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Be sure, today and tomorrow— LC-MS in clinical research

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Evolution in the clinical laboratory

The fabulous scientific advances witnessed in the world of analytical sciences have had a profound influence on the health, comfort and welfare of mankind. However, one can question if there is enough understanding and appreciation of the intensive study in clinical science where the main focus is usually on the *discovery* and not in the *requirements* that made the discovery possible (Sr., 1993).

Mass spectrometry (MS) has been a known technology for almost one hundred years, from the time when its basic principles were first described by Nobel laureate Sir Joseph John Thomson in 1897 (JJ 1897, JJ 1913). Despite some very early research on the use of MS for respiratory gas analysis in the 1950s (KT and P, 1957), the majority of early applications of MS in clinical diagnosis go back to the early 1970s with the application of Gas Chromatography (GC) coupled to MS for determination of a variety of biologically significant molecules.

Because GC requires a certain level of analyte volatility, and since most biologically active molecules are polar, thermolabile, and involatile, elaborate extraction and derivatization protocols had to be devised to implement GC-MS useful enough for the analysis of clinically relevant analytes and samples. To make sample analysis less difficult by MS, a significant amount of R&D

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funding was invested over several decades aimed at coupling Liquid Chromatography (LC) with MS, since High Performance Liquid Chromatography (HPLC) is a much better separation technology than GC for polar thermolabile biologically relevant molecules.

Mass spectrometry and its different flavors

MS is an analytical technique used for determining the elemental composition of samples, quantifying the mass of particles and molecules, and elucidating the chemical structure of molecules. Various types of MS with high specificity, such as Liquid Chromatography (LC-MS), Gas Chromatography (GC-MS), and Matrix-Assisted Laser Desorption/Ionization/Time-Of-Flight (MALDI-TOF MS), are being increasingly valued and utilized as tools in clinical laboratories (FG and AN, 2011).

LC-MS platforms are capable of performing electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), or atmospheric pressure photoionization (APPI) which ionize various semi-volatile, thermally labile, and polar-to-nonpolar compounds, that are usually found in trace levels in complex biological matrices. Ions generated after ionization are transferred through a vacuum interface into the mass analyzer.

These MS techniques overcome the limitations of immunoassays and offer many advantages over earlier approaches. The traditional analytical assays used in Clinical Research laboratories are often found to be lacking desired selectivity and specificity. However, those conventional technologies provided familiarity, a wide reference network/install base, ease-of-use and ease-of-implementation.

As an example, prior to the 1990s, the use of LC-MS for analysis of biological specimens was significantly limited since ionization techniques were available only for low molecular weight compounds (~200 Da or less), and no effective methods existed for easy introduction of biospecimens into the high-vacuum of the mass spectrometer. However, introduction of Electrospray Ionization (ESI) by John Fenn (JB, M et al., 1989), who was awarded the Nobel Prize in 2002 for Electrospray Ionization, showcased the implementation of MS for soft ionization of large biomolecules.

As already indicated in this article, MS has gone through some major evolutionary processes to become a widely used analytical technique in the physical and chemical sciences. In today's world, the most widely used MS technologies comprise two different forms: high-resolution accurate mass (HRAM) and Triple Quadrupole (QqQ) (Figure 1).

