent toxicology departments for reasons of cost-effectiveness.

4.2. Expertise and training

"An expert is a person who has made all the mistakes that can be made in a very narrow field" (Niels Bohr). Highly skilled technicians or people with academic background, is a prerequisite for running LC–MS/MS, especially for method development, assay validation and troubleshooting. For running routine applications, technicians with limited knowledge of LC–MS may suffice, depending on the assay and instruments robustness. Costs for getting technicians or academic personnel trained in all aspects of LC–MS/MS are substantial, and will depend on their familiarity with HPLC. A €5–10,000 investment for dedicated courses is not unusual. Many technicians working with LC–MS/MS have experience in GC and/or HPLC. Their long-term experience is vital for coping with all the potential sources of error in such a hyphenated technique as LC–MS being non-robust and complex. However, reality is that many hospital laboratories have increasing difficulties acquiring technicians, even for most of the routine laboratory activities. The arrival of more integrated LC–MS/MS systems that can be operated like clinical immuno-analysers is much awaited, although this will likely take a decade or more before becoming reality.

4.3. Quality issues, accuracy and regulatory compliance

LC–MS/MS in combination with isotope dilution has the inherent potential to high specificity, accuracy, and sensitivity of measurement. However, a mass spectrometry method does not necessarily make it a reference method. Only after thorough validation of an LC–MS/MS procedure, including proper standardization, the inherent potential is warranted [4]. When possible, a routine LC–MS/MS assay should be traceable to a reference measurement procedure. Whereas commercial immunoassays should meet certain requirements (e.g. European IVD Directive, FDA), this at present is not mandatory for laboratories designing their own LC–MS/MS methods, although these may become subject of FDA regulation in the next several years. Current practice is that laboratories have their own responsibility to properly validate their LC–MS/MS tests, using various documents as guidelines (FDA [20–22]). The outcome is that current MS assays are developed and validated in-house using different procedures, instrumentation, reagents and calibrators. This can lead to differences in individual assay performances and in limitations when comparing results. This is exemplified by the fact that in some proficiency testing programmes the LC–MS/MS inter-method imprecision appears not to be superior to other methodologies [23,24]. The use of common calibrators or commercial kits should lead to improvements in inter-laboratory performance [24–26]. Ideally, MS methods should be calibrated on reference material, when available. However, for many in-house LC–MS/MS methods, standards are not always available for development and validation. Herein may lay the failure so

far for MS methods to improve quality of results between laboratories for an analyte using the same technology [22]. Few LC–MS/MS inter-comparison studies have been published to date [27–30]. Most often, LC–MS/MS methods are compared with immunoassays. Often, discrepancies between LC–MS/MS and immunoassay are attributed to antibody cross-reactivity of the immunoassay, but theoretically it might as well originate from poor calibration of the LC–MS method [30]. If properly calibrated, isotope dilution and specificity of detection using SRM mode allows accurate measurement, even when substantial methodological and instrumentation differences among LC–MS/MS assays are present [30].

Long-term stability of a laboratory test is mandatory for routine practice. Commercial immunoassays may show shifts over time due to reformulation of kits (e.g. change of antibody) resulting in large QC windows between different lot-numbers [31]. Inherent to analytical principles, LC–MS/MS tests prove to be more stable over time than commercial immunoassays, as was recently shown in the DEQAS review 2010 report on 25-hydroxyvitamin D (25(OH)D) testing where the distribution of the same serum pool twice over a 6 months period showed large increase in bias for some immunoassay methods (13.5–23.4%), with little change for LC–MS/MS (0.96%).

5. Method development

Before an analytical method can be used in routine clinical practice, acceptable performance is required. The goals for the method's performance characteristics must be established prior to the development of a new method. Method development usually includes an optimization of all individual steps involved in the sample preparation, chromatographic separation, ionization and mass spectrometric detection. Developing methods for LC–MS/MS analysis is in several ways comparable to that of developing methods for traditional HPLC analysis. Setting up protocols for sample preparation, and optimization of chromatographic procedures are, in broad outlines comparable. There are important differences though. Some of the important steps will be highlighted in the following section. For a thorough review on all issues related to method development, we refer to Honour [22].

When planning to set up a method for a specific analyte, it is important to consider the specific analyte characteristics, e.g. what is the expected concentration, in what way is the analyte present in the matrix (free or bound), are there known metabolites with a same mass, are there specific molecular characteristics that can be used in extraction, can the ionization properties be enhanced etc. These and other data are useful for choosing the right strategy during several steps of method development.

5.1. Sample preparation

Sample clean up procedures are dependent on the origin of the sample. The most commonly used sample matrices in clinical laboratories are plasma, serum, and urine. Less common matrices include saliva, blood spot, ultra-filtrates and other bio-fluids such as interstitial and follicular fluid, and tissue homogenates [10]. In general, sample preparation for clinical testing has to be fast, robust and reproducible. Dependent of the workload, the variety of tests to be performed, and the available budget, automation of the sample preparation can be considered.

Analyte extraction usually requires an initial protein precipitation step. This step needs extra consideration in situations where analytes are bound to binding proteins. Extraction can be performed varying from simple dilution ("dilute and shoot"), solvent extraction (i.e. liquid–liquid extraction (LLE)), solid-phase extraction (SPE) (using SPE-columns or 96-well format), or the automation friendly immobilized liquid (in tips or 96-well format) which requires no centrifugation- or vacuum system (Fig. 2), to alternative techniques such as size-exclusion chromatography, affinity chromatography and immuno-affinity chromatography (IAC) [3,1]. The most commonly used methods for (manual) sample preparation at this moment are liquid–liquid extraction and (off-line) SPE. These procedures resemble those used for conventional HPLC analysis. However, in case of LC–MS/MS analysis extra care should be taken to prevent co-extraction of compounds that cause ion suppression, such as phospholipids. For an extensive review on this, we refer to Kole et al. [1]. In the following section we focus on

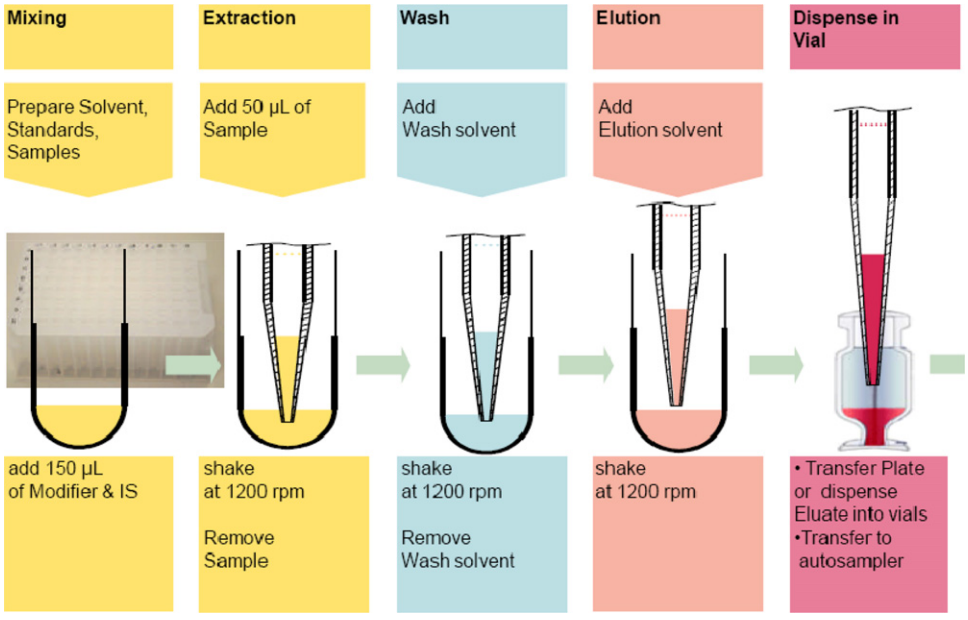

Fig. 2. Sample preparation by immobilized liquid. The wells of a deep well plate are coated with immobilized liquid and used as the extraction device. A similar workflow can be applied to coated inserts stuffed in a disposable tip.
Courtesy of TECAN AG, Männedorf, Switzerland.

