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Reviews

Pitfalls Associated with the Use of Liquid Chromatography–Tandem Mass Spectrometry in the Clinical Laboratory

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BACKGROUND: Novel mass spectrometric techniques such as atmospheric pressure ionization and tandem mass spectrometry have substantially extended the spectrum of clinical chemistry methods during the past decade. In particular, liquid chromatography tandem–mass spectrometry (LC-MS/MS) has become a standard tool in research laboratories as well as in many clinical laboratories. Although LC-MS/MS has features that suggest it has a very high analytical accuracy, potential sources of inaccuracy have recently been identified.

CONTENT: The sources of inaccuracy in LC-MS/MS methods used in the routine quantification of small molecules are described and discussed. Inaccuracy of LC-MS/MS methods can be related to the process of ionization through the insource transformation of conjugate metabolites or target analytes and may also be attributable to ionization matrix effects that have a differential impact on target analytes and internal-standard compounds. Inaccuracy can also be associated with the process of ion selection, which mainly occurs when compounds from the sample matrix share mass transitions with a target analyte. In individual assays, most potential sources of inaccuracy can be controlled by sufficient LC separation–based sample workup before MS analysis.

SUMMARY: LC-MS/MS methods should undergo rigorous and systematic validation before introduction into patient care.

During the past decade, liquid chromatography–tandem mass spectrometry (LC-MS/MS)[3] has substantially extended the methodologic armamentarium of clinical laboratories. It has become one of the essential basic technologies used in laboratory medicine, thereby joining photometry, ligand-binding assays, automated cell-counting techniques, electrophoresis, potentiometric sensors, and nucleic acid amplification techniques.

The mere coupling of LC (with its inherent limited separation power) with simple molecular mass–selective detection by single-stage quadrupole or ion-trap MS instruments (LC-MS) was soon quickly recognized to offer only limited selectivity when used for target analyses of complex biological samples. The introduction of MS/MS, which involves the coupling of 2 quadrupole mass filters with an interposed collision cell, initially seemed to overcome these limitations because the fragmentation pattern of target analytes became incorporated into analyte detection. However, when LC-MS/MS became increasingly used in routine clinical laboratories *(1, 2)*, potential limitations in the analytical performance of this powerful technology became increasingly evident *(3)*. The selectivity of MS/MS detection was one aspect of this technology that was particularly overestimated during the first years of its application to clinical chemistry *(4–6)*. The recent debate about the inaccuracy of 25-OH-vitamin D results obtained by LC-MS/MS *(7–9)* was widely noticed, and has highlighted the need for rigorous quality assurance in clinical MS. Indeed, quality assurance is a particular challenge in clinical LC-MS/MS applications because the end users themselves implement and validate the methods. Only a few commercial LC-MS/MS assay kits are presently available. In addition, the instrument configurations used to run these assays are extremely heterogeneous.

The aim of this report is to provide a systematic review of the common sources of inaccuracy in LC-

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[3] Nonstandard abbreviations: LC-MS/MS, liquid chromatography–tandem mass spectrometry; API, atmospheric pressure ionization; ESI, electrospray ionization; APCI, atmospheric pressure chemical ionization; MPA, mycophenolic acid; SRM, selected-reaction monitoring.

MS/MS methods used in the clinical laboratory. Because relatively few reports or systematic investigations have been previously published on this topic, this overview also is based on personal experiences accrued from the 10 years of experience that 2 university hospital laboratories have had with the use of LC-MS/MS. We have focused on the quantification of small molecules in diagnostic procedures (e.g., hormones, metabolites, and xenobiotics) but have not addressed protein and peptide analyses by LC-MS/MS, although these types of analyses may be routinely used in clinical laboratories in the future *(10–15)*.

Inaccuracy Related to the Ionization Process

MATRIX EFFECTS WITH DIFFERENTIAL IMPACTS ON THE IONIZATION OF ANALYTES

The physicochemical processes involved in ion generation and transfer under atmospheric conditions [atmospheric pressure ionization (API)] are complex and modulated by numerous factors *(16)*. When API is performed by using conventional chromatographic flow rates (i.e., 0.3–1.0 mL/min), it is rather inefficient because only a small fraction of the target analytes becomes ionized and enters the high-vacuum area of the mass analyzer *(17)*.

During the process of API, substantial fluctuations in the ionization yield occur over seconds to minutes *(18)*. Thus, compared to ultraviolet and fluorescence detection, the stability of the signal in LC-MS/MS is rather poor. This high degree of variation in the efficiency of API makes internal standardization mandatory for quantitative LC-MS/MS analyses, and requires compensation to account for potential variations in sample extraction and injection volume. The measurement technique that must be used in these types of internally standardized analyses involves the calculation of the ratio of the peak areas recorded for the target analyte to those of the respective internal-standard compound.

The term "matrix effects" refers to the global impact that the constituents from the evaporated liquid (which originate from the solvents and the sample) have on the process of declustering and the ionization of analytes within the ion source region *(19, 20)*. If the ionization yield observed for an analyte in a complex matrix (e.g., the supernatant of a serum sample after protein precipitation) is lower than that observed when the target analyte is dissolved in a seemingly pure solvent, the term "ion suppression" is used. "Ion enhancement," on the other hand, describes the increase in ionization yield that occurs with the use of a complex matrix compared to matrices termed "pure," "neutral," or "inert" owing to their constituents. It should be noted, however, that HPLC-grade solvents such as methanol, acetonitrile, and water also interact with analytes during ionization, because of the inherent chemical properties of these compounds and the impurities that exist in them *(21–24)*. Thus, no truly matrix-free analyses are possible with the use of LC-MS/MS.

