Review

Applications of mass spectrometry in clinical chemistry

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Abstract Mass spectrometry (MS) is now an essential technology for laboratory medicine. The most successful application of MS in laboratory medicine is the rapid identification of microorganisms using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS. However, the adoption of MS-based assays for routine testing in clinical chemistry is very slow in Japan. In this review, we summarize the current status of clinical mass spectrometry in the US, Europe, and Japan, and discuss the possible reasons why Japan is behind in this regard.

Liquid chromatography-tandem mass spectrometry (LC/MS/MS) is a highly accurate and reproducible analytical technique, but a number of challenges and pitfalls remain for routine use in clinical laboratories, such as the effects of various pre-analytical factors. Indeed, we observed a significant noise peak during routine measurements of vitamin D metabolites, most likely due to a separating gel in blood collection tubes used for particular specimens sent by another hospital. Ion suppression due to matrix effects and problems associated with stable isotope labeled internal standards should also be considered.

MS assays are typically laboratory developed tests at present. As LC/MS/MS procedures become more automated and more MS-related in vitro diagnostics become commercially available, the application of LC/MS/MS to laboratory medicine will be significantly accelerated.

Key words: LC/MS/MS, MALDI-TOF MS, clinical chemistry, matrix effect, pre-analytical factors

Introduction

Advances in mass spectrometer have been remarkable, and increasing numbers of highly sensitive, user-friendly novel instruments are released by instrument manufacturers every year. Mass spectrometry (MS) is now an essential technology for basic biological research and clinical medicine. Rapid progress in MS-related technologies, including sample preparation methods, labeling reagents, stable isotope labeling reagents and peptide synthesis technologies, has led to the evolution of "Proteomics" and "Metabolomics". For example, it was recently reported that the BoxCar acquisition method enables single-shot proteomics analysis of 10,000 proteins in 100 minutes[1]. Over recent years, sophisticated instruments have been developed not only for basic research but also for specialized clinical applications. In the latter cases, sample preparation and LC-MS are integrated into the system. Moreover, advanced ionization process, operating system, and the required reagents including calibrators, quality control samples and ready-to-use solvents are now commercially available. Several ionization methods, such as Desorption Electrospray Ionization (DESI)[2], Probe Electro Spray Ionization (PESI)[3] and Rapid Evaporative Ionization Mass Spectrometry (REIMS)[4] have been used for intraoperative rapid pathological diagnosis and real-time analysis of biological samples. Mass cytometry is a novel technique similar to flow cytometry and is widely used in hematological examinations[5]. Clinical applications of mass spectrometry will become more widespread as technological innovations advance.

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MS is used in various fields in Japan, including newborn screening for inborn errors of metabolism, forensic toxicology, and therapeutic drug monitoring. The most successful application of MS in laboratory medicine is the rapid identification of microorganisms using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS. Indeed, there has been a revolutionary shift in clinical diagnostic microbiology[6]. Since an early report of the application of MALDI-TOF MS-based bacterial identification in clinical laboratory on a routine basis[7], this technology is increasingly used globally. In Japan, as of August 2018, there were around 200 instruments (either MALDI Biotyper: Bruker, USA or VITEK® MS: bioMérieux, France) in operation for routine clinical use. MALDI-TOF MS is particularly useful for rapid bacterial identification in blood culture specimens, providing analyses 1.5 to 2 days faster than conventional methods and thus helping shorten hospital stays and improve overall prognosis. Moreover, a 2018 revision of the Japanese health insurance medical fee system allows additional charges for microbial identification by MS-based methods. This development will accelerate the implementation of MALDI-TOF MS in major hospitals in Japan.

In contrast, the adoption of liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based assays for routine clinical chemistry testing is very slow in Japan and remains limited to large institutions and reference laboratories. Many challenges and limitations prevent LC-MS/MS from being implemented routinely. First, LC/MS/MS involves complex, labor-intensive workflow compared to fully automated immunoassay platforms. Another bottleneck is the absence of officially approved *in vitro* diagnostic reagents (IVD), including calibrators and preparation reagents, although there is an FDA-cleared test kit (SCIEX Vitamin D 200M Assay for the Topaz™ System). As more IVD reagents and kits are officially approved and become commercially available, more LC/MS/MS platforms will be widely used in clinical chemistry laboratories.