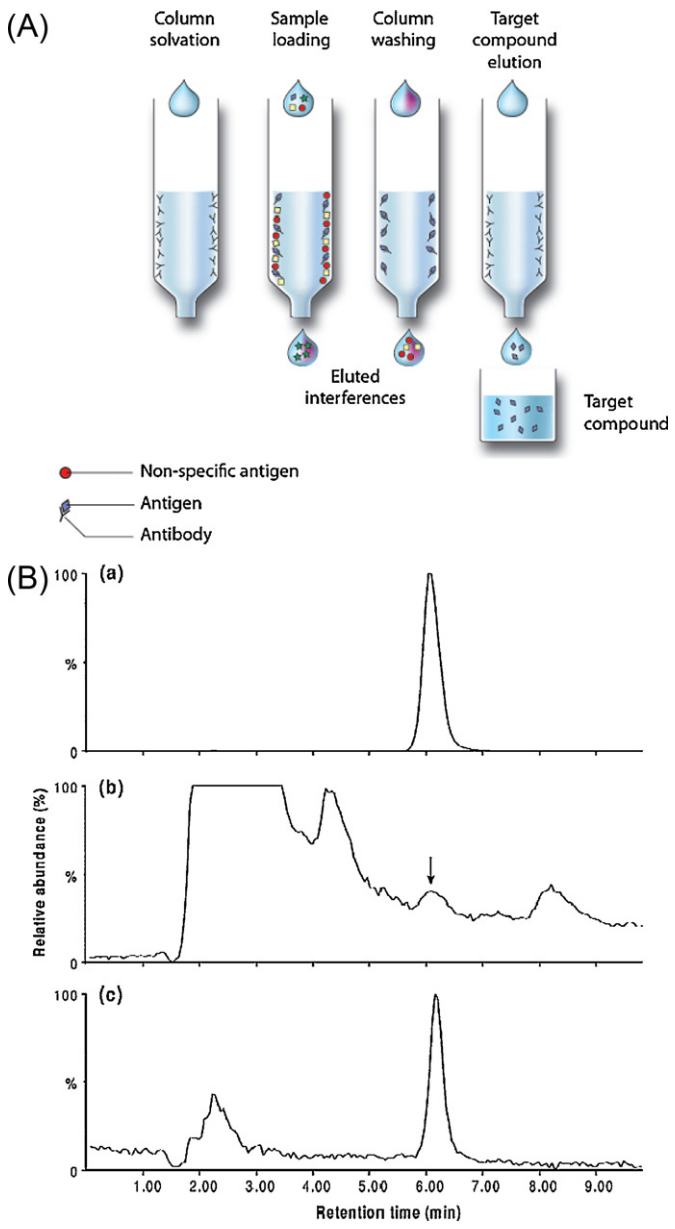

Fig. 3. (a) Immune affinity purification using sorbent bound specific antibodies for analyte specific extraction. The four stages represent conditioning of the column, loading and extraction of the sample, washing of the column and elution of the target compound. (b) LC–ESI-MS chromatogram from the analysis of melatonin standard (a) and human serum sample after SPE (b) and immunoaffinity extraction (c).
Fig. 2 from Rolčik et al. [117] with permission.

novel techniques that potentially enable faster and or more selective extraction, thereby enabling higher throughput and enhance lower detection limits.

IAC is based on the affinity between antibody and antigen, caused by molecular recognition (Fig. 3a). IAC leads to analyte enrichment in addition to removal of interfering sample matrix that would cause ionization suppression in the MS instrument. By using IAC, much cleaner extracts are obtained which results in increased detection sensitivity by LC–MS/MS. IAC can be used for extraction of a single target analyte using a single antibody immobilized on the IAC material (Fig. 3b) or of a group of target analytes by either using multiple antibodies immobilized on the IAC material or by using antibodies displaying cross-reactivity towards a group of target analytes. In the last ten years, IAC has been widely and increasingly used as a sample preparation step for reliable quantitative analysis of endogenous and exogenous biomarkers, drugs and toxins in various biological matrices including human plasma and urine by MS [32]. However, the majority of IAC applications originate from food, pharmaceutical and environmental areas, with very few examples from the field of clinical diagnostics, other than its important contribution in quantitative protein and peptide analysis [33]. In particular analytes that are present at in the picomolar range are attractive candidates for IAC coupled to LC–MS/MS. A recent clinical example is the use of IAC in the determination of low circulating levels of estrone, 17β-estradiol and estrone-3-sulphate in human plasma [34]. The authors could show similar detection sensitivity for 17β-estradiol to a reference measurement procedure using SPE followed by derivatization with dansyl chloride prior to LC–MS/MS [35]. IA extraction, in combination with either lithium adduct formation [36] or derivatization procedure [37], resulted in sensitive LC–MS/MS methods (lower limit of quantification (LLOQ) < 4 pg/ml) for quantification of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$). A major limitation is the absence of commercial sources for IA resins for the majority of low abundance analytes. The availability of such IA-cartridges for use in off-line or in-line sample preparation procedures is likely to boost the number of IAC-based applications. IAC is very compatible with LC–MS/MS and can be automated to a high degree. This is particularly relevant in settings where high volume testing is performed. Issues, still, are the potential high costs for IAC cartridges, the regeneration capabilities of the immuno-beads, and optimization for analyte recognition (in terms of specificity and sensitivity). Notwithstanding these hurdles, IA-LC–MS/MS is likely to gain popularity in the near future in quantification of low molecular compounds.

Recently alternative strategies for sample preparation were introduced. One example is a technique based on core–shell nanoparticles that sequester and enrich LMW biomarkers from carrier proteins directly from biological fluids by simultaneous performance of size exclusion and affinity capture [38]. Another example is the use of molecularly imprinted polymer (MIP) SPE, where tailor-made polymers are used for highly selective extraction [39].

Automation of sample extraction procedures e.g. using the Spark Holland on-line SPE system [14,40–44] offers specific advantages, but its design requires more time and experience than manual, off-line procedures. The on-line SPE system is designed to proceed automatically through a series of programmable routines during which the SPE cartridge is loaded, washed, and eluted. The analytes can subsequently be eluted directly on the analytical column, as is schematically shown in Fig. 4. During chromatographic separation on the analytical column, the SPE cartridge is washed, and subsequent processing of other (plasma) samples can be carried out in parallel. This procedure reduces sample handling by automation, thereby reducing sample handling errors. Using this procedure, more-extensive SPE protocols can be applied without increase of hands on time. The system can handle relatively small sample volumes (50 μl) and can enhance sensitivity because it enables concentration during extraction and point injection after peak focussing, and its effective extraction. Although the system can enhance sample throughput significantly, SPE extraction time becomes a limiting factor in cases where the chromatography time is shorter than the time needed for SPE.