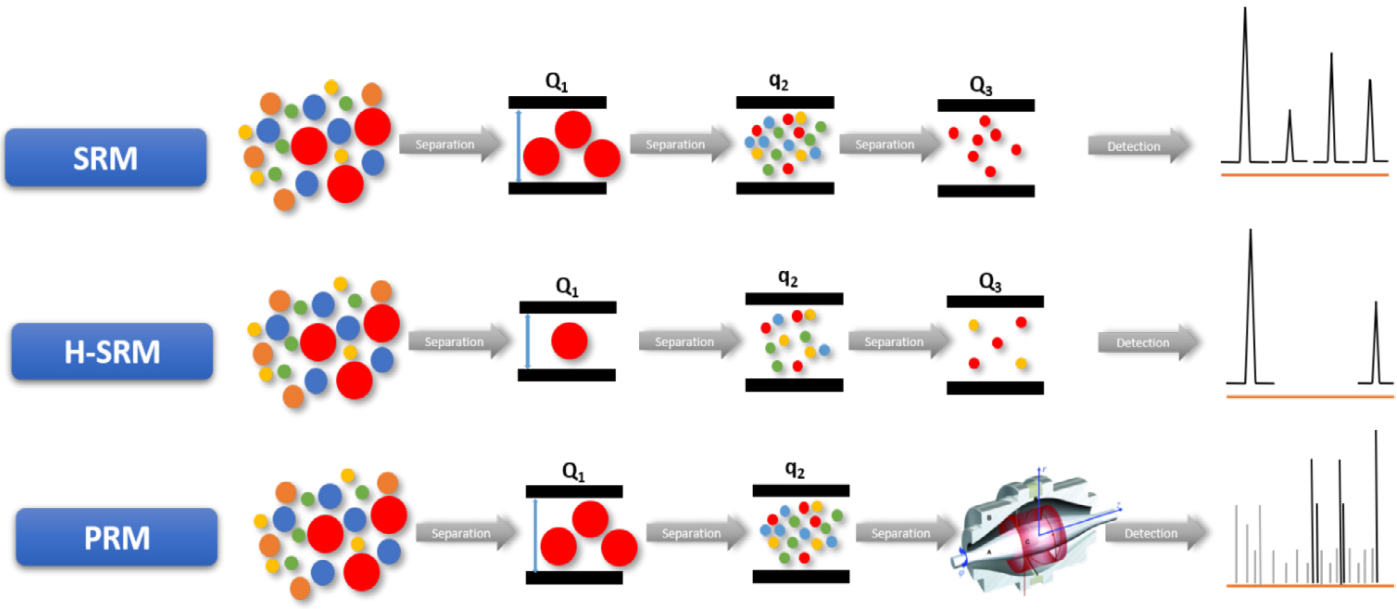

Figure 1. Ion fragmentation schematic in regular QqQ (top) indicated as selected reaction monitoring (SRM), QqQs equipped with high resolution SRM capabilities (H-SRM) where the Q1 and Q3 gap can be minimized to enhance selectivity (middle), and High Resolution Accurate Mass Orbitrap technology showcasing predictive reaction monitoring (PRM) schematic (bottom).

LC differentiates compounds by their physicochemical properties, and MS differentiates compounds by their mass-to-charge (*m/z*) ratio. It is the dual selectivity that makes LC-MS such a powerful analytical tool. Advancements in LC-MS have led to its widespread implementation, delivering high sensitivity and selectivity to ensure isolation of target analyte from samples containing thousands of different molecules.

Typically, MS or LC instruments alone are unable to meet this need as they can only differentiate compounds by their mass-to-charge ratio (*m/z*) or retention time on the column, respectively, which is often less efficient in most practical applications. Therefore, there is a strong requirement for a technique (LC-MS in this case) that utilizes an ideal separation platform and an efficient selective and sensitive platform for detection, analysis, and quantitation. There are several pros and cons of the LC-MS technology, which can be summarized as indicated in Table 1.

As indicated above, there are some significant benefits of LC-MS technology over the traditional analytical techniques, especially for clinical research laboratories. From confirmation of an analyte to sensitive quantitation, LC-MS finds extensive use in almost every clinical research environment.

However, once you arrive at the LC-MS technology, it is important for one to determine the type of MS that will be optimal. The guideline is usually laid out by the nature of analytes, expectation of the assay, etc. Both HRAM and QqQs have their unique set of advantages, and a decision to use one over the other should be governed by critical requirements. Table 2 highlights the benefits and ideal applications for QqQs and HRAMs, which can enable you to decide the right MS platform for your laboratory.

Nuances of the technology and workflow

Improving LC-MS/MS workflows and ease of use

The perceived high level of complexity in operation of LC-MS can be considered as one of the factors impeding a faster adoption of this technology outside of specialized referral and reference laboratories. For smaller laboratories, getting started with LC-MS can be further complicated and laced with hurdles, including (i) the high initial cost of the equipment, which might deter institutions on a tight budget; (ii) the absence of previous MS experience.

It must be anticipated that a laboratory scientist will have to spend 3–6 months, often including training, to acquire a basic skill and comfort level that allows successful implementation of new LC-MS assays. New test implementation and troubleshooting can be difficult until more experience has been acquired. Subtle differences to published methods with regards to equipment, reagents and conditions, which incidentally may not have been listed in enough detail in a publication, can prove frustrating.

Despite these hurdles, which can be formidable for smaller laboratories, the compelling analytical advantages of this technology for many low molecular weight analytes and the sometimes substantial reagent cost savings compared to commercial immunoassays have prompted many smaller, or more general, laboratories to consider LC-MS.

While the performance capabilities of the MS systems have increased in leaps and bounds over the last decade, LC systems have also improved in user-friendliness and integration with the MS, and sample clean-up or extraction has begun to be integrated into several LC front-ends.