Current Status of Mass Spectrometry in Clinical Chemistry

The situation in the US and Europe: Currently, immunoassays are widely used for testing proteins, peptides and hormones in clinical laboratories. However, immunoassays have several limitations and drawbacks, such as being dependent on antibody specificity. Under many circumstances, antibodies are not specific enough and may lead to cross-reactivity between closely related compounds. LC/MS/MS can overcome this limitation and is increasingly used for clinical chemistry, mainly in Europe and the United States. For example, approximately 15% of the papers presented at the scientific poster session of the American Association for Clinical Chemistry (AACC) over the past few years dealt with "Mass Spectrometry Applications." At AACC, a number of MS manufacturers display and introduce their new instruments for laboratory personnel. The Association for Mass Spectrometry Applications to the Clinical Labs (MSACL) has the mission to accelerate the implementation of mass spectrometry in the clinical laboratory with the goal of contributing to the improvement of patient standard-of-care as well as reducing health care costs. Their annual meetings are held twice a year, one each in the United States of America and Europe.

The Clinical Laboratory Improvement Amendments (CLIA) in the United States is a body of United States federal regulatory standards. CLIA certified laboratories can conduct laboratory developed tests (LDT), including LC-MS/MS, in the absence of officially certified IVDs, and health insurance reimbursement is generally possible. More recently, FDA has decided to extend their regulations to LDTs as well. The details of this policy have remained unknown. A provocative editorial was published in the Journal of Clinical Endocrinology and Metabolism (JCEM) in 2013[8] indicating that effective January 1, 2015, manuscripts reporting sex steroid assays as important endpoints must be MS-based assays instead of immunoassays, and the methods used must be reported or cited in sufficient detail. Although this policy has been suspended because of the complexity of the issues, we believe that it will be eventually effective in the very near future.

The situation in Japan: Immunoassays are also widely used in Japan because of the minimal sample preparation required and rapid turnaround time (TAT). Most laboratory tests for outpatients are conducted as a same-day test in hospitals that have their clinical laboratories, which demands short TAT of less than one hour. This requirement for rapid TAT is one reason preventing the widespread use of LC-MS/MS in the clinical chemistry field in Japan.

LC/MS/MS can serve as a gold standard for evaluating the accuracy of other methods. Serum 25-hydroxyvitamin D [25(OH)D] is an indicator of vitamin D status and is generally quantified by immunoassays in Japan. The Japanese

Society of Clinical Chemistry recently evaluated the standardization of six automated immunoassays available in Japan[9] using the LC/MS/MS method[10] developed at Chiba University Hospital as a reference method.

The range of correlation coefficients between the values obtained by LC/MS/MS and each immunoassay was 0.73 to 0.97. The results obtained using six immunoassay kits deviated greatly from the MS assay results (−28.6% to 42.2%). The discrepancies in values obtained using the six immunoassay kits ranged from 3.7% to 18.1%. The researchers concluded that current automated 25(OH)D immunoassays lack standardization and recommended that manufacturers pursue quality assurance so that values obtained using their kits align more closely to the values of internationally accepted standard reference materials.

Accurate measurement of plasma aldosterone concentration is a prerequisite for the diagnosis of primary aldosteronism and is currently conducted by immunoassays. Nishikawa et al. studied the standardization of aldosterone measurements in blood using serum certified reference material[11] and isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC/MS/MS) as the reference method. They reported that the aldosterone values obtained by the representative RIA (radioimmunoassay) using the current calibrator did not agree well with those obtained using certified reference material from the National Metrology Institute of Japan (NMIJ). Given these results, the Japanese Endocrine Society recommended the use of certified reference material to ensure the traceability of each immunoassay. Undoubtedly, the current immunoassays for aldosterone should be improved in terms of specificity, but we believe that LC/MS/MS should eventually take over immunoassays for plasma or serum aldosterone measurements.