Another automated sample preparation method can be seen in turbo-flow applications, in which the supernatant of protein precipitated samples are chromatographed over a micro-porous SPE pre-column. This pre-column only retains small molecules and passes through remnant proteins. Increased specificity of the extraction can be obtained by selection of the different microporous materials available. Besides a protein precipitation step, this method also proceeds automatically, thereby increasing sample

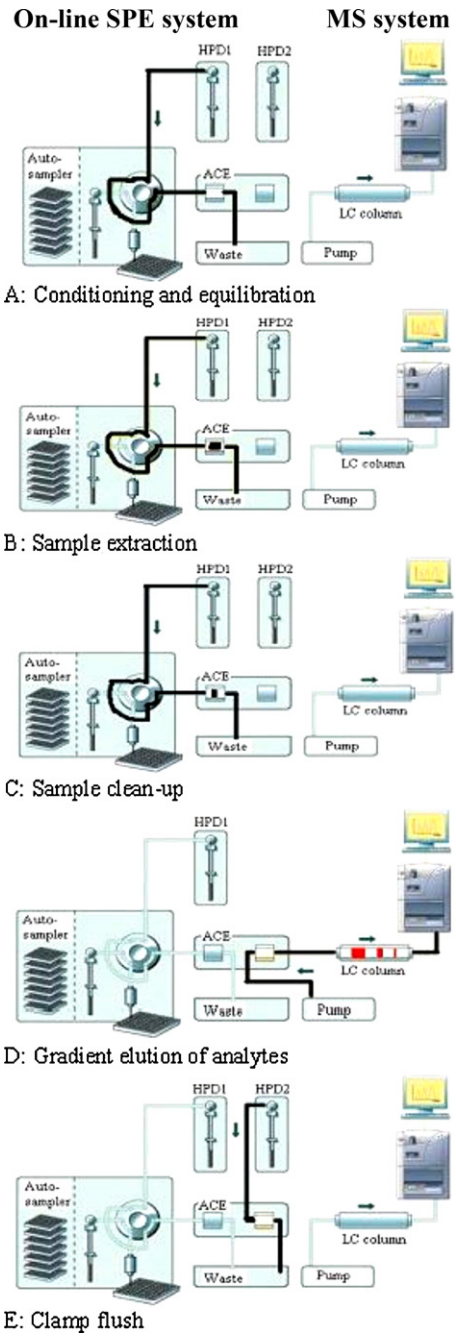

Fig. 4. Schematic representation of the on-line SPE extraction procedure. The different stages of SPE extraction are depicted in the 5 panels of the figure: conditioning and equilibration of the SPE cartridge, sample loading and extraction on the SPE cartridge, washing SPE cartridge, elution of the analytes from the cartridge towards the analytical column, and system and cartridge flush.

throughput capabilities [45–49]. The actual choice for one of these extraction procedures is of course dependent on the specific laboratory expertise, the amount and variety of tests, and financial budget available.

5.2. Choice of internal standard

A significant advantage of using LC–MS/MS, is that the internal standard, needed for correction of extraction losses, can be chosen so that it is chemically almost identical to the analyte of interest, by using stable isotope-labelled compounds. In case of usage of compounds structurally related to the analyte, one should be aware of the fact that ionization efficiency may differentially affect the analyte and its internal standard, in case of (slight) differences in retention time. With increasing demands in the past few years, the amount of suppliers of stable isotope labelled internal standards has increased, resulting in a better availability, mostly being deuterium labelled compounds. C13 labelled compounds are preferable above deuterium labelled ones, but are more expensive with limited availability. Deuterium labelled standards have the potential risk of hydrogen–deuterium exchange. They also show slightly different chromatographic behaviour due to differences in stationary phase interaction between deuterium and hydrogen atoms, which may become particularly relevant when ion suppression is encountered. C13 atoms, on the contrary, are typically located in the backbone of a molecule and are less prone to exchange and differences in chromatographic behaviour. In order to prevent the natural isotope ions of the target analyte contributing to the intensity of the molecular ions of the internal standard, with subsequent underestimation of the true value, it is advised to choose the mass of the internal standard at least 3 amu above that of the analyte [50].

5.3. Ionization and ion selection

In most clinical laboratories that use LC–MS/MS for quantitative target analysis, ions are formed by either ESI or APCI and sorted usually by SRM analysis. Triple quad derives its name from the fact that the detection principle is based on three subsequent steps. In the first step, compounds separated in LC are ionized and then selected in the first quadrupole according to mass-to-charge ratio (m/z). The second step is when the selected precursor ion enters a collision cell containing inert gas molecules (erroneously named as the second quadrupole) and fragment ions are produced. Finally, the fragmented ions are selected again by mass analysis in the second quadrupole. This three-part strategy make triple quadrupole MS highly selective and presently the most sensitive MS instrument for quantitative analysis. Multiple reaction monitoring with careful selection of a precursor $[M+H]^+$ molecular ion and a subsequent product ion selection after collision induced dissociation, creates specificity which is sufficient in the majority of clinical applications, especially when used in combination with appropriate sample preparation and chromatography. It is highly recommended to monitor more than one precursor/fragment transition (quantifier and qualifier) to secure the specificity of the method. Optimization of ion selection as qualifiers and quantifiers can be done using onboard software utilities in most systems, but care should be taken that the automatically selected m/z transitions are indeed the most intense and specific ones. The use of multiple and highly specific transitions, and avoidance of non-specific MS/MS transitions, such as loss of water, for both quantifier and qualifier ions will reduce the chance that specificity problems arise [51]. SRM is selective, however it is still vulnerable to isomeric and isobaric constituents in the sample (see below).

For those compounds that are unstable, show low ionization efficiency or difficulty to fragmentation, derivatization could ad value to enhance the LC–MS/MS capabilities [52,53]. Derivatization is the modification of the chemical structure of an analyte, generally to enable or improve the suitability of an analyte for separation or detection in chemical analysis. One prerequisite for the performance of a derivatization procedure is that there must be at least one reactive functional group in the target compound and the corresponding reaction group(s) in the derivative reagent. Disadvantages often include more laborious sample preparation requirements and (in some cases) reduced analytical specificity. Derivatization enhances the poor ionization efficiencies of steroids, leading to higher sensitivity and more specific detection. Depending on the functional group different derivatization reagents

are used. Examples in the clinical laboratory field are for the detection of MMA (dipentafluorobenzyl derivative [54], or butylation derivatization [17,18,55]), testosterone using oxime derivative [56,57], estrogens using dansyl chloride derivatization [35,58,59] and vitamin D metabolites using Cookson type reagents [60–62]. Recently, a variant Cookson type reagent with a quaternary amine as ionization enhancing group (QAO-Cookson) has been used for the quantification of 1,25(OH)2D achieving an approximately 200 fold sensitivity enhancement [63]. Commonly, the introduction of basic chemical groups, including permanently charged (quaternary ammonium and pyridinium) and easily ionizable moieties, will significantly increase the response in the positive ESI mode [52] as shown here for the vitamin D example.

5.4. Chromatography and specificity issues

In the early years of the use of LC–MS/MS in bioanalytical and clinical laboratories, selective sample clean-up and chromatographic separation were considered rather unnecessary owing to the preconceived notion that MS/MS spectrometers were extremely selective detectors, and would permit minimal chromatographic resolution as well as retention times that are close to the void time of the chromatographic systems. Over the years, increased awareness has risen about the importance of appropriate sample preparation and chromatographic separation before selective MS/MS detection for accurate measurement. Despite the fact that MS/MS are highly specific detectors, especially when compared to traditional HPLC detectors, ignorance of specificity issues is one of the major pitfalls in mass spectrometry [4]. In particular when facing isomeric and isobaric compounds, SRM might not be enough and more extensive chromatographic separation using dedicated columns sometimes is compulsory [8,64]. An illustrative example is the overestimation of true 25(OH)D concentrations by most current LC–MS/MS methods from co-elution of the 3-epi-25(OH)D metabolites. As the C3-epimers give rise to the same MS/MS ion pairs chromatographic separation of 3-epi-25(OH)D from 25(OH)D is needed. Methods requiring either lengthy runtimes using cyano-propyl (SB-CN) [65–67] or chiral columns [68] have been described as well as specific derivatization procedures [62]. By using a pentafluorophenyl (PFP) column the 3-epi-25(OH)D_3 metabolite can be separated from 25(OH)D_3 within an acceptable run time (Fig. 5) making it an attractive approach for routine measurement of 25(OH)D [69,70]. The pentafluoro-bonded phase exhibits strong dipole potential (polar interaction) from the carbon–fluorine bonds, pi–pi interaction potential and the ability to interact via charge-transfer interactions due to electro-negativity of the fluorine atoms [71]. Another example is that of cortisol, prednisolone and their respective metabolites. It appears that