It is now conceivable to imagine that soon we will have integration of liquid handling and sample extraction/clean up in a single MS-front-end, which, in turn, will be highly integrated with the MS instrumentation, all being interfaced bi-directionally to a Laboratory Information Management System (LIMS). In the continued development process of adoption of LC-MS in the clinical laboratory, one must keep in mind that there are several nuances of the LC-MS technology, if not the entire workflow. Some of them can be highlighted as follows:

Sample preparation

A host of sample preparation techniques are known and used across all clinical laboratories performing quantitation of critical analytes in biological matrices. While protein precipitation is used for many assays such as immunosuppressants, other sample preparation techniques that offer higher sensitivity or cleaner extracts come into consideration, such as solid-phase extraction (SPE), supported liquid extraction (SLE) and liquid-liquid extraction (LLE). Although derivatizing analytes is primarily used with Gas Chromatography, it is also used in certain situations for achieving higher sensitivity in LC-MS (U, B et al., 2011, M, K et al., 2012). A recently published review demonstrated the importance and diversity of sample preparation (L, 2012).

Table 1. Pros and cons of adapting LC-MS technology for clinical research.

Pros	
Selectivity	Combining LC and MS technologies results in additional information. Selectivity, which is inherent to this technology allows ideal isolation of the analyte of interest from the complex background matrix/matrices. Since analytes are separated by their *m/z* ratio, this technique allows for the use of isotopically labeled internal standards, which may not be separated by LC, but can be separated by their mass difference.
Speed of LC	Since the mass spectrometer will distinguish compounds based on mass, the chromatographic method does not have to separate every single component in the sample, so coelution of non-isobaric analytes is possible. This allows fast LC analysis times and reduced sample preparation, which helps with method development and high throughput sample analysis.
Speed of MS	Both HRAM and QqQs offer high speed detection capability, with the QqQ being faster between the two. LC instruments tend to be slower than the MS detection speed, resulting in the MS system being idle during the LC analysis of a sample. Hence, faster detection of MS can ensure higher productivity of assays.
Sensitivity	Mass spectrometers are inherently sensitive. Good selectivity also leads to reduced noise, allowing easy development of highly sensitive assays.
Cons	
Expense	Compared to other widely used technologies, LC-MS can potentially be more expensive, having a negative impact on an organization's cost/sample goals.
Complexity	Individually, either LC or MS can be difficult to optimize, so optimizing the two together requires an even more complex co-dependent synergy. The ionization mechanism can be especially complicated—often several charged species are formed in the ionization source, and multiple charging of ions can occur. Care must be taken to choose conditions for optimum sensitivity and reproducibility.
Dynamic Range	Compared to other quantitative techniques, LC-MS can have a limited range where the response is linear with respect to concentration.
Excessive Selectivity	In quantitative analysis, it is usual that the mass spectrometer is set to only detect specific analytes. This results in a very "clean" looking chromatogram, and it may be easy to forget that there can be many components still present, but not seen. These components can cause challenges in achieving reproducible quantitation and can be difficult to trace if they are not being looked for in the analysis.

Table 2. Features and benefits of QqQ and HRAM in clinical research.

Parameters	QqQ	HRAM
Productivity	Fast acquisition modes with MS/MS sensitivity offer excellent reproducibility for quantitative analysis at the limit of detection (LOD) or quantitation (LOQ).	Outstanding resolution offers clarity of the analytes in environments not seeking the ultimate in high-throughput.
Robustness & Reproducibility	Highly demanded in a targeted quantitation environment for quantitation of one to hundreds of analyte(s) in complex matrices	Provides information concerning the elemental composition and molecular weight of an analyte. Accurate mass of fragments may not be sufficient to elucidate the structure.
Sensitivity	Applicable for a host of molecule types in complicated biological matrices	Sensitivity is not necessarily the driving force—comprehensive structural identity with some sensitivity is the typical expectation.
Cost/Sample	Fast, robust, reproducible workflows for sample analysis, every day.	Retrospective search—Full Scan MS is an information-rich mode that can be analyzed post-acquisition.
Regulatory Requirements	Address regulatory requirements—from regulated environments to established methods; easy method development for all molecule types.	Growing footprint—while some laboratories are validating HRAM to perform studies in a regulated environment, they are mostly used in upstream discovery work.
Selectivity	Offers high-resolution selected reaction monitoring (SRM) capabilities; records nominal mass.	Outstanding resolution—obtain better information about your sample with the ability to distinguish between two peaks with similar ion-to-charge ratio.

