# Current and Future Applications of Mass Spectrometry to the Clinical Laboratory

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## Abstract

*Mass spectrometry is an analytic technique with high specificity and a growing presence in laboratory medicine. Various types of mass spectrometers are being used in an increasing number of clinical laboratories around the world, and, as a result, significant improvements in assay performance are occurring rapidly in areas such as toxicology, endocrinology, and biochemical genetics. This review serves as a basic introduction to mass spectrometry, its uses, and associated challenges in the clinical laboratory and ends with a brief discussion of newer methods with the greatest potential for clinical diagnostics.*

Methods to analyze any analyte by mass spectrometry (MS) can generally be divided into 3 steps: (1) sample preparation, (2) chromatographic separation (if needed), and (3) mass spectrometric analysis. In-depth discussions of each of these steps are available in textbooks[1-3] and journal articles referenced throughout. In addition, readers are directed to materials prepared by the Clinical and Laboratory Standards Institute for detailed recommendations about the use of mass spectrometry in the clinical laboratory.[4,5] The purpose of this article is to provide a brief and nontechnical introduction to the use of mass spectrometry in the clinical laboratory.

## Sample Preparation

Various methods exist for preparing a patient sample before analysis by mass spectrometry. The selection of which method to use relies primarily on the chemical characteristics of the analytes being measured (eg, acidic or basic), whether the analytes are heavily protein-bound, and the sample type chosen for analysis (eg, serum or urine). Sample preparation typically involves one or a combination of the following techniques: protein precipitation, liquid-liquid extraction, immunoaffinity purification, dilution, and solid-phase extraction (SPE).

The sample matrix is of outstanding importance in mass spectrometry and is a collective term referring to everything present in the sample not including the analytes of interest. Analytes measured from whole blood or prepared blood fractions typically require the greatest extent of sample preparation owing to the complexity of the sample matrix and

the relatively low concentration of most analytes compared with matrix components. Analytes present in urine, however, are often compatible with simple dilution protocols (so-called dilute and shoot methods) and are often present at much higher concentrations owing to the concentrating effect of the kidneys during urine production. Regardless of the sample matrix, the difference in behavior between the analytes and the matrix components in the downstream analysis will ultimately dictate the choice of sample preparation.

### Solid-Phase Extraction

SPE is an affinity-based method for separating analytes of interest from the unwanted matrix components. SPE uses a liquid mobile phase and a solid stationary phase. The analytes of interest, and any other molecules present in the sample, must continuously choose to stay in the liquid phase or to associate temporarily with the solid phase. The amount of time the analytes or matrix components spend interacting with the stationary phase will be based on their individual characteristics (eg, charge and polarity). By using a binding and wash solvent that is different from the elution solvent, analytes can be separated from the unwanted matrix components. For example, matrix components may have a very low affinity for the solid phase in the binding and wash buffer, whereas the analyte has a high affinity and spends so much time interacting with the solid phase that it appears to "stick" to the column under these conditions. The analyte can then be eluted in the elution solvent in which it has a much lower affinity for the solid phase. The end result of SPE is a crude separation of analytes from potentially interfering matrix components.

### Immunoaffinity Purification

Immunoaffinity purification (also referred to as immunoextraction) uses antibodies bound to a solid phase to allow for separation of the antibody-bound analytes from the nonbound matrix components. Direct detection of analytes by mass spectrometry, which is generally a much more specific method than competitive or sandwich immunoassays, overcomes the lack of specificity often observed with antibody-antigen interactions and results in a powerful means of measuring a broad range of analytes. Thus, immunoextraction provides purification and concentration of the analytes of interest with a significant reduction in unwanted matrix components.

### Dilute and Shoot

For less complicated matrices or for analytes present at high concentrations, dilution of the sample provides a simple but effective means to reduce matrix components. Comprehensive screens and confirmatory testing for drugs in urine commonly use this method of sample preparation.