Factors that cause ion suppression or enhancement include the presence of salts and hydrophilic small molecules that occupy or provide ions (i.e., hydrogen, sodium, or ammonium ions) as well as the presence of compounds that affect droplet formation as surface-active compounds in the matrix. In addition, late-eluting sample constituents, such as phospholipids, may cause ion suppression. This problem is particularly relevant if gradient elution is used.

During method development and validation, ion suppression effects are often investigated through post-column infusion of the dissolved target analyte into the eluent from the HPLC column via a T-piece *(25)*. This infusion generates a sustained background signal in the multiple-reaction–monitoring tracing of the target analyte. In this setup, the HPLC injection of analyte-free biological matrix (e.g., deproteinized plasma) typically leads to a decrease, lasting from several seconds to several minutes, in the baseline signal generated by analyte infusion*(19)* (Fig. 1). F1

If an investigated analyte elutes during this period of baseline depression, it is subject to ion suppression. This experimental setup can provide only a rough qualitative description of the ion suppression characteristics of an individual analytical method. Furthermore, the ion suppression profile can vary substantially between different human serum samples *(26)*. Regarding the HPLC settings, the general desire is to use chromatographic conditions that avoid elution of the target analyte during the period of ion suppression within a chromatographic run *(27)*. Avoiding such elution, however, requires increased run times, which leads to diminished sample throughput and increased instrument costs per analyzed sample.

The sample preparation protocol used in the method contributes to the ion suppression duration in a chromatographic run. Typically, laborious methods such as solid-phase extraction and liquid-liquid extraction achieve short periods of ion suppression, whereas simple protein precipitation protocols are often associated with more extended periods of ion suppression due to early eluting, low molecular weight matrix constituents.

Matrix effects depend on the mode of ionization used as well as on the polarity of the selected ion. Generally, positive-ion electrospray ionization (ESI) is prone to ion suppression, whereas negative-ion ESI (which is applicable only for a minority of target analytes), atmospheric pressure chemical ionization

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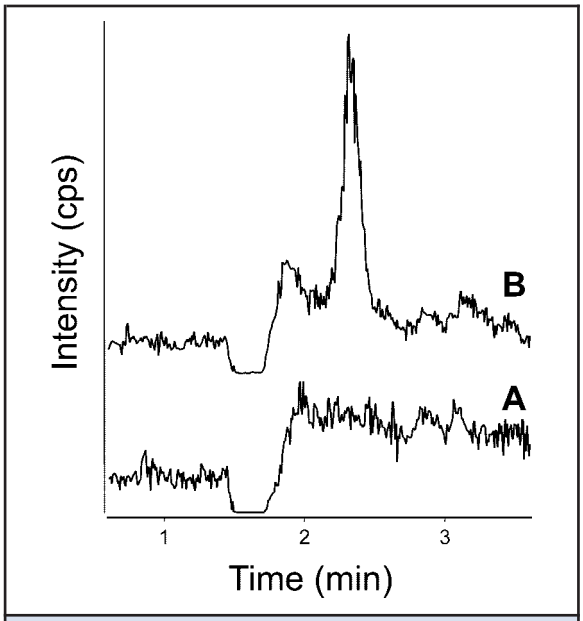

Fig. 1. Monitoring of ion suppression via the postcolumn infusion method.

(A), A selected-reation–monitoring (SRM)-based tacrolimus ion trace (821.6 > 768.6), which was steadily infused into the LC eluent of a blank whole-blood extract obtained by protein precipitation. A strong loss of ions (i.e., the occurrence of the ion suppression effect) marks the breakthrough of the sample solvent, whereas a slight ion-yield–enhancement effect can be observed during the effluent of the sample matrix. (B), Adding tacrolimus (5 μg/L) to a whole-blood sample allows the elution window of this analyte, which is clearly separated from the ion suppression event, to be recorded.

(APCI), and atmospheric pressure photoionization are less affected *(20)*.

Ion suppression (as a negative matrix effect) has been discussed mainly in the context of its impact on the sensitivity and the lower limit of quantification of an assay. We emphasize, however, that short-term variations in ion yields, particularly those due to matrix components, can also compromise the accuracy of analyses. The accuracy of the testing method is compromised whenever the variation of ion yield has a differential impact on the target analyte and internal standard. Thus, the reliability of LC-MS/MS analyses is affected by both differences in the way in which ion suppression or ion enhancement affects the ionization of the target analyte and the respective internal-standard compound, and differences in the way that the matrices of calibrator samples and actual patient samples behave with respect to the modulation of ionization efficiency. The problem of differential ionization matrix effects can affect an entire batch of samples if systematic differences in the ionization modulation properties of calibration materials and actual patient samples are present. This problem can also nonsystematically affect the analysis of individual patient samples.