We often observe discrepancies between the values obtained by immunoassays and LC/MS/MS in the method development process. Serum estradiol (E2) levels are mostly measured by immunoassay in Japan. Although E2 immunoassays are useful when fast TAT is required, they are not specific enough for post-menopausal subjects and in the diagnosis of sex hormone-related disorders. We compared serum E2 levels obtained by LC/MS/MS with those by two representative immunoassays available in Japan[12] and found that both immunoassays exhibited a remarkable positive bias, particularly at low E2 levels.

In the case of vitamin D measurements, we have demonstrated that the discrepancy resulted from a cross-reaction between the antibody and other low-abundance metabolites with similar structures[10]. We speculate that a similar explanation will be possible for the discordant results of E2, but exact reasons remain to be clarified.

Challenges and pitfalls in clinical applications of LC-MS/MS

LC-MS/MS is a highly accurate analytical technique increasingly used in clinical chemistry, but a number of challenges and pitfalls remain for its routine use in clinical laboratories[13]: specifically, the effects of various pre-analytical factors, ion suppression due to matrix effect, the problems associated with stable isotope labeled internal standards, and lack of automation and standardization.

Pre-analytical factors

Quality assurance of IVDs is conducted in many ways. Interferences by bilirubin, hemoglobin, and chyle have to be checked in LC/MS/MS as well. When antibody use is included in the first step of sample preparation as in the case with 1α,25-dihydorxyvitamin D measurements[14], effects of a rheumatoid factor (FA) to specific antibody for the analyte have to be excluded. Antibodies are often used in targeted proteomics to quantify low abundant peptides (on the order of pg/mL) in blood[15].

Blood collection tubes currently used in clinical chemistry labs are designed basically for colorimetric assays and immunoassays and are not supposed to be used for LC/MS/MS. Therefore, it is possible that unexpected results are obtained when MS-based assays are carried out using these tubes. Wang et al. reported that testosterone levels determined by LC-MS/MS in sera collected in particular tubes gave results 4-fold higher than those collected in the plain tubes which do not contain any additives[16]. It was most likely that any component of separating gel was responsible for the interference. We experienced a similar case when we changed the transition in our LC/MS/MS method for serum 25(OH)D_3[17] . After the change of transition, a strong noise peak was detected in the vicinity of the 25(OH)D_3 peak in clinical specimens provided by Hospital A (Fig. 1a). The noise peak disappeared in the transition before the change (Fig. 1b)[17]. This noise was observed in every specimen from Hospital A, but not in specimens collected in our hospital. Although the specific reason is yet unknown, we suspect that some component in the blood collection tubes used in Hospital A caused the noise peak.