Kits and calibration for clinical research

Quantitative MS is often plagued by lack of robust, reliable, and reproducible calibrators, which are often prepared in the research laboratory for assays such as quantitation of immnosuppressants, vitamins, drugs of abuse, etc. It has been reported that if sufficient attention is paid to remove matrix effects and ion suppression caused by matrix components, matrix matching may not be necessary (AK 2011).

Currently, some ready-made kits are available for purchase that promise an 'out of the box' solution. However, there can be disadvantages to reliance on such kits, considering there can be significant lot-to-lot variation. In addition, the ability to address the critical goal of reducing cost/sample faced by every clinical research laboratory can be further challenged by the price of the ready-made kits.

Moreover, as the diversity of clinical research projects continues to grow, it could become complicated to address each and every application type with a dedicated sample preparation kit. The traditional protein precipitation, liquid-liquid extraction procedures often serve as a good and economical starting point for any research project focused on quantitation of analytes in biological matrices.

Finding the right analytical column

Finding the right column for the quantitative assay can be the most critical part of LC-MS method development. On one hand, the chosen column should demonstrate desirable separation of the analyte from any isobaric and non-specific interferences. The same column, on the other hand, must also enable separation of the analyte(s) from any compounds that might cause ion suppression. Also, the right time of elution should be determined for each of the compounds (TM, 2003). The choice and use of internal standard is critical to ensure quantitative efficiency and reduce potential matrix effects, especially when the use of deuterated stable isotopes can still result in differential matrix effects (DR, El-Khoury et al., 2014).

Sample clean-up techniques (comprising SPE, LLE, SLE, PPT, phospholipid removal plates) have become a part of the standard operating process in most quantitative environments. These techniques are used for the removal of matrix interferences and compounds that cause ion suppression (CR, Z et al., 2004). Regardless of the techniques that are employed, the removal of compounds causing undesired interferences is incomplete. Chromatography plays a major role in this regard, and there is a large, continuously evolving variety of chromatography columns available from each manufacturer.

Increasingly, columns are expected to resolve complex analytes in a mixture from complicated matrix interferences at relatively short run times. Some of the recent developments in column technology, such as the Thermo Scientific™ Accucore™ Biphenyl Reversed Phase LC column (2018), which is based on core enhanced technology, can enable every clinical research laboratory with high quality data. These columns provide unique selectivity for aromatic and moderately polar analytes and offer a rugged platform for a variety of matrices.

Diversity of matrices

LC-MS enables the analytical scientist to have additional flexibility resulting in efficient analysis (from characterization to quantitation) of analytes in different biological matrices. In addition to the known biological matrices, such as, blood, plasma, serum, oral fluid, urine, drug analysis is also carried out on hair samples. Drugs can be detected in hair for much longer time periods than in other matrices, and hair analysis can also give information on previous drug abuse (JE, BG et al., 2015).

Analysis in saliva is often viewed as less invasive than blood, and it can be very useful for getting multiple samples over the course of time (e.g., analysis of hydrocortisone or testosterone). Dried blood spot analysis has been used for many years for newborn screening and is now used for other assays due to the stability demonstrated by many analytes in dried blood spots compared to whole blood or serum (BG, 2011). Other matrices for which MS assays are reported include fibroblasts, bile, tears, and cerebral spinal fluid.

Improving throughput

The advancements in LC-MS technology and easier implementation of critical methods and workflows have reduced the challenges and barriers that were cited as weighing against adoption of LC-MS as the primary technology. However, despite showcasing the ease-of-use, LC-MS (regardless of use of HRAM or QqQs) often has difficulty addressing some pressing and critical challenges, such as increasing productivity by improving sample throughput. Depending on the test(s), or mix of tests, an LC-MS system, with a single channel LC,

can perform between 70 to 250 tests per working day, assuming a two-shift (14–16 hours), 5–6 days per week schedule (or half that number for a single shift laboratory) (KG and J, 2011).

While these numbers might seem respectable to some, volumes of clinical samples in most clinical diagnostic laboratories can range between the high hundreds to the thousands. Some of the common tests that result in high volume samples include 25-OH Vit D_2 and 25-OH Vit D_3, testosterone, estradiol, and cortisol. Consequently, many laboratories continue to use automated immunoassays for these analytes, despite the often compelling analytical and financial advantages of LC-MS. Those laboratories that do use LC-MS/MS for high volume tests face the dilemma of recurring, costly instrument purchases, increasingly cramped laboratory space, and escalating use, storage and disposal of the toxic and flammable solvents used in LC-MS workflows.