ROLE OF INTERNAL-STANDARD COMPOUNDS

In general, the accuracy of LC-MS/MS analyses will be good if the physicochemical properties of the target analyte and internal-standard compound are very similar. Stable isotope-labeled compounds are ideal internal standards because they have almost identical overall physicochemical properties compared to their unlabeled counterpart, the target analyte. With the use of MS, these 2 species can be distinguished by their differing molecular weights. In labeled compounds, typically either ^{1}H (hydrogen) is exchanged for ^{2}H (deuterium) or carbon ^{12}C is exchanged for ^{13}C in several molecular positions. Ideally, more than 3 atoms are exchanged during the labeling process. Nonetheless, the physicochemical behaviors of labeled and unlabeled compound are not identical. The term "isotope effects" refers to these minor differences. In GC-MS, the small differences in the retention times of the labeled and unlabeled compounds can be explained by isotope effects. Nevertheless, isotope-dilution GC-MS is considered to be a nearly matrix-independent reference method that has an extremely high degree of accuracy. This is because all matrix-related effects likely have a very similar impact on naturally occurring target analyte molecules and on labeled internal-standard molecules *(28)*. In contrast to GC-MS, substantial isotope effects in the ionization efficacy can be observed with LC-MS/MS. This disadvantage of LC-MS/MS has been demonstrated via observed differences in the quantification of carvedilol *(29)* and piperaquine in plasma *(30)*. In the first case, the internal standard did not completely coelute with the analyte, which led to major differences in the ion suppression effects during the analysis of some specimens. In the latter case, residual amounts of triethylamine that had remained in the sample solution after solid-phase extraction differentially suppressed the signals of piperaquine and its 6-fold deuterated internal standard, leading to large errors in the calculation of analyte concentrations.

If high ionization temperatures are employed (particularly when APCI is used), hydrogen-deuterium exchange of deuterated internal-standard compounds can occur during the ionization process *(31)*. Carbon atoms are typically located in the backbone of a molecule and are not prone to exchange. Therefore, ^{13}C atoms are considered more reliable than deuterium as labels for isotope dilution internal standardization.

Notably, stable isotope-labeled internal standards are not currently available for the majority of potential

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small-molecule analytes. This problem applies particularly to therapeutic drugs, whose concentrations are monitored by use of this technique. In such cases, compounds having similar molecular structures (i.e., homologs or analogs) are typically used as the internal standards. However, because the ionization properties of a molecule can be impacted by its functional groups, the ionization behavior of compounds with very similar overall molecular structures may differ significantly. The differential clustering of sodium, ammonium, or formate ions, which are often present in mobile phases, may also impact the parity of the ionization yields between a target analyte and its internal standard. Hence, the availability of an appropriate internal standard is crucial for the development of reliable LC-MS/MS methods *(32)*.

ROLE OF CALIBRATOR MATERIALS

A fundamental requirement for LC-MS/MS calibration materials is that the matrix effects exerted by these materials must be as similar as possible to the matrix effects exerted by patient samples. Lyophilization, virus inactivation, and other procedures used during the industrial production of calibration and control materials can markedly affect the ionization behavior of extracts from such samples and can result in differences in the impact that matrix effects have on calibrator samples vs patient samples. If the internal-standard peak areas found for calibrator samples differ systematically from those found in patient samples, one should suspect that an inappropriate calibration material was used. However, we previously observed that calibration materials from different commercial sources led to inaccurate tacrolimus results in an instrument-specific manner without exhibiting obvious deviations in the internal-standard peak area. This effect was most likely related to the occurrence of ionization enhancement that affected the target analyte but not the homolog internal standard (ascomycin). This resulted in a systematic underestimation of tacrolimus results of clinical samples analyzed on 1 instrument using 1 specific calibrator lot *(33)*.

Substantial matrix effects can also be specific to individual patients' samples and are often due to the coadministration of other medications or the presence of xenobiotic compounds. In generally, attempts are made to detect such samples by assessing the peak areas of the internal-standard compound over a series of samples. Thus, the presence of outliers with respect to the peak area of the internal standard may indicate the occurrence of unusually pronounced matrix effects in an individual sample (Fig. 2). In such cases, it is unclear whether the analyte and the internal standard are affected to an identical degree by the evident matrix effects. The analyst must therefore decide whether the quantification results of such samples are correct and can be reported. Reanalysis after dilution can be useful in cases in which matrix effects are evident, as long as there is a sufficiently high analyte concentration present in the respective sample.

Such decisions are always more or less arbitrary because the normal variation of internal-standard peak areas between subsequent samples in a series can be substantial in many routinely used LC-MS/MS instruments. Moreover, it is always uncertain whether the matrix effects in an individual sample impact the target analyte and the internal standard compound to a similar degree. This consideration underscores the importance of the use of appropriate internal-standard compounds to ensure the reliability of LC-MS/MS results.

Matrix-related modulation of ionization will remain an important issue in clinical LC-MS/MS. To minimize the effects of this phenomenon, a multistep strategy should be applied:

1. Matrix effects must be detected by systematic experiments during method development.

2. Efforts should be made to minimize such effects via adequate sample preparation and chromatographic separation.

3. Because it will not always be possible to completely avoid the matrix-related modulation of ionization during elution of target analytes, particularly with the use of metabolomic multianalyte methods, an attempt must be made to compensate for these effects through the use of the most appropriate internal-standard materials.