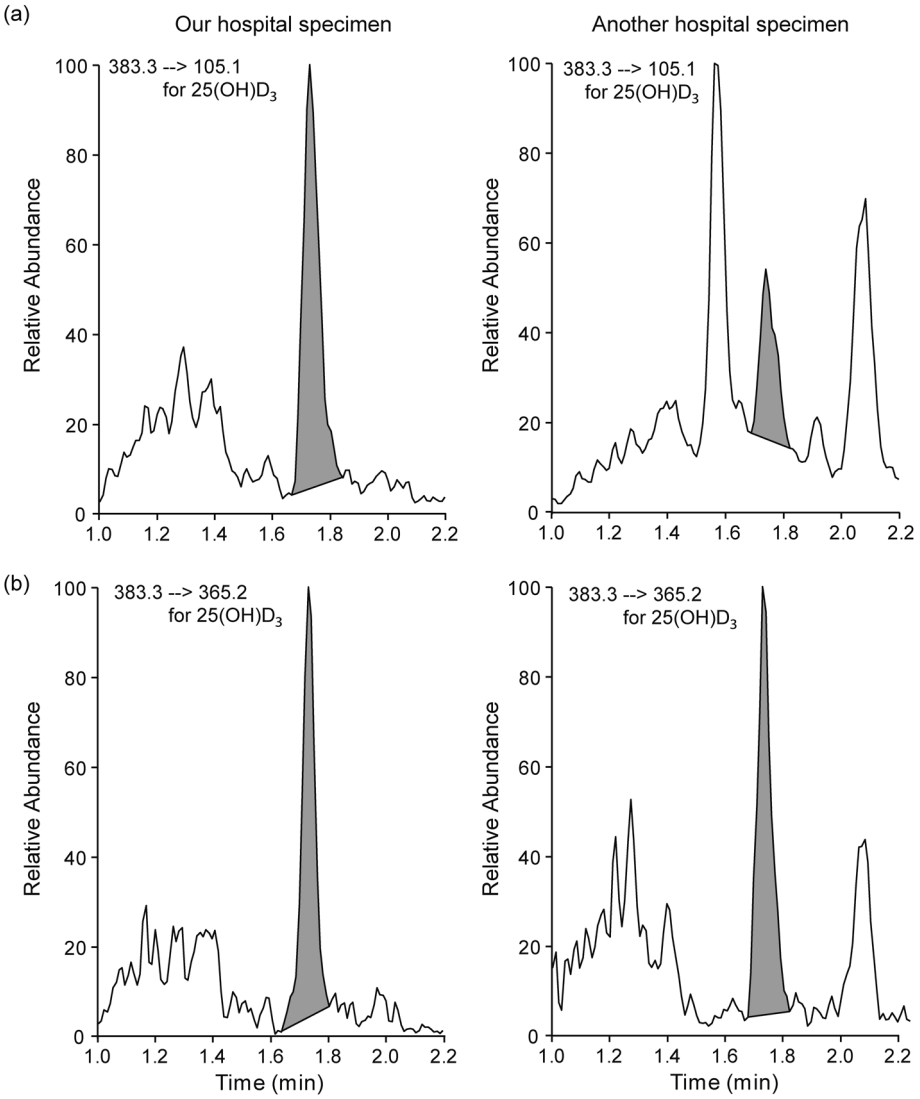

Fig. 1. SRM chromatograms for 25(OH)D_3 in serum samples collected in two types of blood collection tubes.
The left chromatograms were obtained by LC/MS/MS of a serum sample collected by Chiba University Hospital. The right chromatograms are of a serum sample collected by Hospital A. (a) The transition was set at 383.3→105.1 for 25(OH)D_3 measurement. (b) The transition was set at 383.3→365.2 for 25(OH)D_3 measurement.

As we reported previously[18], the time intervals between venipuncture and serum separation have a significant impact on serum peptide profiles. As we demonstrated more recently, it is essentially important in serum biomarker exploration to consider the influence of storage temperature and storage period[19]. Those who are involved in clinical mass spectrometry have to consider that a series of operations from sample collection to analysis greatly affect the test results.

Role of sample preparation for reduction of matrix effect

Appropriate sample preparation is required to accurately measure a target analyte in biological samples whose components are extremely complex. "Matrix effects" means the global impact that the presence of coeluting substances have on the process of declustering and the ionization of analytes within the ion source region[20]. In general, the matrix effect is mitigated by appropriate sample preparation and chromatographic separation. Moreover, insufficient sample preparation not only shortens the life of the columns for liquid chromatography but also increases the downtime risk of the equipment, because crude samples were injected directly into the equipment. The matrix effects can be confirmed through post-column infusion of the target analyte solution into the eluent from the HPLC column via a T-piece[21]. Phospholipids are one of the most well-known factors of matrix effect, and its elution pattern