Dilute and shoot

The most obvious factor responsible for the limited throughput of LC-MS is the time required for sample introduction into the LC and the subsequent time necessary for chromatography. Once a chromatographic system has been fully optimized to minimize the time needed to remove interferences and to separate analytes from solvent fronts, there is no room for further improvement in throughput from a chromatographic standpoint.

The only way to save additional time in a simple LC setup is to dispense with analytical chromatography altogether. In such a technique, the sample is simply diluted and injected directly into the MS, with or without in-line sample clean-up or guard column. For this so called 'dilute and shoot' approach, the new speed limit is determined by the combination of the mechanical speed of the autosampler and the time it takes for the sample to traverse tubing, pumps and any guard columns or in-line clean-up columns/loops, if present.

Depending on the setup and instrumentation, this translates into a time of 45–120 seconds per injection for a theoretical throughput of about 400–1300 samples per 14–16 hours working day (or half that number for a single shift). Increased instrument cleaning and maintenance, due to the large amounts of sample matrix injected, tend to cut the theoretical throughput figure by around 50%, still a sizable improvement on the baseline throughput of single channel LC and LC-MS/MS systems (KG and J, 2011).

Multiplexing technology

An innovative technology for increasing throughput is multiplexing. Multiplexing is the ability to simultaneously run multiple LC systems into a single mass spectrometer. This novel technique takes advantage of the time a MS typically spends idle by time-staggering the LC methods so that each one elutes in succession. The MS is dedicated to each LC elution, thus providing the same quality data as a single LC/MS method. A typical LC/MS method using multiplexing will gain a two- to four-times throughput increase while still using a single MS. Therefore, productivity is increased substantially, cost increase is low, and there is no negative effect on data quality. Note that some multiplexing products do not time-stagger the samples into the MS. These LC systems alter the pump pressure or sample the eluting stream. Both result in poor chromatography or reduced data (J, 2009).

The ability to run multiple methods on a two- or four-channel parallel system provides flexibility to meet today's demanding lab workflows. In an illustrative example, a method for simultaneous analysis of opiates and benzodiazepines for forensic toxicology testing was set up utilizing a Thermo Scientific™ Transcend™ TLX-2 system. The system was used to run two LC-MS/MS methods, one for each class of compound. While different analytical columns were used for each method, the system utilized one set of solvents and one MS.

Using Thermo Scientific™ Aria™ operating software, multiplexing technology and data windowing were used to enable these methods to be run simultaneously and robustly, leading to increased throughput with minimal operator intervention.

Along with multiplexing, these methods also utilized Thermo Scientific™ TurboFlow™ technology for automated sample preparation, allowing direct injection of the biological matrices into the LC-MS system. Multiplexing products can help customers to substantially increase their LC-MS throughput without any loss in data quality and with a single MS. The technology also allows customers the flexibility to validate multiple methods on a single LC-MS multiplexing system, allowing scientists to choose and run separate methods without any change-over or setup on their MS. These separate methods can be started at different times and will still run simultaneously to increase throughput.

Ion production and fragmentation

Electrospray ionization (ESI)(CM, RN et al., 1985) and atmospheric pressure chemical ionization (APCI)(EC, DI et al., 1974) are the two most common ionization techniques used in clinical laboratories. In ESI, the eluent from the column passes through a stainless-steel capillary to which a charge is applied. A heated gas, usually nitrogen, is directed along the outside of the capillary, resulting in the solvent being nebulized at the end of the capillary (Figure 2).

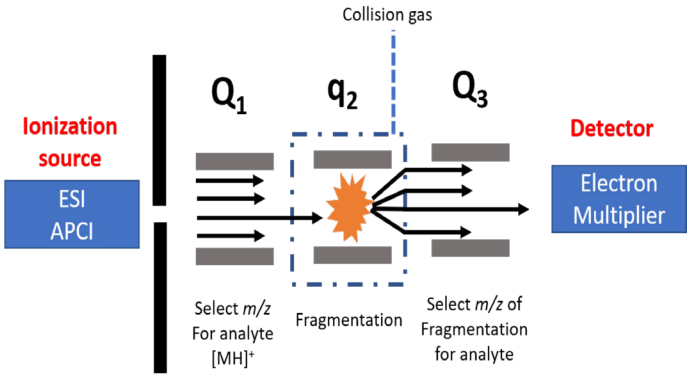

Figure 2. Schematic showing ESI and/or APCI source fragmentation in QqQ.