4. As a final step, the developers of new generations of MS/MS analyzers should seek to decrease matrix-related effects on ionization in the development of new instruments. We must note that orthogonal modes of ion selection (e.g., as realized in ion-mobility MS) separate interferent ions from the analyte ion only before their detection. This process acts after ion formation, and hence it does not prevent the occurrence of ionization-related matrix effects *(34)*.

Insource Transformation

API techniques used in LC-MS/MS, such as ESI and APCI, are generally considered to be "soft" analytical techniques. Nevertheless, if weak bonds are present, molecules disintegrate during the process of ionization, before entering the actual mass spectrometer. In bioanalyses such "insource transformations" are particularly prominent if conjugated metabolites (e.g., glucuronide- or sulfate-conjugated metabolites) of target analytes are present in a sample *(35)*. Analytical inaccuracy can occur whenever a target analyte coelutes with its conjugate metabolites, for example, if mycophenolic acid (MPA) coelutes with MPA-

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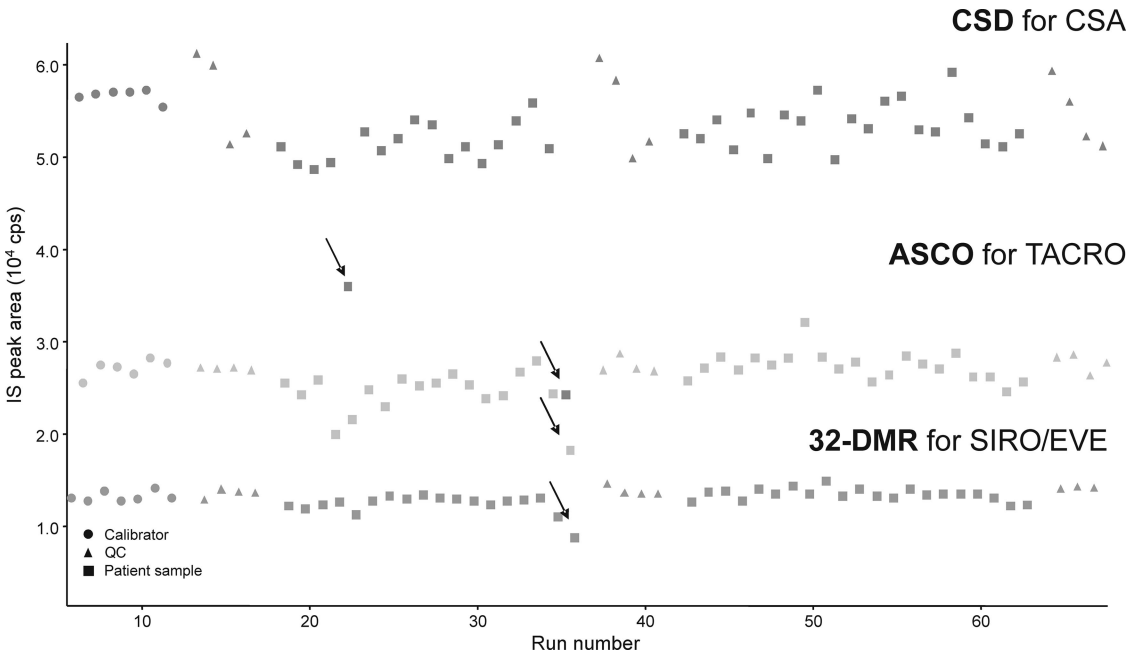

Fig. 2. Routine SRM-based LC-MS/MS immunosuppressant–therapeutic drug-monitoring assay [Seger C et al. *(64)*] internal standard (IS) plot showing the detection of severe ion suppression effects in 2 individual patient samples (arrows).

The IS peak areas of cyclosporine D (CSD) [1233.9 > 1198.9; IS for cyclosporine A (CSA)], ascomycin (ASCO) [809.6 > 756.3; IS for tacrolimus (TACRO)], and 32-desmethoxyrapamycin (32-DMR) [901.6 > 834.8; IS for sirolimus (SIRO) and everolimus (EVE)] are depicted. Circles represent the calibrator samples, triangles represent the controls, and squares represent the patient samples.

glucuronide *(5)*. In such cases, analyte molecules may be generated from the conjugate metabolites within the ion source by fragmentation. It is not possible to compensate for this effect through the subsequent MS analysis of these ions once they have been generated. In general, the risk of inaccuracy due to insource transformation increases whenever the selectivity of the chromatographic process is too low to separate a target analyte from its more hydrophilic conjugate metabolites.

Analyte-specific tuning of ionization conditions favoring the ionization of nonconjugated analytes over the ionization of conjugate metabolites may reduce the impact of insource transformation for an individual instrument. Maintenance procedures that involve retuning, however, require subsequent revalidation of the method used to compensate for signal interference caused by insource transformation. Hence, although it requires additional analysis time, target-analyte–specific optimization of the chromatographic resolution is the most reliable approach that can be used to avoid inaccuracy due to insource transformation (Fig. 3).

Insource transformation effects can be detected during method development only if extracts of biological samples are analyzed via extended chromatographic runs. If one or more additional peaks are observed in the mass transition trace of the target analyte (in comparison to a reference solution of the analyte), the process of chromatography should be accelerated only to such a degree that these peaks remain separated from the true analyte peak at baseline.