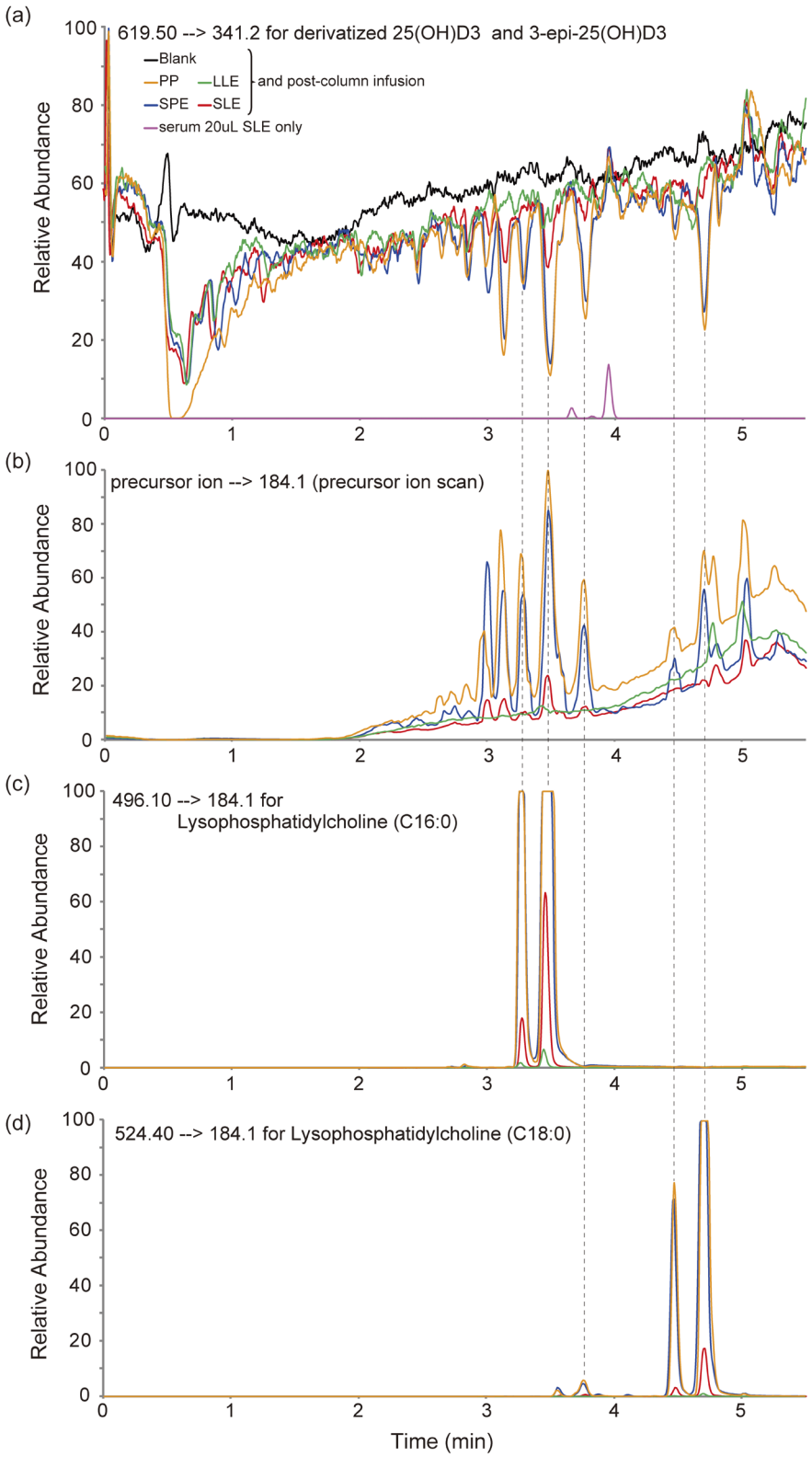

Fig. 2. Matrix effects (most likely due to serum phospholipids) during sample preparation using four methods for derivatized 25(OH)D_3 and 3-epi-25(OH)D_3.

(a) SRM chromatograms obtained using post-column infusion of a solution containing derivatized 25(OH)D_3 and 3-epi-25(OH)D_3 at a concentration of 750 and 250 ng/mL, respectively. The infusion rate was 10 μL/min. 20 μL of pooled serum sample was prepared using each method and injected onto the column using an autosampler. (b) Precursor ion scan at 184 *m/z* of each prepared sample. (c) SRM chromatogram of lysophosphatidylcholine (C16:0). (d) SRM chromatogram of lysophosphatidylcholine (C18:0).