## Sample Delivery

Methods for introducing a sample into the mass spectrometer range from direct infusion to multidimensional chromatographic separation. Use of a chromatographic method results in the staggered delivery of analytes and matrix components to the mass spectrometer, allowing for more effective use of analyzer time by limiting the portion of the sample being analyzed to only the fraction or fractions containing the analytes of interest and sending the other fractions to waste.

### Gas Chromatography

Gas chromatography (GC) uses a gaseous mobile phase such as helium or hydrogen to push molecules through a column that serves as the stationary phase. Manipulation of the column temperature changes the affinity of molecules for the stationary phase, providing a means to separate analytes from matrix components. GC is predominantly limited to volatile, heat-stable compounds lacking polar functional groups; however, chemical modification with derivatizing agents allows for functional groups to be masked, producing less polar, GC-compatible compounds. GC is the method most often used for comprehensive drug screening in the clinical laboratory using a combination of GC and mass spectrometry (GC-MS).

### Liquid Chromatography

Liquid chromatography (LC) is an increasingly popular choice for the chromatographic separation of samples before MS analysis due in large part to the broad range of compatible analyte types and a diminished need for derivatization. The mobile phase is a combination of aqueous and organic solvents, with adjustment of the aqueous/organic ratio used to alter the distribution of analyte and matrix components between the mobile and stationary phases. Use of LC systems capable of producing higher pressures, termed ultra–high-performance liquid chromatography, can be combined with a stationary phase composed of extremely small particles (< 2 μm) to provide fast, effective methods for separation of samples before analysis by MS. A high degree of similarity exists between the LC and the SPE methods described, with the most important distinction being the more precise separation of analytes by LC.

## Ionization Methods

Mass spectrometers are capable of detecting charged analytes only in the gas phase. An ionization method is, thus, required to convert analytes in the liquid phase to corresponding gaseous ions for analysis. The hardware required to ionize

analytes can be run in positive or negative ion mode, which selects for analyte ions carrying 1 or more positive or negative charges, respectively. The following discussion is limited to electrospray ionization and atmospheric pressure chemical ionization because these are the 2 primary ionization methods used in the clinical laboratory.

## Electrospray Ionization

Electrospray ionization uses a combination of voltage, heat, and air to produce successively smaller droplets from the liquid eluting off a chromatographic column. The continuous loss of solvent concentrates these droplets, resulting in a dramatic increase in charge per unit volume. Ions that have accumulated at the droplet surface desorb from the liquid into the gas phase, allowing these gas phase ions to enter into the mass spectrometer for analysis. In addition, complete evaporation of the solvent liberates large ions, such as proteins, producing the necessary gas phase ions for analysis.

## Atmospheric Pressure Chemical Ionization

Atmospheric pressure chemical ionization produces ions by using a combination of heat to completely vaporize the sample and plasma produced by an electrical discharge, commonly referred to as a corona discharge. The corona discharge ionizes the evaporated solvent, and, through physical interaction with gaseous sample components (including the analytes of interest), formation of negative or positive ions occurs.

## Types of Mass Analyzers

Mass spectrometers use a given molecule's mass/charge ratio ($m/z$) to provide a means to uniquely identify components present in the sample. For example, a molecule with a molecular weight of 400 and a charge of +1 will have an $m/z$ of 400. Furthermore, a molecule with a molecular weight of 15,000 and a charge of +3 will have an $m/z$ of 5,000. The mass range limit, analysis speed, percentage of ions analyzed, mass accuracy, and resolution are characteristics that distinguish the various types of mass analyzers and are summarized in ▌Table 1▌.