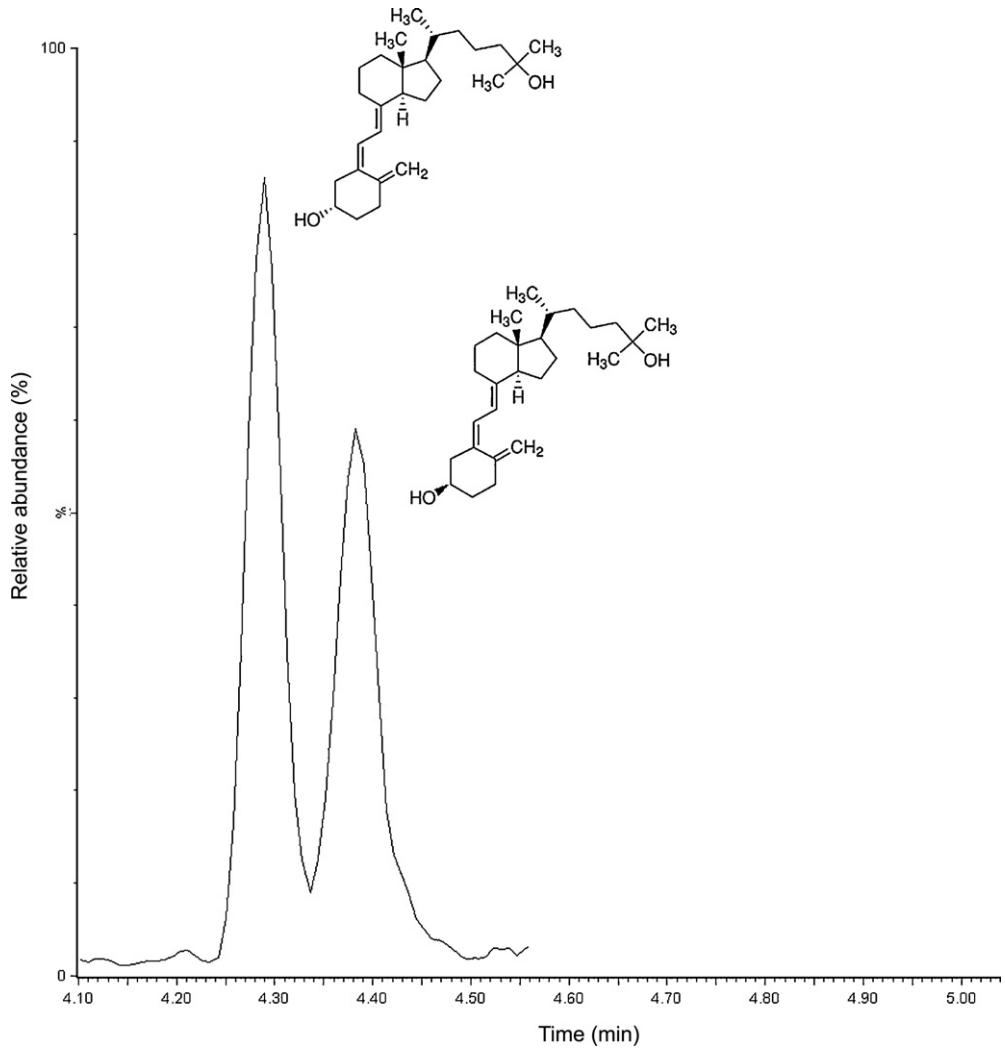

Fig. 5. Chromatographic separation of 3-epi-25(OH)D_3 (Rt 4.42 min) from 25(OH)D_3 (Rt 4.32 min) in an infant serum sample with a relative content of 44.6% 3-epi-25-(OH)D_3 using SRM with (m/z) transition 401.5 → 159.2. Chromatographic separation was by use of a UPLC CSH™ fluoro-phenyl column (Acquity 1.7 μm, 2.1 mm × 100 mm, Waters). Note the subtle difference in orientation of the C3-hydroxylgroup between 3-epi-25(OH)D_3 (α-position) and 25(OH)D_3 (β-position).

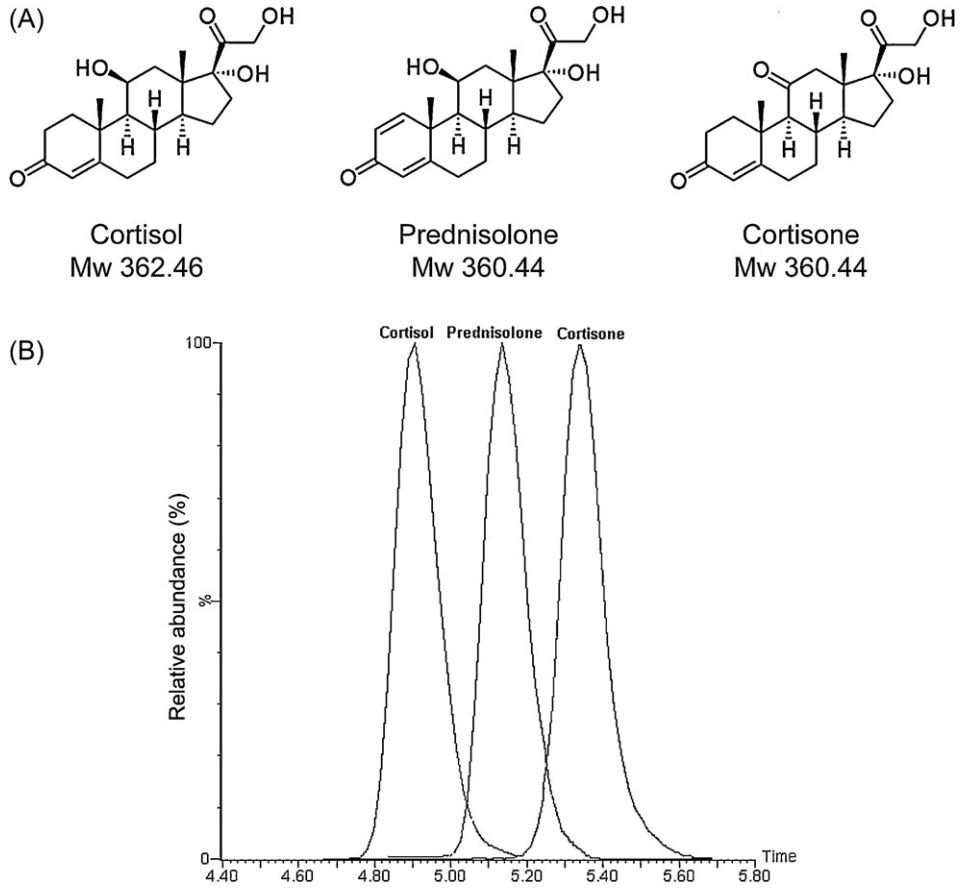

Fig. 6. (a) Molecular structures of cortisol, prednisolone and cortisone. (b) Representative chromatogram showing the separation of cortisol ((*m*/*z*) 363 → 121), prednisolone ((*m*/*z*) 361 → 147) and cortisone ((*m*/*z*) 361 → 163) standards on a UPLC CSH™ fluoro-phenyl column (Acquity 1.7 μm, 2.1 mm × 100 mm, Waters).

cortisol, cortisone, and prednisolone have similar fragmentation patterns. Fragments from the M+2 and M isotopes of prednisolone contribute in the SRM channels of cortisol and cortisone, respectively. Thus these need to become chromatographically separated in order to eliminate inherent interferences. This can be achieved by the use of a Zorbax-SB Phenyl column under isocratic conditions [64] or by using a PFP column as shown in Fig. 6.

Additional selectivity can be generated by monitoring and quantitating on the MS/MS/MS fragmentation pattern rather than just the MS/MS pattern. By monitoring the SRM^3 transition, the selectivity of the assay is increased eliminating the presence of endogenous interferences, co-eluting contaminants or high background noise, and effectively increasing the Signal to Noise ratio (S/N) of the assay. MS/MS/MS may contribute to the selectivity of analytes with higher molecular masses, a limitation though, is that when testing low molecular weight components Q3 fragments become so small that they lose specificity and end up among numerous endogenous matrix constituents (product ion redundancy).