can be identified by precursor ion scan at 184 *m/z*. We compared four sample preparation methods [protein precipitation (PP), liquid-liquid extraction (LLE), solid phase extraction (SPE) and supported liquid extraction (SLE)]. In this comparison, LC separation was carried out using a column of a reverse phase distribution system, which separates analytes based on the lipid solubility. Fig. 2 shows the matrix effects observed using each preparation method for derivatized 25(OH)D_3 and 3-epi-25(OH)D_3 transition (619.50→341.2). The HPLC and MS parameters were set according to our previous report for measuring four metabolites of vitamin D[10]. Strong ion suppression at the indicated elution times was detected using PP and SPE whereas LLE resulted in minimal ion suppression. When the precursor ion scan was performed using the same sample, elution of phospholipids was detected at the elution time when strong ion suppression occurred (Fig. 2b). Molecular species of phospholipid can be presumed by setting multiple transitions with the precursor ion of the phospholipid and a product ion of 184 *m/z*. Lyso-PC species containing palmitic acid (C16:0) or stearic acid (C18:0) were detected with the same elution time as the species detected by a precursor ion scan of 184 *m/z* (Fig. 2c and 2d). The matrix effect was not effectively removed by SPE, which is most likely because the same reversed phase separation mode was used for SPE and LC column. For effective pretreatment, it is important to use SPE with a separation mode different from that used in LC columns, such as ion exchange and mixed-mode. HybridSPE®-Phospholipid columns (Sigma-Aldrich, Germany) packed with zirconia-coated silica is effective for removing phospholipids by SPE[22]. LLE gave the weakest ion suppression because its separation is based on the polarity, unlike the reverse phase distribution mode. Because SLE is the same separation mode as LLE, it effectively removed the matrix effect. However, LLE is not suitable for large-scale studies because this technique is not automated. On the other hand, SPE, SLE and HybridSPE®-Phospholipid can be easily automated. Automated sample preparation enables consistent sampling procedures. Eventually, complete automation, including data analysis and interfacing, will be the key for further acceleration of the clinical application of LC/MS/MS.

Degrees of matrix effects have different degrees of influence depend on the ionization mode. Ion suppression is often observed well in ESI positive mode, but it is hardly seen in ESI negative mode, atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI)[20]. It was recently reported that a new commercial atmospheric pressure ionization (API) source, UniSpray™ (Waters, USA), slightly reduces the matrix effect compared with ESI, depending on the target analyte[23].

Stable isotope-labeled internal standards

Internal standards (ISs) are essential to compensate for matrix effects and correct potential variations during sample preparation steps. The Clinical and Laboratory Standards Institute (CLSI) C62-A lists seven recommendations for selecting ISs and strongly recommends that the IS be a stable isotope-labeled form of the analyte being tested[24]. Deuterium, ^{13}C, ^{15}N and ^{18}O labeled compounds are generally used as ISs, although the elution time of a deuterium-labeled IS is slightly faster than that of the target analyte on reversed-phase separation, requiring careful attention to matrix effects caused by phospholipid and other compounds. On the other hand, ^{13}C, ^{15}N and ^{18}O labeled ISs may be more useful than deuterium labeled IS because they elute almost adjacent to the target analyte. It is noteworthy that hydrogen-deuterium exchange may occur, depending on the labeled position, resulting in inconsistent IS responses[25]. Thus, stable isotope-labeled ISs are indispensable for clinical mass spectrometry, but deuterium-labeled ISs are not widely available for the majority of potential analytes.

Calibrator- and serum-based certified reference materials

Calibrators serve as references for converting raw data to the concentration of the target analyte. The use of faulty calibrators leads to erroneous results. A New York Times article on January 7, 2009 shook the LC/MS/MS society[26]. A spokesperson from Quest (one of the distinguished private medical laboratories in the US) admitted that some erroneous 25(OH)D results had been reported because of the problems with calibrators. Although it is generally believed that inter-laboratory variation in LC/MS/MS measurements is small compared with immunoassays, that is not necessarily the case based on the results of the 25(OH)D College of American Pathologists (CAP) survey presented at a workshop held by the FDA in May 2016[27]. A total of 8 immunoassays were included in the 25(OH)D CAP survey, and the method-specific between-laboratory CVs ranged from 6.4 to 11.6%. As many as 74 laboratories