### Time-of-Flight Analyzers

Time-of-flight (TOF) mass spectrometers use an electric field to accelerate bundles of gas phase ions toward a detector. The $m/z$ of an ion will determine how long it takes for it to travel from the source to the detector, with low $m/z$ ions traveling faster relative to high $m/z$ ions. Several designs of TOF analyzers exist, some using a linear flight tube and others using 1 or more reflectrons that change the direction of ion flight and improve resolution or the ability to distinguish 2 $m/z$ ratios from one another. TOF analyzers have an essentially unlimited $m/z$

▌Table 1▌
**Characteristics of Selected Mass Analyzers**

|  | Time of Flight | Quadrupole |
| --- | --- | --- |
| Ion introduction | Pulsed | Continuous |
| Mode of separation | Velocity and flight time | Flight path stabilization |
| Sensitivity | High | Very high |
| Mass accuracy | High | Very high |
| Percentage of ion transmission | High | Low |
| Range of mass/charge ratio | Unlimited | Limited |
| Dynamic range | Limited | Wide |

range and very high sensitivity, mass accuracy, and percentage of ion transmission, but a limited dynamic range.

### Quadrupole Analyzers

Quadrupole analyzers have 4 parallel rods arranged in a square formation. At any given time of analysis, one diagonal pair of rods is positively charged and the other diagonal pair is negatively charged. These charges alternate at a set frequency such that balanced attraction and repulsion of the ion of interest maintains a stable flight path between the pairs of rods. The amount of positive or negative charge and the frequency at which the charges alternate is optimized for each analyte and can be rapidly changed throughout the duration of the analysis to allow for sequential detection of numerous analytes. How long the analyzer remains at a given voltage and frequency is referred to as the dwell time, and during that dwell time (on a millisecond timescale), 1 $m/z$ is detected. Some analyzers are limited in how fast they can switch between combinations of voltage and frequency, which limits the number of different ions that can be monitored in a given sample. Quadrupole analyzers have a limited $m/z$ range, high sensitivity and mass accuracy, but low percentage of ion transmission.

### Tandem Mass Spectrometry

Identification of a given analyte using $m/z$ alone does not always confer the needed specificity, illustrated by an analysis for morphine and hydromorphone, 2 distinct compounds with identical positive ions of approximately 286 $m/z$. Tandem mass spectrometers use multiple sets of quadrupoles arranged in series. A common tandem mass spectrometry (MS/MS) analyzer will have 3 quadrupoles (referred to as a triple quadrupole mass spectrometer or "triple quad"), denoted Q1, q2, and Q3. Q1 and Q3 are true mass analyzers using combinations of voltages and frequencies as described. The second quadrupole, denoted as q2, functions as a collision cell and uses a combination of an inert gas such as nitrogen and varying frequency. Ions entering q2 will collide with the inert gas, causing fragmentation of the precursor ion into smaller product ions, which will then pass through Q3 to strike the detector.

## Methods of Analysis

Several methods of analysis are possible with single and multiple mass analyzers with nearly all conceivable combinations of quadrupole, TOF, and tandem instrumentation. The following discussion will focus on methods of analysis using linear quadrupoles because this is currently the type of mass spectrometer most frequently encountered in the clinical laboratory. Methods of analysis, summarized in ❚Table 2❚, differ based on the desired selectivity for ions that enter and successfully traverse through the mass spectrometer to the detector. Q1 and Q3 can introduce the desired selectivity, and, to some degree, increased selectivity is possible with q2 as well ❚Figure 1❚.

### Full Scanning

The acquisition of full-spectrum data from a sample can be helpful in situations such as a comprehensive drug screen in which the analytes of interest have yet to be determined. The lack of restrictions on the $m/z$ of ions entering and traversing the mass spectrometer allows for finding unknown components in the sample (eg, >200 different drugs and metabolites) with a concomitant increase in the complexity of data interpretation (Figure 1A). The lack of specificity and increased complexity in the full mass spectrum reduces the usefulness of this method for routine clinical testing.