5.5. Sensitivity issues

Early applications for LC–MS/MS, such as newborn screening did not require high sensitivity assays as compounds as these are present in the micromolar to millimolar range (Fig. 1). However, in order to analyse LMW compounds that are present at much lower concentrations (picomolar to nanomolar) range, the analytical sensitivity became important and required more sophisticated approaches, both in sample preparation and in mass spectrometric detection. Enhanced detection was achieved by instrumental progress, e.g. with improved sensitivity due to improved ion sources, travelling wave technology, or other strategies to enhance detection. On-line SPE and IAC are examples of sample preparation strategies that result in more efficient extraction and/or reduction of matrix influences, thus reducing ion suppression. In selected cases derivatization (see above) or adduct formation [48] proved to enhance ionization. Even though many instruments have shown a significant increase in sensitivity, this has not in all cases resulted in lower detection limits. An important limitation appears to be in the ionization efficiency and in the selective extraction of analytes from their matrix as stated above. More than the limitations in the MS detector-sensitivity, these factors contribute to a lowered signal to noise ratio. Optimizing the quality of mobile phase solvents can contribute significantly to the chromatographic or mass spectroscopic properties of the analyte as well as the overall detection limits of the instrument [72].

6. Method validation

Contrary to what might have been expected on the basis of its high analytical sensitivity and specificity, MS-based methods also demand thorough validation. A number of items that need to be explored are similar to those for other diagnostic methods and include an evaluation of sensitivity, selectivity (matrix interference), imprecision, accuracy, linearity, recovery, carry-over, robustness, acceptable sample types, collection tubes and anticoagulants, sample storage conditions and establishment of reference ranges.

The essential parameters required according to the FDA guidance [20] are selectivity (matrix interference), sensitivity, accuracy, precision, reproducibility, and stability. In addition, it is advisory

to determine several other parameters during validation, such as extraction efficiency, calibration range, response function, and dilution specificity.

Most of these items are well known from validation of conventional (HPLC) analyses. Ion suppression, however, is an additional important factor to consider in the LC–MS/MS validation process.

Matrix effects are factors adversely affecting the accuracy, precision and lower limit of quantification of quantitative bio-analytical method. One of the main factors are co-eluting substances altering ionization efficiency [73]. In ion suppression, the analyte signal in the ion source becomes suppressed due to competition with other sample components and can be caused by the presence of non-volatile compounds such as salts, ion-pairing agents, endogenous compounds, such as phospholipids and drugs or metabolites. The two main techniques used to determine the degree of matrix effects on a LC–MS/MS method are post-extraction addition and post-column infusion [73]. Because of the fact that ionization efficiency is not constant, this item must be addressed during validation. It is known that analyte response may decrease with increases in the amount of mobile phase (although this is a constant factor once a method has been developed). Besides this, higher masses of co-eluting compounds will suppress ionization of smaller molecules, and more polar analytes are more susceptible to ionization process. Ion suppression can to a certain extent be circumvented by using more selective sample pre-treatment and/or by improved chromatographic separation of analytes from co-eluting phospholipids [74–76,2]. Other ways to reduce detrimental matrix effects can be achieved by using smaller injection volumes or by increased dilution of samples. The use of an isotopically labelled internal standard prevents differential ion suppression of the analyte and its internal standard, although a reduction in assay sensitivity still may occur.

As LC–MS/MS enables the use of stable isotope labelled internal standards, increased accuracy is inherent to this method compared to conventional (HPLC) methods. Important in this respect is, however, the availability of certified reference materials. Accuracy can be determined by performing analysis of certified materials with defined concentrations, performing method comparison to a preferably higher-order RMP, or by recovery studies where analytes are spiked.

The robustness of a method can be assessed by analysing many patient samples for a significant period of time. A ratio of concentrations determined from different mass transitions greater than ±20%, broadening of chromatographic peaks, split peaks, or an increase in the background can be interpreted as potential interference [10].

Finally, post validation surveillance requires careful monitoring of analyte response ratio's, longitudinal QC data, stability in intensity and retention time of analyte and IS, and results from external quality assessment schemes. Monitoring of all these factors will ensure accurate measurement in daily routine practice.

7. Sample throughput

Driven by financial restraints and/or high test volumes laboratories seek for ways to increase the throughput on LC–MS/MS systems. Increased productivity can be achieved by innovations in sample preparation, chromatography as well as in mass detection.

Automation of sample preparation can be achieved by parallel processing using on-line SPE formats as discussed above. Alternatively, a batch-wise approach may be preferred using semi- to fully automated instrumentation. These consist of relatively simple to highly complex liquid handling systems [3], essentially robotizing manual SPE steps. Such systems are costly however, and require expertise for implementation and programming, and are especially of interest for laboratories dealing with high numbers of single tests (e.g. 25(OH)D or testosterone). Smaller laboratories might prefer manual to semi-automated approaches, using vacuum or positive-pressure manifolds for 96-well plates extraction.

Besides the time needed for sample preparation, the analytical run-time of the LC–MS/MS itself is the most obvious limitation to LC–MS/MS throughput. Chromatographic run-time can be shortened by using ultra-high-performance liquid chromatography (UHPLC) coupled to MS. UHPLC is a variant HPLC technique that allows the system to handle the high backpressure resulting from the stationary phase with sub-2 μm particles, offering advantages in chromatographic resolution, speed, and sensitivity over conventional HPLC systems [77]. An alternative approach to alleviate the restrictions on LC–MS/MS throughput, is the use of a multiplexed LC system, in which multiple separate LC-systems operate simultaneously, but in a staggered fashion, allowing analytes to enter the MS only at the time when the peak of interest elutes, thereby increasing throughput up to four-fold when compared to a single LC system [49,78,79]. Approaches which help to reduce chromatographic run-time to a minimum might show promise in the near future. In a novel sample introduction method, called laser diode thermal desorption/atmospheric pressure chemical ionization (LDTD/APCI) coupled to MS/MS, the chromatographic step even is completely eliminated [80].

Finally, increased throughput can be obtained using a sample-multiplexed format. In this case, more than one specimen per injection is introduced, up to the number where the electronic switching time of the MS/MS detector becomes limiting. In order to achieve such multiplexing of samples, each sample needs to become chemically modified in a different way, allowing unique identification by the MS instrument. Using this approach, a five-fold increased sample throughput has been achieved in the measurement of 25(OH)D in human serum [81]. When combining some of the above mentioned approaches, sample throughput can be increased even further. Such solutions, however, will only be profitable for a few larger laboratories performing hundreds to thousands of the same test each day.

8. Diagnostic metabolites

We here illustrate some LC–MS/MS applications for the measurement of biochemical markers of (neuro)endocrine and metabolic diseases that have gained routine status in the clinical laboratory setting due to their high diagnostic value. These examples highlight the advantages of LC–MS/MS over other existing methodologies, as well as illustrate some of the critical aspects in terms strengths and weaknesses as mentioned earlier.

8.1. Methylmalonic acid and vitamin B12 deficiency

Methylmalonic acid (MMA) can be regarded as the most sensitive and specific marker of cellular vitamin B12 status [82,83]. It accumulates upon a vitamin B12-deficiency due to reduced transition from methylmalonyl-CoA to succinyl-CoA by methylmalonyl-CoA-mutase, a vitamin B12-dependent enzyme. Despite the facts that MMA is a better marker of functional vitamin B12 deficiency, being more stable and present in higher concentrations than vitamin B12, it is not routinely measured in most clinical chemistry laboratories. The reason for this is the lack of simple and cheap analytic methods for MMA measurement, originating from difficulties of its low endogenous concentration and potential interference from other low molecular weight organic acids, especially from the naturally occurring isomer succinic acid (SA), that is present in physiological concentrations approximately 50 times higher than MMA. For many years, GC–MS has been the gold standard for MMA determination in serum, plasma or urine. Due