Because in most cases pure samples of conjugate derivatives of target analytes are not available, ruling out assay inaccuracy due to insource transformation effects is challenging. This emphasizes the importance of the use of patients' samples that contain relevant metabolites for reliable validation of LC-MS/MS methods, because interference by insource transformation will not become evident if only spiked QC materials (which do not contain endogenously formed metabolites) are used.

Inaccuracy Related to the Process of Ion Selection

ISOBARIC MASS TRANSITIONS AND ISOMERS

Isobaric compounds are either structural isomers of the target analyte that share its elemental formula or structurally unrelated compounds that have the same nominal molecular mass as the target analyte. In single-stage MS (i.e., GC-MS or LC-MS with low-resolution

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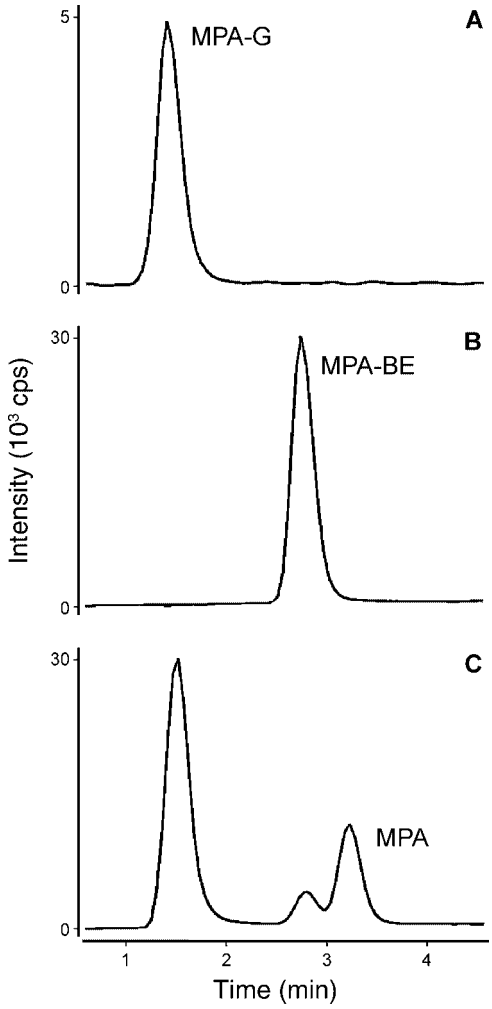

Fig. 3. Three LC-MS/MS ion traces recorded from a clinical sample collected from a patient receiving MPA therapy.

(A), The MPA-glucuronide (MPA-G, MPA metabolite) SRM-trace (514.0 > 206.9) with MPA-G peak. (B), The MPA-butyl ether (MPA-BE, internal standard) SRM-trace (421.0 > 206.9). (C),The MPA SRM-trace (321.0 > 206.9) with MPA eluting at about 3.2 min. Because of in-source fragmentation, both MPA-G and MPA-BE gave rise to ions with an SRM transition of 321.0 > 206.9 and consequently appeared in the MPA SRM. Hence, accurate quantification of MPA by LC-MS/MS requires the baseline separation of MPA from its conjugate metabolites.

single-quadrupole or ion-trap detectors), for which the analyte detection (i.e., in the selected ion-monitoring mode) is based solely on its molecular weight, potential inaccuracies due to the presence of isobaric constituents in complex biological samples have been well recognized *(36)*. In LC-MS/MS, the probability that an analyte or its internal-standard compound shares both precursor and product-ion masses with other unrelated compounds present in the sample is far lower than the probability that isobaric effects will occur in single-stage LC-MS.

Nevertheless, it has been recognized that isobaric mass transitions are an important potential source of inaccuracy in clinical LC-MS/MS applications *(15, 37, 38)* owing to the extreme complexity of metabolomic *(39)* and proteomic *(40)* components present in biological sample matrices such as serum and urine.

An apparent isobaric effect may actually also be attributable to the presence of a multiply charged ion of an unrelated molecule with a several-fold mass of the target analyte, because MS is based on the assessment of mass-to-charge (*m/z*) ratios. As a hypothetical example, a 2-fold charged xenobiotic compound molecule with a molecular mass of 724 Da would have an *m/z* ratio of 363 ($[M+2H]^{2+}$), which is identical to the *m/z* ratio of a 1-fold charged cortisol ion ($[M+H]^{+}$). Therefore, these 2 ions could not be distinguished with a single low-resolution MS unit as realized in ion-trap or single-quadrupole instruments. In addition, triple-quadrupole instruments operated in the selected ion-monitoring mode will also fail to distinguish these ions. Furthermore, the shared mass transition of analyte and a potential interfering compound may not necessarily be one of the most favored mass transitions under the latter conditions. If the interfering compound is present in much higher concentrations than the target analyte, substantial inaccuracy may result even if only a less abundant fragment ion of the interfering compound shares the *m/z* ratio of the target analyte.