There are a couple of proposed models for ion formation for ESI:(i) the ion evaporation model (S and JB, 2007) where the droplet forms an ideal state to assist in desorption of solvated ions, and (ii) the charged residue model (M, L et al., 1968) where the charge is carried by the solvent and as the solvent evaporates, the charge remains on the gaseous analytes. The conditions and solvents can be optimized for the formation of positive or negative ions. APCI utilizes a plasma region for desolvation and is a harsher ionization technique; however, it is felt that this is more efficient at removing matrix components.

In tandem (MS/MS) mass spectrometry analysis, a parent (precursor) ion of interest formed in the ionization source is selected for fragmentation using, for example, a quadrupole mass filter. Once the parent ion has been selected, the ions enter a collision cell for fragmentation (Figure 2). There are several designs of the collision cell, and all are filled with collision gas. The energized collision gas collides with the analyte and results in fragmentation.

Improving sensitivity

LC-MS (whether HRAM or QqQ) systems are usually available at multiple price/performance levels—entry level, mid-range, and high-end. An optimal choice of MS with the right LC results in addressing the sensitivity requirements in analytical laboratories. In quantitation of trace analytes in biological matrices, the desired detection range of analyte concentrations usually range between high picomolar to nanomolar, micromolar, or occasionally even millimolar range.

However, as is often observed in clinical research assays, some analytes (e.g., steroid hormones) circulate at low picomolar concentrations (or less). Similarly, desired detection limits for free thyroid hormone is in the low picomolar range. Owing to advancements in LC-MS technology, most of these challenging limits of detection (LOD) are now frequently, if not routinely, achieved. Significant advancement in both HRAM and QqQ technology results in sensitive, reliable, reproducible, robust quantitative assays.

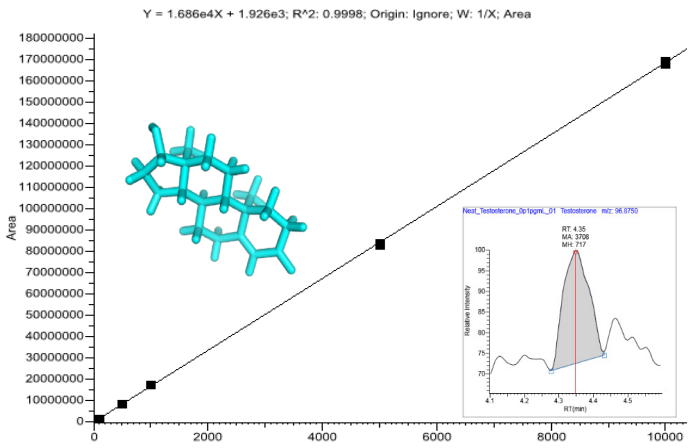

Figure 3. LLOQ of 5 fg on column for quantitation of Testosterone in human plasma observed with Thermo Scientific™ Vanquish™ Flex Binary UHPLC System and Thermo Scientific™ TSQ Altis™ triple-stage quadrupole mass spectrometer.

When it comes to sensitive, robust, and targeted quantitation of analytes in complex biological matrices, the performance of today's QqQs has increased significantly. While the developed ion-optics and detector performances are not limiting factors for increased sensitivity, as described above, the biological sample matrix negatively impacts detection sensitivity, either through elevating the non-specific background signal, or through interferences that obscure analyte peaks, or through suppression of analyte ionization.

Ion suppression

The mechanisms of ion suppression are not well understood. They are mainly observed with ESI, but can also be found with APCI (LL and DA, 2006, A, K et al., 2009, D, DK et al., 2010, M and C, 2010). In ESI, the analyte competes with itself and various matrix components for the relatively limited amount of excess charge that is available for full ionization, as well as for space within droplets. Saturation of droplets with surface-charged analyte or matrix components can also interfere with the ejection of the analyte trapped within the droplets. Typically, these factors that can result in ion suppression in ESI do not affect APCI, since the heated gas stream used in APCI obviates the need for charge-initiated volatilization.

Regardless of the mechanism, ion suppression can occur if a sample contains high concentrations of basic components that elute in the same time window as the analyte and have a similar mass. Ion suppression results in a significant amount of signal loss, and hence should be proactively addressed during the method development process. As described above, the most common approaches to address ion suppression are (i) sample clean-up, and (ii) optimization of chromatography.