❚Table 2❚
**Methods of Analysis**

|  | MS | SIM | MS/MS | MRM |
|---|---|---|---|---|
| Quadrupoles | 1 | 1 | 3 | 3 |
| Specificity | Low | High | High | Very high |
| Selectivity | Low | High | Low | Very high |
| Data complexity | High | Low | High | Low |
| Ions detected | Precursor ions | Precursor ions | Fragments | Fragments |
| Application | Comprehensive drug screening | Very long chain fatty acid analysis | Metabolomics | Drug confirmation testing |

MRM, multiple reaction monitoring; MS, mass spectrometry; MS/MS, tandem MS; SIM, selected ion monitoring.
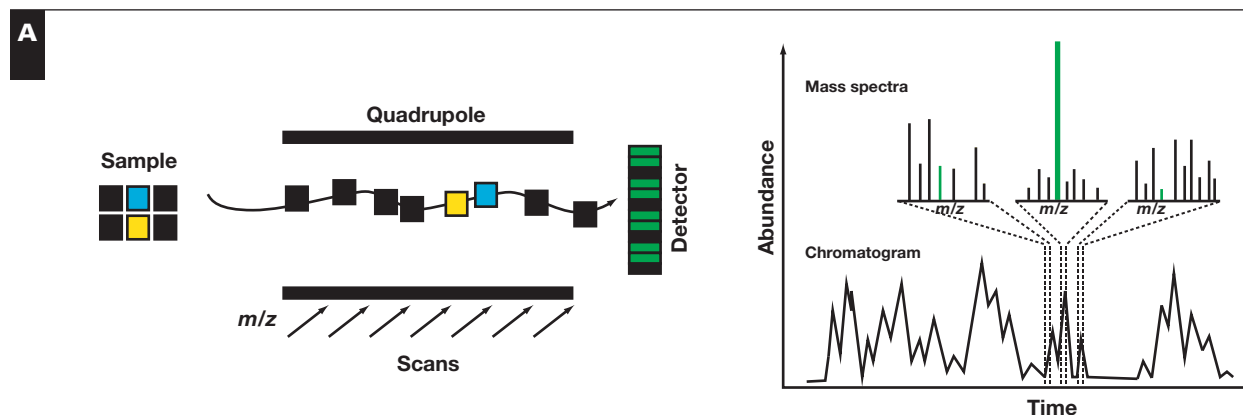
❚Figure 1❚ Mass spectra and chromatograms for a fictional sample undergoing chromatographic separation before 4 separate methods of mass analyzer analysis. In this example, the sample of interest contains 2 analytes of identical mass (blue square and yellow square) in addition to various other components constituting the sample matrix (black squares). The chromatogram for each method is accompanied by mass spectra selected from several time points demarcated by dotted lines. **A**, In the mass spectrometry (MS) mode, the single quadrupole constantly scans across the entire mass/charge ratio ($m/z$) range as the sample elutes from the chromatographic column into the mass spectrometer. Molecules of all $m/z$ within this scan range reach the detector, producing a complex chromatogram and mass spectra. The inability of the detector to distinguish between the analytes of interest with identical masses is indicated by a green mass spectral peak (yellow + blue = green). Note that only the middle mass spectrum selected at the time point when the analytes of interest are eluting from the chromatography column contains an $m/z$ signal belonging to the analytes of interest, indicated in green. The presence of green mass spectra at alternative time points indicates other compounds that match the selected $m/z$ but that are chromatographically resolved and, therefore, separated in time from the analytes of interest.