MS approaches to identify isobaric interferences in individual samples may rely on the acquisition of several mass transitions of a target analyte and of the internal standard, a topic extensively reviewed by Kushnir et al *(41)*. If, for example, a precursor ion of a target analyte in a pure solution exhibits an *m/z* ratio of 582 and gives rise to 2 major fragment ions by MS/MS, these 2 fragments will exhibit a characteristic fragment-intensity ratio (e.g., the fragment with an *m/z* ratio of 188 is 3 times more intense than a fragment with an *m/z* ratio of 210, yielding a 3:1 ratio). If these 2 fragment-ion species are found to have an intensity ratio ("branching ratio") *(41)* of 1:1 in a patient's sample, it should be suspected that an isobaric compound that has undergone a mass transition of 582 *m/z* to 210 *m/z* is coeluting with the target analyte. The use of "quantifier" and "qualifier" ions, and the acceptance of results according to predefined branching ratios, is common in GC-MS. This principle is often applied to quantitative LC-MS/MS applications as well, especially

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in strictly regulated environments such as forensic toxicology or pesticide and mycotoxin analysis in animal feed and food products *(42)*. In contrast, in routine clinical LC-MS/MS applications, this approach is only rarely used at present *(41, 43, 44)*. Besides the fact that, for many analytes, collision-induced dissociation generates only a single product ion of sufficient intensity to be detected, this technique is not widely used, mainly because of the practical reasons described below. Quantitative fragmentation patterns, which determine branching ratios, are a result of complex individual instrument characteristics *(45–47)*. These patterns typically differ between instruments and even can show distinct matrix effects *(48)*. Consequently, accepted branching ratios must regularly be assessed with matrix-matched solutions of the target compound.

As an alternative approach for result verification, 2 separate quantifications of the target analyte can be performed based on simultaneously acquired differential mass transitions of the target analyte and of the internal-standard compound, respectively. The accepted degree of deviation between the 2 results is arbitrary (as are acceptance criteria for branching ratios of a compound). Whenever discrepancies do occur, it is not evident which result is correct.

If a target analyte and an interfering compound share the same nominal mass but differ in their elemental formula, their exact masses differ because of differences in the pattern of naturally occurring elemental isotopes *(49, 50)*. Consequently, MS instruments with a very high mass-resolving power can potentially distinguish compounds with identical nominal masses but different elemental formulas. Such platforms rely on sophisticated software algorithms and can be used to predict the elemental formula of unknown compounds based on a single accurate mass measurement. However, some limitations of this technique must be taken into account *(38, 51, 52)*, and fragment ions must be recorded to allow unequivocal analyte identification *(53)*. LC-MS hyphenations offer such exact mass determination *(50)* [e.g., those that are equipped with time-of flight *(54)*, Fourier transform ion cyclotron resonance *(55, 56)*, or orbi-trap *(57, 58)* analyzers], but these are still rarely used in clinical laboratories.

In the case of analytes with identical elemental formulas (true isobars), such as positional or geometrical isomers (e.g., 11-hydroxycortisol and 21-hydroxycortisol or testosterone and epitestosterone), discrimination between an analyte and an interfering compound is not possible with any mass analyzer, even one that has a very high mass-resolving power. It is possible that the disintegration patterns of a pair of isomers may be different, which would therefore allow for analytical discrimination between the 2 compounds, but in most cases, the chromatographic baseline separation of an analyte and its isomer before their MS/MS detection is required for unequivocal quantitative measurements to be performed *(44)* (Fig. 4). F4

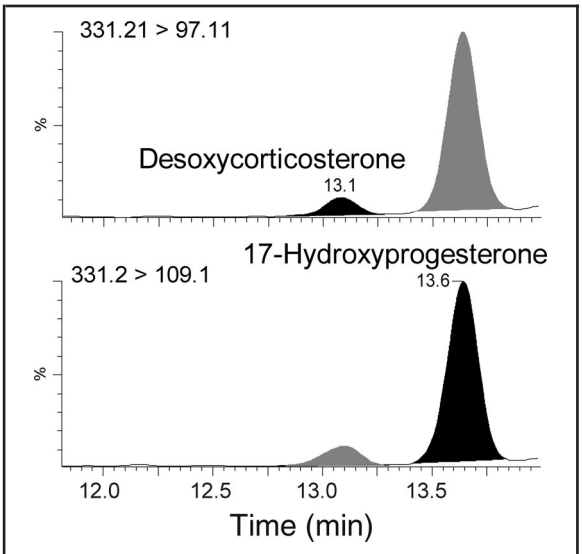

Fig. 4. Section of an HPLC-MS/MS-derived adrenal steroid profile derived from a patient sample.

Because of their structural similarity, isobaric steroid congeners, [i.e., 17-hydroxyprogesterone (OHP) and deoxycorticosterone (21-OHP)], interfere with each other (gray peaks) in the SRM ion traces used for analyte quantification (black peaks). Hence, chromatographic analyte separation must be achieved to allow accurate quantification of 17-OHP and 21-OHP. Reprinted from: Carvalho VM et al, Simultaneous quantitation of seven endogenous C-21 adrenal steroids by liquid chromatography tandem mass spectrometry in human serum. J Chromatogr B Analyt Technol Biomed Life Sci 2008;872:154–61 *(44)*, with permission from Elsevier. AQ: D

Examples of potential interference by isomers include the quantification of testosterone (biologically inactive isomer: epitestosterone) *(59)*, 2-arachidonoylglycerol (biologically inactive isomer: 1-arachidonoylglycerol) *(60)*, cortisone (synthetic isomer: prednisolone) *(61)*, methylmalonic acid (isobaric endogenous metabolite: succinic acid) *(41)*, and mannitol (stereoisomer: sorbitol) *(62)*. The last example demonstrates that LC-MS/MS may ultimately fail in specifically quantifying known compounds because the separation of these 2 sugars by use of LC is extremely difficult.