Improving specificity and selectivity

The ability to have an ideal combination of sensitivity with high analyte specificity resulted in the enthusiastic acceptance of LC-MS technology in clinical laboratories. Generally, the biggest benefit of this combination can be observed in SRM (selected reaction monitoring) mode with QqQs. However, similar to sensitivity, the promise of high specificity and selectivity cannot be realized without proper attention to sample preparation and chromatography.

Isobaric compounds, where the precursor and the product ions have identical *m/z*'s, often cause some significant issues resulting in reduced assay performance. This is a particularly significant issue faced during the analysis of steroid hormones. The concentration range of the lowest to the most abundant steroids in serum/plasma can range across several orders of magnitude, and there can be vast differences in observed absolute and relative concentrations of groups of steroids between different groups of healthy individuals.

To add to the woes, the presence of inherited disorders of steroid metabolism or previously dosed drug treatments can further complicate the analytical process. Being closely related to each other, many steroids depict very similar fragmentation patterns resulting in frequent observation of isotopic cross-talk. While a careful development and optimization of chromatography can help avoid these issues, recent advances in QqQ technology allowing for high resolution selected reaction monitoring (H-SRM) can be of great help (Figure 1).

As observed in Figure 4, H-SRM, which allows the unique capability to reduce the Q_1 width to 0.2 Da FWHM from its fully open width of 0.7 Da FWHM, results in increased selectivity without compromising the signal-to-noise ratio. As can be observed for the peptide, GPSVFPLAPSSK in human plasma, reducing the Q_1 width results in increased selectivity. While the area count was significantly less at reduced Q_1 width, the signal-to-noise ratio increased almost by a factor of 4 owing to significant reduction of matrix interference between the fully open Q_1 (Figure 4, middle spectrum) and reduced Q_1 (Figure 4, top spectrum).

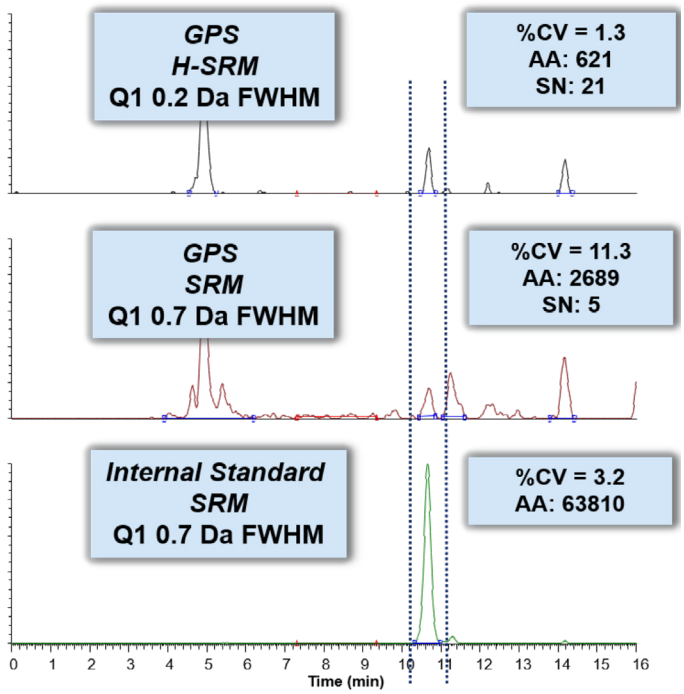

Figure 4. H-SRM observed with the TSQ Altis triple-stage quadrupole MS shows increased selectivity with reduced matrix interferences for the 25 ng of peptide (GPSVFPLAPSSK) with internal standard.

Known clinical applications with MS

LC-MS technology finds widespread popularity in clinical research laboratories for unknown screening, untargeted quantitation, and targeted quantitation of critical analytes in biological matrices. These assays enable research programs to better understand several disease states and metabolic disorders, while also helping to monitor drug therapy, identify drug toxicity and poisoning, and discover new biomarkers.

Limitations of immunoassays, such as nonspecific binding of the antibody and cross-reactivity with metabolites that often result in overestimation, have made the more accurate LC-MS methodologies the assays of choice (Saint-Marcoux, Sauvage et al., 2007). LC-MS is also regularly used for the quantitation of steroid hormones to better understand endocrine disorders. Also, while immunoassays lack the specificity to distinguish between 25-OH vitamins D_2 and D_3, LC-MS can measure these levels separately so the contribution of each to the total can be determined.