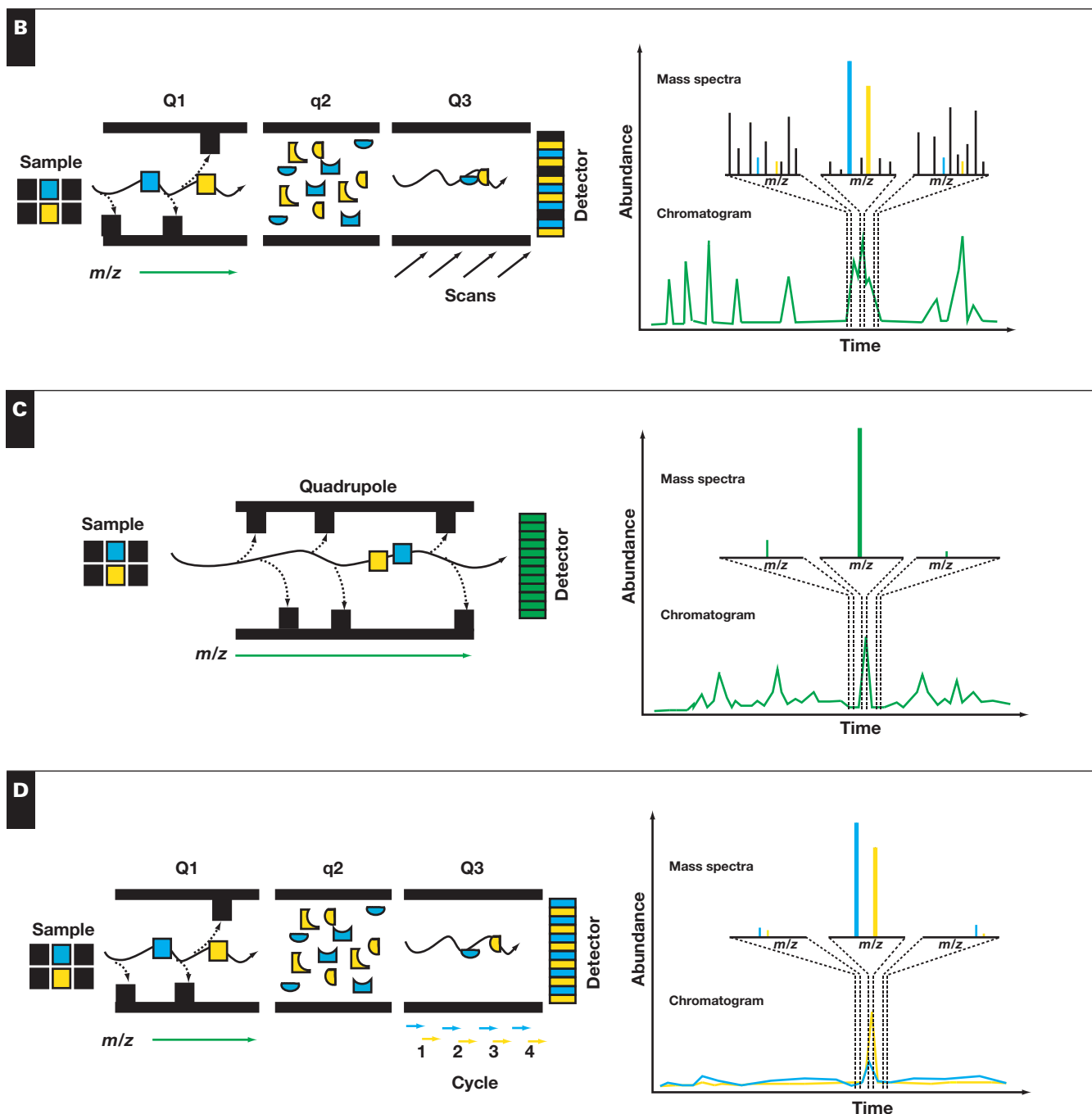
**B**, In tandem MS mode, Q1 is set to transmit only ions with a specific *m/z* that matches the analytes of interest. The collision cell, q2, produces fragments from these transmitted ions, while Q3 constantly scans across the entire *m/z* range, allowing all fragments produced in q2 to reach the detector. The recording of all fragments results in a complex chromatogram and mass spectra but has allowed for the unique identification of each analyte at the appropriately sampled time point, indicated in blue and yellow. **C**, In the selected ion monitoring mode, the quadrupole is set to transmit only ions with a specific *m/z* that matches the analytes of interest. Because only ions matching the selected *m/z* are transmitted, the resultant chromatogram and mass spectra are cleaner and easier to interpret. However, because the 2 analytes of interest have identical *m/z*, the mass spectrometer is unable to differentiate between the 2 analytes, and, in this example, a green mass spectrum results. **D**, Using a multiple reaction monitoring method of analysis, Q1 is set to transmit only ions with a specific *m/z* that matches the analytes of interest. The collision cell, q2, produces fragments from these transmitted ions, while Q3 continuously cycles between 2 specific *m/z* settings corresponding to 1 unique fragment from each of the analytes of interest. This provides individual chromatograms for each transition monitored and unique mass spectra at the appropriate time point.