We previously demonstrated potential interference in the measurement of cyclosporine A due to the insource decay of a cyclosporine A metabolite. This

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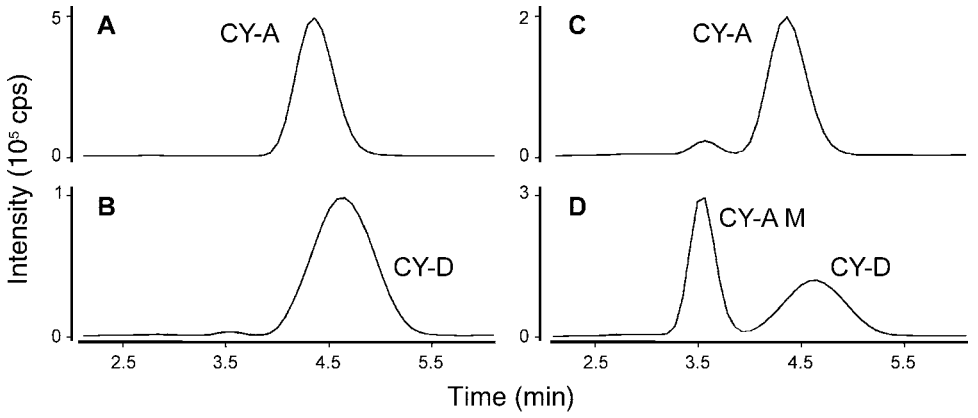

Fig. 5. Ion-source–mediated metabolite decay leading to additional peaks in patient samples (C,D) compared to reference samples (A,B).

(A,C), SRM-transition–derived ion trace (1220.0 > 1203.0) of cyclosporine A (CY-A) and (B,D), SRM-transition–derived ion trace (1234.0 > 1217.0) of its internal standard, cyclosporine D (CY-D). Calibrator materials yielded clean peaks for cyclosporine A (A) and cyclosporine D (B), whereas in patient samples, an additional prominent peak can be detected in the cyclosporine D channel (D). It originates from a cyclosporine A metabolite (CY-A M) undergoing an ion-source decay-promoted water loss.

breakdown results in a fragment that is isobaric to cyclosporine D, which is a widely used internal standard for cyclosporine A measurement *(6)*. This case demonstrates that in therapeutic drug monitoring that employs LC-MS/MS, potential interfering compounds that are related to isobaric metabolites can be identified only in postdose patient samples that contain relevant endogenously formed drug metabolites (Fig. 5). F5

Notably, isobaric interferences may also occur sporadically in individual samples owing to the presence of xenobiotic compounds, which are not typically assessed during assay validation.

Although the acquisition of multiple mass transitions is probably the best way to identify isobaric interference, the most efficient approach to avoid isobaric interference is the use of a sufficient degree of chromatographic fractionation, which again comes at the price of a prolonged analysis time. When extended chromatographic run times are used, the probability of isobar coelution is reduced, but cannot be ruled out with certainty.

CROSS-TALK

The mass spectrometric effect of cross-talk may occur if several mass transitions with identical product ions are acquired (e.g., 455 > 97 alternating with 316 > 97). If the collision cell is not emptied completely within the very short time between different transition settings (the interscan delay), spurious signals are recorded that will appear in the subsequently acquired mass transition trace (Fig. 6). Cross-talk may be particularly relevant in the quantification of several different metabolites of 1 drug in which the detected fragment ions are identical. F6

Collision-induced dissociation creates, in addition to the desired analyte product ions, a variety of additional fragment ions that originate from the target analyte, isobaric precursor ions, and chemical background noise. Hence, it is not at all evident which potentially interfering ion species are actually present in the collision cell. Consequently, cross-talk effects can arise from unintentionally monitored fragment ions.

In the newest generations of instruments, the occurrence of cross-talk effects seems to be minimized, but particular methods that involve the simultaneous acquisition of a very high number of mass transitions and short interscan delay times may still be prone to such interference. Although few reports address cross-talk (which should not be confused with carryover in the HPLC module) as a relevant source of inaccuracy in clinical LC-MS/MS assays, it should nonetheless be considered as a potential cause of inaccuracy.

INTERACTIONS BETWEEN CHROMATOGRAPHIC RESOLUTION AND MS/MS DETECTION

In the early years of the use of LC-MS/MS in bioanalytical and clinical laboratories, chromatographic separation was considered rather unnecessary owing to the preconceived notion that MS/MS spectrometers were extremely selective detectors *(63)*. Thus, the use of many LC-MS/MS methods that employ a minimal degree of chromatographic resolution as well as analyte