reported results obtained using LC-MS/MS and the CV was 11.4%. It is likely that inter-laboratory variation can be improved if a common calibrator is used[28]. Considering that the results of 25(OH)D submitted to the International Quality Assessment Scheme (DEQAS) have shown poor interlaboratory agreement, Carter and Jones tried to answer whether the use of a common standard could reduce the discordance[29]. They demonstrated that use of a commercially available common standard (Chromsystems Inc., Germany) reduced the mean inter-laboratory precision (CV) for $25(OH)D_3$ from 16.7% (in-house standard) to 8.5% (common standard). On the other hand, it was reported that calibration alone is not the cause of variation in LC/MS/MS testosterone measurement[30]. Although it is desirable that the matrix for the calibrator be the same as that for real samples, this is not possible when the target analytes are endogenous compounds. Successful quantitation of endogenous analytes in biological samples by LC/MS/MS without a true blank is challenging. One approach is to pretreat sera samples to remove as much the particular endogenous analyte as possible, which is not satisfactory under most circumstances. An alternative approach is to use a deuterium-labeled compound as a surrogate analyte for generating calibration curves[31].

BSA solutions (4−7%) are often used as a matrix substitute, but the possibility that BSA solutions contain any binding proteins to the target analyte should be noted. Large deviations in $25(OH)D_3$ values might be caused by vitamin D-binding protein contaminants in BSA[29]. Furthermore, we have detected significant amounts of $25(OH)D_3$ in commercially available non-recombinant BSA (data not published). Commercially available recombinant human serum albumin (HSA) does not contain detectable amounts of $25(OH)D_3$ and can be used as a matrix. It is important to note not only the type of matrix used for the calibrator but also the purity of the standard materials per se. Purity index may differ depending on the determination method (for example, HPLC and quantitative NMR).

Clearly, it is impossible to obtain accurate calibration curves using impure materials, thus highlighting the importance of certified reference materials (CRMs)[32]. Solvent-based standard materials (SRM 2972a) and reference sera (SRM 972a) are currently available. The National Institute of Standards and Technology (NIST) supplies reference sera with values assigned by LC-MS/MS, and these are helpful in assuring the accuracy of 25(OH)D measurements. These calibration problems are not unique to vitamin D measurements and are also important in the measurement of steroid hormones and other low molecule analytes. Currently available serum-based CRMs other than vitamin D metabolites are limited and include SRM971 for cortisol, testosterone, progesterone, total T3 and total T4; BCR576−578 for 17beta-estradiol; ERMDA192−193 for cortisol; ERMDA347 and BCR348R for progesterone; NMIJ CRM 6401-b for cortisol; and NMIJ CRM 6402-a for aldosterone. More serum-based CRMs are needed to accelerate the implementation of MS-based assays into clinical laboratories. CLSI released a standardized approach for LC-MS assay development and verification in its 2014 guidance document, mainly for low molecule compounds. Similar guidance documents for proteins and peptides are required.

Data analysis and instrument control software

The use of updated LC/MS/MS platforms, and defining the peak detection method, allow automatic peak detection and quantification. Several different peak detection algorithms are adopted for available MS instruments, but regarding the validity thereof, it is necessary to check whether the results obtained automatically agree well with those obtained manually by LC/MS experts. LC/MS experts check not only peak area ratio of analyte and IS but also analyte area of all QC samples and IS area of all specimens independently. Few software programs automatically plot the area value of the analyte and IS, and thus this must be done manually at present.

Making LC/MS/MS platforms more applicable to routine clinical laboratory analyses will require total automation, including automated sample preparation, sophisticated software for data review, and interfacing with laboratory information systems. Mass spectrometry platform manufacturers conduct their standard performances tests with easy-to-handle analytes to ensure the function of the instrument but do not go beyond that. Indeed, a considerable in-house capability for troubleshooting and maintenance of the MS instrument is currently required. As pointed out by Vogeser and Seger, software-based troubleshooting and maintenance instruction tools would be effective but are not currently available[33].

At present, MS assays are mostly laboratory-developed, labor-intensive, and require considerable expertise in method development and validation. As LC/MS/MS procedures become more automated and more MS-related *in*

vitro diagnostics become commercially available, the application of LC/MS/MS to laboratory medicine will be significantly accelerated.

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Conflict of Interest

All authors declare that they have no conflict of interest.

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