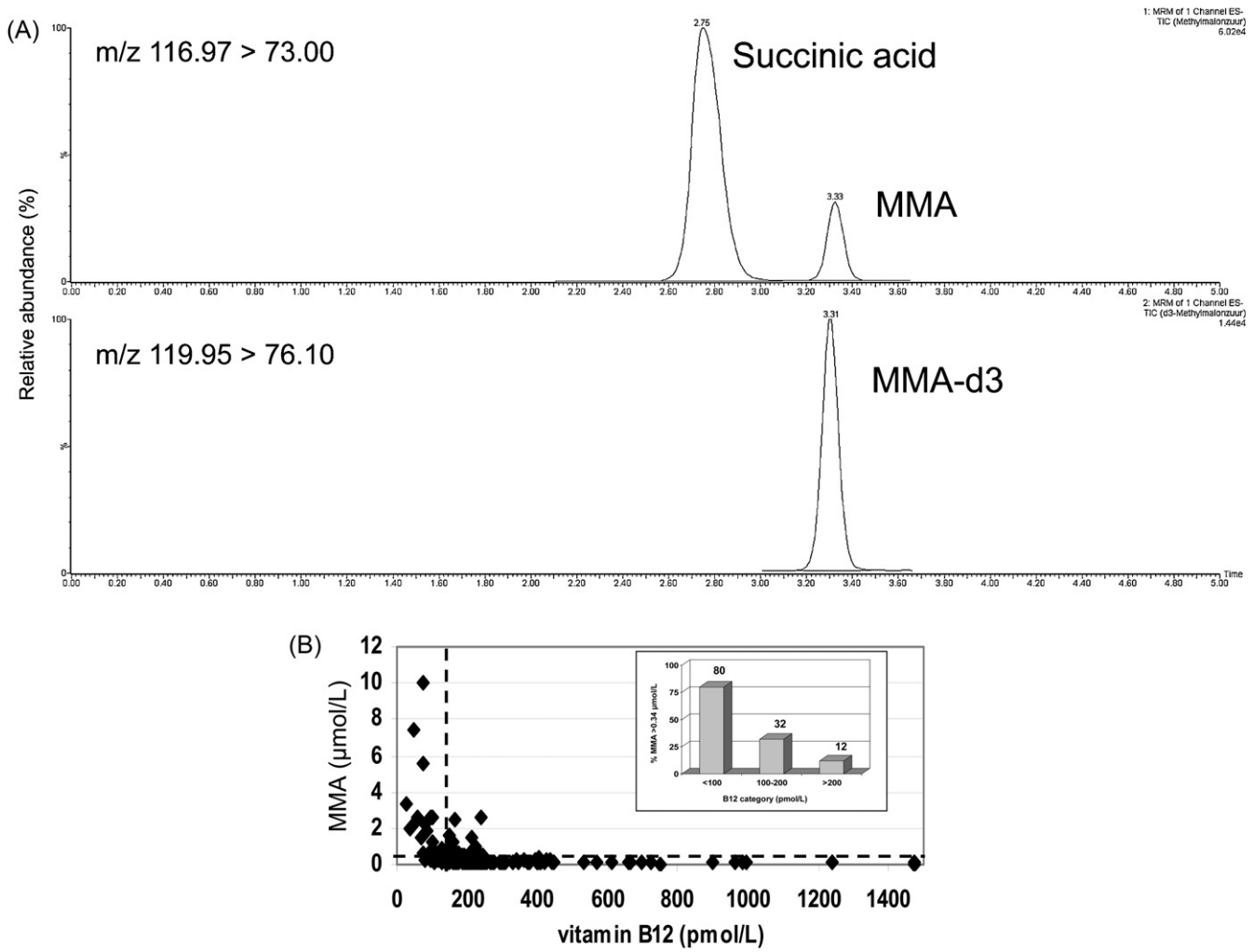

Fig. 7. (a) Typical LC–MS/MS chromatogram showing base-line separation of MMA and succinic acid (SA). The retention time of MMA and MMA-d3 are 3.33 min and 3.31 min, respectively, and that of the isomer SA is 2.75 min. (b) Relationship between serum vitamin B12 (abscissa) and MMA (ordinate) concentrations in human serum samples with normal kidney function ($n = 360$). Dotted lines represent reference range cut-offs for vitamin B12 (150 pmol/L) and MMA (0.34 μmol/L), respectively. Insert shows the percentages elevated MMA according to vitamin B12 category.

to the fact that it requires extensive sample preparation including derivatization, with long run-times generating low throughput, GC–MS is now being replaced by LC–MS(/MS). LC–MS/MS has higher speed of analysis when compared to GC–MS, but early protocols still required labour-intensive sample preparation for reasons of sensitivity and specificity including derivatization to either form butyl esters [17,18,55] or dipentafluorobenzyl derivatives [54]. More recently, protocols using a single protein precipitation step, without the need for derivatization, have been reported using C18 or HILIC in negative ionization mode achieving equal sensitivity (LLOQ of approximately 0.1 μmol/L) and specificity (no interference from SA) [16,84].

In our own laboratory we use a simple LC–MS/MS method for MMA measurement in serum and urine, consisting of a simple protein precipitation, solvent evaporation and reconstitution step (water + 3% formic acid), without the need for derivatization [85] (Fig. 7a). MMA and SA are base-line separated using an UPLC HSS T3 Column (2.1 mm × 30 mm, 1.8 μm), followed by SRM in the negative ionization mode. Functional sensitivity is 0.1 μmol/L. MMA is employed as a reflex test for intermediate serum vitamin B12 concentrations (100–200 pmol/L) [86] (Fig. 7b). Only approximately 32% of the sera with intermediate vitamin B12 levels show elevated MMA concentrations (>0.34 μmol/L), for which vitamin B12 treatment appears indicated. The quality issues related to correct diagnosis and the downstream clinical costs of multiple patient visits justify the wider use of MMA measurements. Reflex testing for MMA can easily be performed on the serum tube that is already present in the laboratory from initial measurement of vitamin B12. Only with progress in the degree of automation for MMA analysis by LC–MS/MS, reducing costs per MMA result, MMA may well become the first-line test for vitamin B12 deficiency in the near future.

8.2. Plasma free metanephrines and pheochromocytoma

Pheochromocytoma is a potentially life threatening endocrine tumour that is characterized by excessive secretion of catecholamines. Highly sensitive and specific biochemical tests are required for correct clinical chemical diagnosis, avoidance of false-negative results, and follow-up of patients. Traditional biochemical tests for the diagnosis of pheochromocytoma are based on the presence of several compounds in the catecholamine metabolic pathway. Some of these analyses, such as urinary homovanillic acid (HVA) and vanillylmandelic acid (VMA) have limitations with respect to analytical or diagnostic sensitivity or specificity and analysis time [87]. Recent studies have highlighted the higher diagnostic accuracy of plasma free metanephrine measurements over tests that quantify catecholamines [88]. Because