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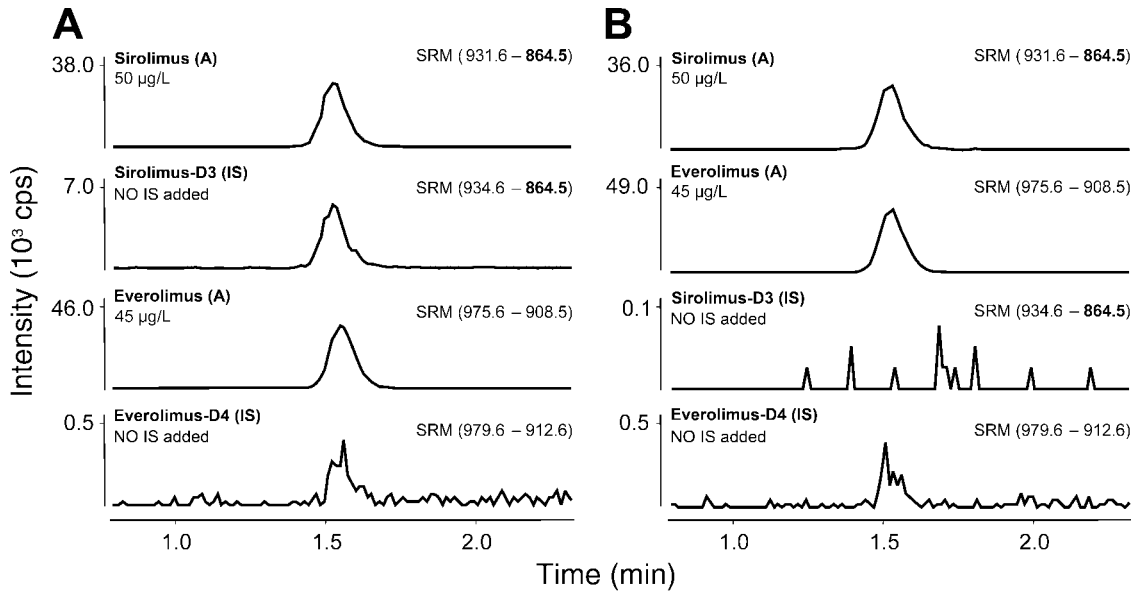

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Fig. 6. SRM-related MS/MS cross-talk in a sirolimus and everolimus analytes (A) LC-MS/MS assay performed by using deuterated (sirolimus-D3, everolimus-D4) internal standards (IS).

(A), Strong ion-trace cross-talk is observed in the blank sirolimus IS ion trace if it is recorded after the sirolimus analyte-ion trace, because both SRM reactions end in the identical product ion. Consequently, this effect is not observed for everolimus SRM reactions, which do not share an identical product ion. (B), Changing the order of the SRM experiments successfully prevents sirolimus cross-talk effects from occurring.

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retention times that are close to the void time of the chromatographic systems have been described (i.e., "dilute and shoot" methods). Because of the issues discussed thus far, however, the requirements for appropriate sample preparation and chromatographic separation before selective MS/MS detection have become evident. Minimized resolution is generally associated with an increased risk of interference due to insource transformation, isobaric compounds, and differential matrix effects. Thus, it has become increasingly accepted over the past several years that efficient chromatography is required to ensure reliable and accurate quantitative LC-MS/MS analyses of many, if not most, analytes.

The recent introduction of stationary HPLC phases $<$2-μm particle size has made it possible to optimize analyte separation with very short chromatographic runs. Such methods are characterized by very narrow peaks, and this technique requires the use of "high-end" MS/MS analyzers that allow for very fast data acquisition to ensure that an adequate number of data points ($>$10–15) are recorded over these narrow peaks. Moreover, the use of very small particles leads to very high back pressures and requires very efficient sample preparation because residual matrix components limit the lifespan of these columns much more than they limit the lifespan of conventional columns.

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FURTHER SOURCES OF INACCURACY

There is a preconceived notion that LC-MS/MS analyses are always highly reliable, but this innovative technology is subject to gross handling errors as well as the general pitfalls of quantitative chromatographic analyses. Sources of unreliable results include errors that occur secondary to inappropriate vial labeling, inaccurate manual pipetting, insufficient equilibration of the internal standard before protein precipitation, incomplete hemolysis in the quantification of immunosuppressant concentrations, inappropriate placement of vials in the autosampler, autosampler carryover, contamination, incorrect peak integration, and errors in manual data transfer *(3)*. Indeed, because of the lack of automation in LC-MS/MS, the risk of such human-related gross errors can be assumed to be greater than that associated with the use of automated clinical chemistry analyzers. Automation of the processes involved in LC-MS/MS methods with the final goal of developing fully automated MS/MS-based analyzer systems is not only a prerequisite for more widespread use of this powerful technology in clinical laboratories

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but is also essential to increase the reliability of the results of these assays.

LC-MS/MS shares with all other methods in clinical chemistry the need for appropriate quality-assurance policies and accurately specified calibration materials. These requirements have become evident in the current debate about incorrect 25-OH-vitamin D results that were reported by large laboratories employing this technology *(7–9)*.

Conclusion

The use of LC-MS/MS can enable the performance of highly accurate analyses. However, the application of this technology is not necessarily translatable into accurate results. The pitfalls of this technology must be recognized and systematically addressed. In particular, interferences from insource transformation of conjugate metabolites, matrix compounds sharing mass transitions with the target analyte, and the differential impact of matrix effects on the analyte and the internal standard can lead to inaccurate results in LC-MS/MS analyses. Additional technological developments will likely help to make LC-MS/MS assays more robust and decrease the effect of such interferences, but clinical chemists must remain watchful for potential sources of inaccuracy even with these types of powerful and fascinating technologies.

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