## Selected Ion Monitoring

With the identities of the analytes known, optimized voltage and frequency parameters for the desired $m/z$ ratios can be used as a "filter" to reduce the number of unwanted ions entering the mass spectrometer and eventually reaching the detector (Figure 1C, selected ion monitoring [SIM]). The rate at which the instrument can move between ion-specific settings (ie, the scan speed) determines the maximum number of unique ions the mass spectrometer is capable of detecting during a single analysis. This mode of analysis requires a single quadrupole and only the $m/z$ of a precursor ion because no fragmentation is induced. For example, SIM analysis using GC-MS is useful for targeted determination of disrupted steroid hormone biosynthesis and various other metabolic syndromes when the analytes of interest are known.

## Multiple Reaction Monitoring

The use of multiple reaction monitoring (MRM) provides a definitive demonstration of the unparalleled specificity obtainable by mass spectrometry. Optimizing the parameters for a precursor ion and an associated product ion allows for 2 increasingly unique forms of identification. In the example of the problematic compounds morphine and hydromorphone (having identical precursor ions of 286 $m/z$), use of a triple quadrupole mass spectrometer can reliably differentiate these compounds using MRM (Figure 1D). The first quadrupole (Q1) is set to a combination of voltage and frequency to promote passage of precursor ions with an $m/z$ of 286 (ie, morphine and hydromorphone). Entrance into the second, gas-filled quadrupole (q2, or the collision cell), produces stable product ions ($m/z$ of 153 for morphine and 157 for hydromorphone) unique to each precursor ion. Setting the voltage and frequency in Q3 to first transmit the product ion for morphine and then changing the voltage and frequency to transmit the product ion of hydromorphone results in an effective strategy to independently measure the 2 compounds. The precursor ion and product ion pair is referred to as a transition, and in this specific example, the $m/z$ transition for morphine would be 286 > 153. Furthermore, the use of MRM allows the mass spectrometer to effectively scan faster as scanning is reduced to discrete $m/z$ set points rather than scanning a broad $m/z$ range.

## Common Challenges and Techniques in Clinical Mass Spectrometry

### Ion Suppression

Molecules present in the sample matrix, coeluting compounds, and $m/z$ cross-talk are common causes of ion suppression or enhancement.[6] Simply stated, ion suppression occurs when something present in the sample interferes with the ionization process of the analytes. For example,

nonvolatile or less volatile components found in the sample can reduce droplet formation and the efficiency of solvent evaporation, both leading to a reduction in ion formation. Several techniques for determining the degree of ion suppression exist and are described in detail elsewhere.[6]

### Internal Standards

Several methods exist to reduce or eliminate ion suppression, including the sample preparation techniques outlined earlier, modifications to the current chromatographic method to better resolve matrix components and analytes, and mobile phase additives to aid in ionization. Internal standards behave similarly to the analytes of interest throughout sample preparation and analysis and can reduce the deleterious effects of ion suppression on quantification.

It is important to note that the internal standards must be different, typically by increasing their molecular weight, to reduce overlap in the mass spectrometer with the endogenous analytes. For example, replacement of 6 hydrogen atoms (molecular weight, 1 atomic mass unit each) with 6 deuterium atoms (molecular weight, 2 atomic mass units each) on a molecule will increase the net mass by 6. This produces an internal standard with similar extraction and chromatographic performance but a different $m/z$. Spiking this internal standard into calibrators, standards, and samples at an equal concentration provides a means to normalize for variability in sample preparation and mass spectrometric analysis. Dividing the measured peak area of an analyte by the peak area of the internal standard provides a response value. Use of the normalized response significantly reduces variability for the calibration curve and subsequent quantification as the internal standard accounts for sample-to-sample fluctuations in extraction efficiency, chromatography, and ionization.