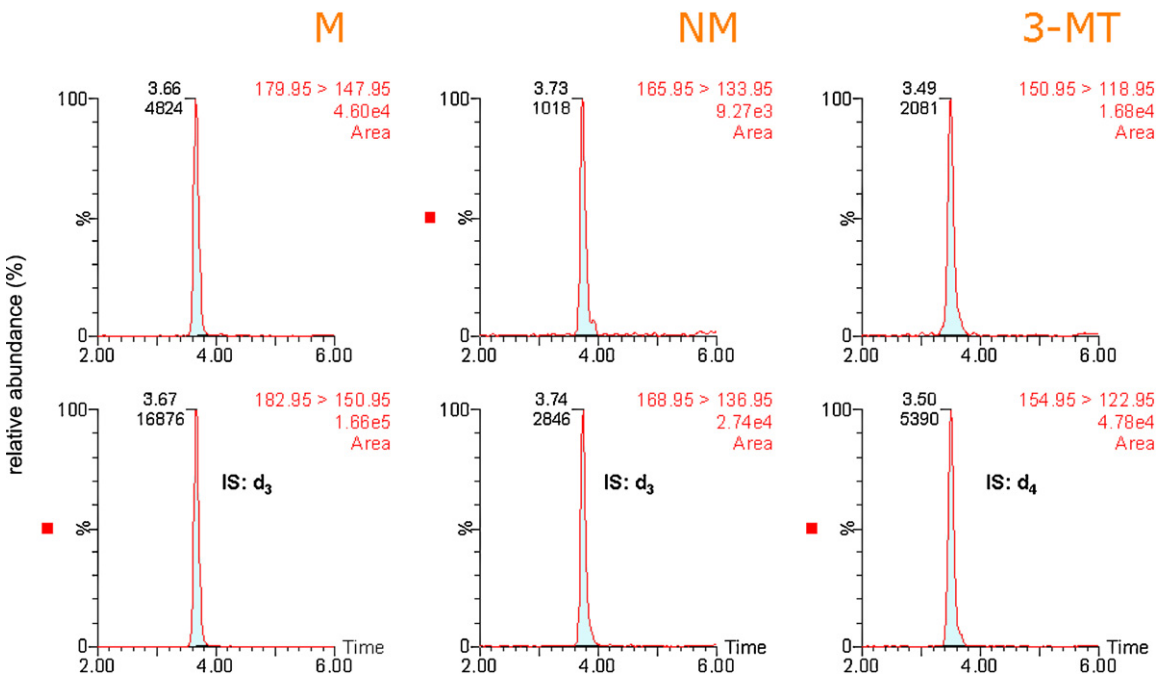

Fig. 8. Chromatograms of free metanephrine (M), normetanephrine (NM) and 3-methoxytyramine (3-MT) and their deuterated internal standards in a normal plasma sample, as obtained by on-line solid phase extraction and HILIC chromatography and SRM tandem mass spectrometry as described in de Jong et al. [14].

plasma metanephrines occur in low nanomolar concentration in a complex matrix and their chemical characteristics are not unique, developing an assay for these compounds is analytically challenging. For long, HPLC using amperometric or coulometric detection was the method of choice [89]. However, data interpretation may be complicated because of co-eluting compounds, and improvements can be made in reducing labour-intensive sample cleanup and total analysis time. Alternatively, rapid measurement of plasma free metanephrine can be achieved by the use of immunoassays [90,91]; however, cross-reactivity and non-specific binding may lead to erroneous results. In addition, the lack of an internal standard in such assays reduces confidence in reported values. GC–MS methods address some concerns regarding sensitivity and specificity. Nevertheless, sample preparation procedures for these methods involve derivatization and extraction, which are laborious and time-consuming. LC–MS/MS methods using either LLE or on-line SPE appear to be superior to GC–MS methodologies in terms of both sensitivity and sample throughput. Because metanephrine (MN), normetanephrine (NMN), and 3-methoxytyramine (3-MT) contain the same functional charged amino group, selective SPE processes can be achieved using cation exchange. Weak carbonyl cation-exchange material (WCX), retain strong bases like metanephrines, at pH > 5, permitting SPE cartridges to be washed with both water and 100% acetonitrile without elution of the analytes of interest. Strong cation exchange (SCX) media proved not suitable for quaternary amines, since elution by neutralization is difficult. The use of hydrophilic interaction chromatography (HILIC) for the analysis of polar bases proved to enhanced analytical sensitivity compared with traditional reversed-phase methods when using electrospray ionization. For the desolvation process, an organic solvent is more efficient and with HILIC, metanephrines are eluted in a high proportion of organic solvent (>80%) (Fig. 8). The principal is normal-phase separation in a reversed-phase manner with a polar stationary phase and an aqueous–organic mobile phase. This mode of chromatography is especially suitable for the separation of polar compounds from possible matrix interferences [14,92,93].

8.3. Salivary cortisol and Cushing syndrome

Cushing syndrome is characterized by a chronic state of cortisol excess. Biochemical screening studies for Cushing's syndrome include measurement of 24 h urinary free cortisol production, morning cortisol in serum after low-dose dexamethasone suppression testing, and more recently late-night cortisol in saliva. Late-night salivary cortisol is an excellent indicator of the biologically active, free cortisol concentration in the serum and has a high sensitivity and specificity (92–100%) for diagnosing Cushing's syndrome [19]. Salivary cortisol, rather than serum cortisol, can be collected non-invasively on an out-patient basis and samples are stable at room temperature for weeks. LC–MS/MS is the method of choice, as automated immunoassays lack the sensitivity to detect the low midnight salivary cortisol concentrations (0.3–3.0 nmol/L) [94–97]. Salivary cortisone concentrations, being approximately four times higher than salivary cortisol concentrations, are a consequence of the salivary glands expressing 11β-hydroxysteroiddehydrogenase type 2 which converts cortisol to cortisone. The presence of relatively high concentrations of cortisone in saliva constitutes a risk for cross-reactivity using direct immunoassays, as well as the presence of other endogenous cortisol precursors, metabolites and exogenous glucocorticoids (e.g. prednisolone). LC–MS/MS provides the specificity required to eliminate cross-reactivity by the related steroids, but only when adequate chromatographic separation is achieved between cortisol, cortisone and prednisolone, having nearly identical molecular masses (Fig. 6). This is because the inherent interference of fragments derived from the M+2 and M isotopes of prednisolone contribute in the SRM channels of cortisol and cortisone, respectively [64].

8.4. 25(OH)D and vitamin D deficiency

With annual doubling of requests for 25(OH)D over the last 3–5 years, it has become the number one LC–MS/MS test in many clinical chemistry laboratories worldwide. The high prevalence of vitamin D deficiency, combined with the recognition of its

importance in skeletal as well as in non-skeletal disease, including autoimmune-, infectious- and cardiovascular disease, has resulted in increased clinical testing for 25(OH)D [98]. Vitamin D3 (cholecalciferol) is produced from its precursor 7-dehydrocholesterol in the skin upon exposure to sunlight, whereas vitamin D2 (ergocalciferol) is obtained from dietary intake or supplements. Vitamin D is metabolised in the liver to form 25(OH)D, which is further metabolised in the kidney to form the active metabolite $1,25(OH)_2D$. Measurement of 25(OH)D, being the major circulating vitamin D metabolite, is accepted as a reliable clinical indicator of vitamin status, which is important in the diagnosis of vitamin D deficiency and for monitoring supplementation therapy. Most 25(OH)D testing is done by immunoassays, either semi- or fully automated, using antibodies recognizing 25(OH)D or by competitive protein binding assay. Alternatives are HPLC and LC–MS/MS [99]. Measurement of 25(OH)D is difficult due to its lipophilic nature and strong protein binding properties which are likely to contribute to the large inter-method variability. Also, adequate standardization is lacking at present [100]. Automated immunoassays seem attractive for reasons of high throughput capabilities. However, the majority of immunoassays have limited sensitivity and dynamic range and show cross-reactivity towards other major circulating vitamin D metabolites (e.g. $24,25(OH)_2D_3$). The automated immunoassays are delicately balanced in getting 25(OH)D displaced from its binding protein without destroying the 25(OH)D antibody at the same time. The net effect is that these methods are rather sensitive to matrix effects which can lead to marked discrepancies in individual serum samples when compared to other techniques such as HPLC or LC–MS/MS [101,102]. LC–MS/MS methodology is far more robust as it uses extensive sample preparation, including protein precipitation and LLE or SPE, combined with chromatographic separation and SRM using isotope dilution. Additional strength is its potential to measure $25(OH)D_3$ and $25(OH)D_2$ independently. The number of laboratories that use LC–MS/MS for measurement of 25(OH)D is steadily increasing, currently representing 11% of all participants in the Vitamin D External Quality Assessment Scheme (DEQAS) (Vitamin DEQAS January 2011 report) [103]. The current inter-method variability among LC–MS/MS methods and bias to reference measurement procedures [66,67] is likely to improve over the next years when LC–MS/MS methods get standardized against serum-based reference material (SRM972). A limitation, though, is the overestimation of most current MS methods from co-measurement of the 3-epi-25OHD metabolite. This metabolite has shown to be present in high concentrations in infants < 1 yr of age, as well as in sera from adults, although at lower levels [104,65,69,70]. Ideally, an LC–MS/MS method should be used that separates 3-epi-25(OH)D from the main 25(OH)D peak.

9. When to expect LC–MS/MS becoming routine technology?

LC–MS/MS has been around for over 20 years now. From a highly research environment it is starting to become a routine technology in the clinical setting. However, LC–MS systems still have the handling characteristics of research instruments. They will have to mature to user friendly routine instruments in order to achieve a breakthrough of this powerful technology in the setting of clinical laboratories. The strengths of current instruments is, that it are versatile systems allowing to customise parameter settings for optimal analysis of certain biomarkers. However, their versatility can also be interpreted as its weaknesses when pursuing user-friendly and robust instruments. Still many manual handling steps have to be taken care of (e.g. sample-lists, manual data entry). Dedicated instruments for glycated haemoglobin and haemoglobinopathy analysis have shown it is feasible to automate HPLC and transform these into robust auto-analysers for which no longer highly skilled technicians are needed. Ultimately, it is desirable to have an LC–MS platform capable of performing random-access analysis with user-friendly operation and data handling.

It is a fact that progress in automation takes many years of step-wise improvements. Automation of ligand-binding technologies was not less of a challenge compared to the automation of LC–MS/MS and took substantial years of stepwise improvements. The first radioimmunoassay was developed in 1959 [105] and it was not until 1980 before the first automated immunoassay platforms arose. These platforms needed manual handling, had low throughput, and were batch oriented. It took another 10–20 years towards instruments with near complete automation, with random access and high capacity. Despite the fact that volumes of ligand-binding assays are substantially lower when compared to those for routine clinical chemistry tests, the higher test margin makes it a profitable business for IVD companies. On the contrary, the relatively low volumes for LMW compounds might have detained big IVD companies from major investments in MS technology. With the perspective of quantitative protein analysis on a routine basis using MS, the number of tests that can be performed on an MS instrument may reach a critical mass for companies to adopt MS technology, even though some LC–MS/MS assays can be seen as competitors to some of their own immunoassays.

It remains unforeseen whether current MS vendors will enter the arena of developing instruments for clinical laboratories or whether the MS technique will be adopted by one of the major in vitro diagnostics (IVD) companies in clinical chemistry. Entry into the IVD market requires a huge commitment for MS companies having limited experience in regulatory aspects that are common sense in the field of clinical diagnostics (e.g. IVD directive (98/79/EC). On the other hand, traditional IVD companies have limited experience with MS technology. Ideally, a company takes responsibility for the development of random access MS-based platforms as well as for the development of reagent kits. In this respect the introduction of the first commercially available MS-based clinical analyser by a major IVD company for biological organism identification and use in human forensic studies shows promise.

Reagent kit manufacturers face long developmental pathways before final market approval is obtained. On the other hand, the innovations in instrument refinement travels at an enormous speed, largely driven by sensitivity needs which poses serious challenges in maintaining “cleared” assays. For reagent kit companies re-validating assays on a two to three year cycle is not viable for reasons of speed, labour intensity and costs, besides all the dynamic regulatory hurdles. This would lead reagent kits to be more or less out-dated at the moment of release. From that point it would be desirable to have longer life cycles of MS-instruments for which it becomes worth developing bio-analytical kits. Freezing instruments design and instrument configurations would help to reduce revalidation of routine assays and re-training for users. It would decrease regulatory compliance burden, enable expansion of vendor-developed test menus with enhanced service and support capabilities. For comparison, the electrochemiluminescence (ECL) technique used by one of the major IVD companies was introduced more than 15 years ago and still is the key technology for their immunoassay instruments. By freezing the instrument’s technology, platform stability and longevity for both vendor and customer are guaranteed. For numerous bio-analytical compounds current MS systems meet the performance criteria for accurate analysis in clinical diagnostics. Only for those compounds, circulating in the pico-molar range, or for those which suffer from specificity problems, improvements in instrument sensitivity

and/or resolution is desirable. Alternatively, improvements may come from more sophisticated sample preparation to decrease sample matrix effects eliminating the need for more sensitive MS detectors.

10. Future directions

10.1. Innovations in sample preparation and instrument throughput

LC–MS/MS develops at a significant speed, showing a doubling in sensitivity nearly each year. This gain in sensitivity either by instrumental innovation (increased sensitivity and speed to collect multiple SRMs in a single run for triple quads) or by introduction of novel ion sources will result in better analysis. Enhanced detection specificity can be expected from novel features such as ion mobility. Besides the increase in sensitivity and selectivity from instrumental innovation, significant advances in both sensitivity and specificity can be expected to come from more sophisticated sample preparation procedures (e.g. IAC, MIP-SPE), or multi-tagging/plexing strategies to increase throughput. The availability of ready-to-use reagents kits is likely to contribute to a wider acceptance of LC–MS/MS in clinical laboratories. At present most LC–MS/MS assays deal with single components. Increase in sensitivity and advances in sample preparation will enable to profile metabolically related compounds, thereby significantly contributing to a more integrated insight in patho-physiological processes.

10.2. Protein and peptide quantification

As quantification of low molecular analytes has become common practice, a growing and exciting area is the use of LC–MS/MS for quantitative determination of relevant peptides and proteins in human serum or urine. Examples are LC–MS/MS assays for urinary albumin [106], serum thyroglobulin [107], parathyroid hormone [108], apolipoproteins [109], and plasma renin activity [110]. This analytical technique measures a surrogate peptide of the target protein (assuming molar equivalence between the protein and the peptide) via stable isotope dilution internal standardization. The mass spectrometer is capable of providing high selectivity and low limits of quantification, especially when it is coupled with immunoaffinity enrichment [33]. Many hurdles in sample preparation still have to be overcome, including proteolytical variability upon digestion by trypsin and peptide degradation, as well as limitations in sample throughput, before quantitative protein analysis by mass spectrometry can be used in routine clinical measurement [111,112]. Nevertheless, we are in an exciting era, which prevails the growing applicability of LC–MS/MS in the clinical laboratory field.

10.3. High resolution mass spectrometry

Most LC–MS/MS methods in clinical use are SRM methods for selective and sensitive analysis of single, or multi-analyte parameters. However, this targeted analysis approach has the major disadvantage to be blind to non-target analytes.

In contrast, a full-scan approach using accurate mass, high-resolution LC–MS/MS (hrMS) measurements will enable screening of targeted analytes as well as non-a priori selected substances with high-selectivity [113–115]. Moreover, a posterior detection of untargeted substance is possible without having to rerun an analysis since all the information is already stored in the acquired spectrum. High-resolution accurate full-scan and MS2 scan at high or low resolution can be done in a single run. At present, almost all hrMS instruments are being used in academic environment, with none in a routine clinical laboratory. Given the fact that these instruments are available at similar costs of a triple quad instrument, and provided there are further improvements in quantification performances, there is no doubt such hrMS instruments will find their place in clinical laboratories in the near future. Recently an hrMS method has been developed for the quantitative analysis of intact insulin-growth-factor-1 (IGF-1) [116]. hrMS instruments will yield superior performance in specificity with potential of simplified method development, although limitation at present is the limited linear range when compared to a triple-quadrupole instruments and suboptimal sensitivity.

11. Conclusion

Over the past ten years LC–MS/MS has undergone an enormous growth in terms of laboratories who have started using these instruments for routine diagnostic measurements in clinical diagnostics, as well as in growth in technological improvements. Despite its great potential, application has been limited to a certain extent by the reality and or perception of expense, availability, sensitivity, throughput, robustness, and validation when in competition against readily available immunoassays. So, for LC–MS/MS to fulfil its promise in the clinical diagnostic arena, all critical steps in the total process must become more integrated as they are in conventional clinical analysers. In addition, the availability of ready-to-use reagents kits, eliminating efforts needed for method development and extensive validation, are likely to contribute to a wider acceptance of LC–MS/MS in clinical laboratories. Current evolution of LC–MS/MS instrumentation is extremely fast, with continuous improvement in instrument sensitivity. Growing applicability of LC–MS/MS in the clinical laboratory field is expected from quantitative protein analysis. Finally, with further improvements in quantification performance, high-resolution MS will certainly finds it place in clinical laboratories in the near future.

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