### Ion Ratios

Analytes present in complex matrices or analytes with large numbers of structurally similar compounds can be challenging despite high specificity methods such as MRM. Because interference is possible due to coeluting molecules with identical precursor mass, it is possible to monitor multiple $m/z$ transitions for each analyte. The ratio of the chromatographic peak area of the more abundant fragment to the peak area of a less abundant fragment produces a ratio of the 2 $m/z$ transitions. Use of this type of ion ratio can further enhance the specificity for individual compounds by ensuring that the ratio of the chromatographic peaks matches that expected for the analyte of interest.

## Applications in Toxicology

Many clinical laboratories offer toxicologic screening methods that are immunoassay-based. Fewer laboratories offer

GC-MS comprehensive drug screens, and even fewer offer confirmatory testing by LC-MS/MS or GC-MS. Screening assays are qualitative and have cutoffs set to provide adequate specificity at an acceptable level of sensitivity. Typical screening assays will identify a class of compounds (eg, opioids) but are not designed to make a final identification (eg, morphine vs hydromorphone). Confirmatory testing is generally quantitative and performed using a method that is sensitive and specific, such as SIM or MRM. Confirmatory testing, such as that using LC-MS/MS, is critical when the true identity of the compound or a greater degree of sensitivity is needed.

## Applications in Endocrinology

Continuous optimization of antibody-mediated detection methods has reduced but unfortunately not fully eradicated the intrinsic faults of immunoassays for the measurement of small molecules[7] and proteins.[8] Cross-platform variability, inadequate specificity, antireagent antibodies, and the hook effect are characteristics of many modern-day immunoassays that negatively impact their usefulness in clinical diagnostics.

The increase in testing for vitamin D has resulted in a dramatic amplification in interest and implementation of clinical mass spectrometry.[9] LC-MS/MS methods for vitamin D are able to separate vitamin $D_2$ from vitamin $D_3$[10,11] and provide information on the epimeric form of vitamin D,[12] neither of which is currently possible with existing immunoassays.

The rate of development of steroid hormone assays for diagnostic and forensic testing continues to increase.[13,14] A lack of specificity and accuracy at low concentrations in many steroid immunoassays has contributed to mounting frustration in the diagnosis of endocrine disorders[15] and has led several medical groups to recommend mass spectrometry as the preferred method of analysis. It is important to note, however, that the application of mass spectrometry to steroid analysis has not been free of challenges[16,17] and requires a high degree of technical competence, skill, and experience to provide the needed improvement for measures of endocrine function.

## Future Directions

### Clinical Protein Analysis

The success of mass spectrometry in the measurement of small molecules has pushed clinical mass spectrometry toward the analysis of peptides and proteins for diagnostic testing. A more targeted approach to quantitative protein analysis has emerged that differs significantly from the attempts to identify all proteins in a complex sample (termed shotgun proteomics). Quantitative clinical proteomic methods typically use enzymatic digestion of a complex sample, isotope dilution for normalization, and *m/z* transitions with tandem mass spectrometry. Quantitative analysis of proteins by mass spectrometry is an exciting area of laboratory medicine that faces several challenges before implementation.[18]

### Metabolomics

Clinical diagnostic measurements of the genome and the proteome are intertwined with measurements of the metabolome to fully comprehend the differences between healthy and diseased states. The basic metabolic panel is one example of many in which measuring the resultant output of the underlying genome and proteome aids in the diagnosis and monitoring of disease. Newborn screening is an area in which metabolomics by mass spectrometry has greatly expanded, resulting in multiplexed assays for organic acids,[19] amino acids,[20] fatty acid oxidation,[21,22] and other inborn errors of metabolism.[23]

## Summary

The implementation of mass spectrometry in the clinical laboratory has resulted in significant advancements in clinical pathology while adding a new layer of complexity to an already overwhelming aspect of medicine. Now, as always, extensive communication between laboratory directors and clinicians is a vital component of effective laboratory medicine and patient care. Mass spectrometry is increasingly prominent in the clinical laboratory, and a basic understanding of the technology is important to help support the important, productive interactions between clinicians and laboratory staff to aid in the diagnosis and monitoring of disease.

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