Development of the capability to measure thyroid hormones by QqQ has helped overcome the issues associated with immunoassays (SJ, N et al., 2005). QqQ is the preferred MS for targeted toxicology screening and for toxic drug quantitation (E, R et al., 2010). In newborn and prenatal screening programs, ESI with QqQ and HRAM has made it possible for the identification of inborn errors in metabolism or genetic defects so that preventive and medical intervention can be implemented promptly to relieve or treat the disease. Another area of increasing interest is the use of automated MALDI-TOF MS for rapid identification of microorganisms in clinical microbiology laboratories (S and M, 2010).

However, there are challenges faced in the implementation of LC-MS in the clinical laboratory, including sample preparation, online extraction, throughput, automation, laboratory information system interfacing, inter-instruments standardization and harmonization, and FDA regulation. With emerging technologies in MS, we expect to see more robust and reliable MS applications with a broad menu of tests that will become routine diagnostic tools in clinical laboratories soon.

In addition to the most common assays (described above), there are several other examples of the most prevalent uses of LC-MS in clinical laboratories, such as screening of newborns for congenital metabolic diseases, including aminoacidopathies, organic acidurias, and fatty acid oxidation disorders (MS, MP et al., 1997), (DH, TA et al., 2003), (DH, 2009), multi-analyte therapeutic drug monitoring (TDM), especially for the administration of cocktail therapies involving immunosuppressants (M, C et al., 1995), (PJ, A et al., 1996) (Z, Y et al., 2005), oncology drugs (L, 2001), anti-virals (DJ, SH et al., 2001) (J and SJ, 2007), etc.; toxicant and drugs-of abuse screening where samples can be screened and validated in a single run (-L, Saint-Marcoux et al., 2006b), (HH, 2007) the analysis of endogenous peptides especially where different isoforms exist; and the analysis of steroid hormones (JP, SJ et al., 2007).

With respect to steroid hormones, there has recently been a growing level of interest in the application of LC/MS/MS to clinical diagnosis in endocrinology to the point where the American Endocrine Society has issued a statement recommending LC-MS for the determination of endogenous levels of steroid hormones, such as testosterone, over more traditional technologies, including immunoassays (W, RJ et al., 2007). The rationale for this position has been the superiority of analytical results obtained by LC-MS, especially for low levels of these analytes (JP, OP et al. 2004, T, M et al., 2004). The reader is referred to a recent review article on the use of LC-MS for a variety of endocrinology applications (M and KG, 2007). Despite these useful applications and the rapid growth of LC-MS in clinical diagnosis, the number of QqQ or HRAM systems in use in routine diagnostic laboratories is relatively small compared to more traditional biochemical or immunological analyzers.

LC-MS in clinical research: the next step

Addressing upcoming analytical challenges is one of the main requirements for any analytical laboratory. In the constantly evolving world of clinical research, the next challenge for LC-MS technology is peptide/protein analysis. One can anticipate that this will result in similar growth of clinical protein/peptide LC-MS/MS as has been seen for low molecular weight applications (KG and J, 2011).

One of the new ways that MS technology is being used is in proteomics for quantitative identification of small amounts of proteins or molecules in blood to serve as biomarkers. This has opened up the discovery of new tumor markers as a potentially promising area of application of MS (A and SM, 2013). The intensive research into metabolomics, including the assessment of endogenous metabolites as new disease biomarkers, is another promising applications of MS, which has been demonstrated to be very well suited to discovery and clinical application of metabolite profiles (N, DD et al., 2011).

The next goal and challenge for LC-MS is to address limitations of immunoassays for protein and peptide analysis. While extensive use of MS-based research with proteins and peptides has yielded a significant repository of data, experience, and expertise, it would be important to translate this information to clinical practice.

There have been a number of translational attempts in the high profile fields of cancer and cardiovascular diseases. As a result of this work, several multi-marker profiles with qualitative patterns were developed. Unfortunately, during validation of these profiles, flawed signals were discovered, which were due to pre-analytical errors or inconsistent sample preparation between different sites (D, WE et al., 2008). However, there are several reports of successful LC-MS analyses using QqQs for established peptides and proteins, including protein biomarkers. This is particularly valuable for scenarios where the following occurs:

a. Analyte has multiple isoforms

b. High variability can be observed between different assays of the same analyte

c. Existing workflows are difficult resulting in low reproducibility

d. Existing immunoassay fails to answer all or some of the critical challenges, and is also subject to frequent interferences

The H-SRM feature observed in the next generation QqQs offers some significant advantages in this regard.

As evident in Figure 5, targeted quantitation assays experienced a remarkable difference in performance quality between the SRM and H-SRM modes of data

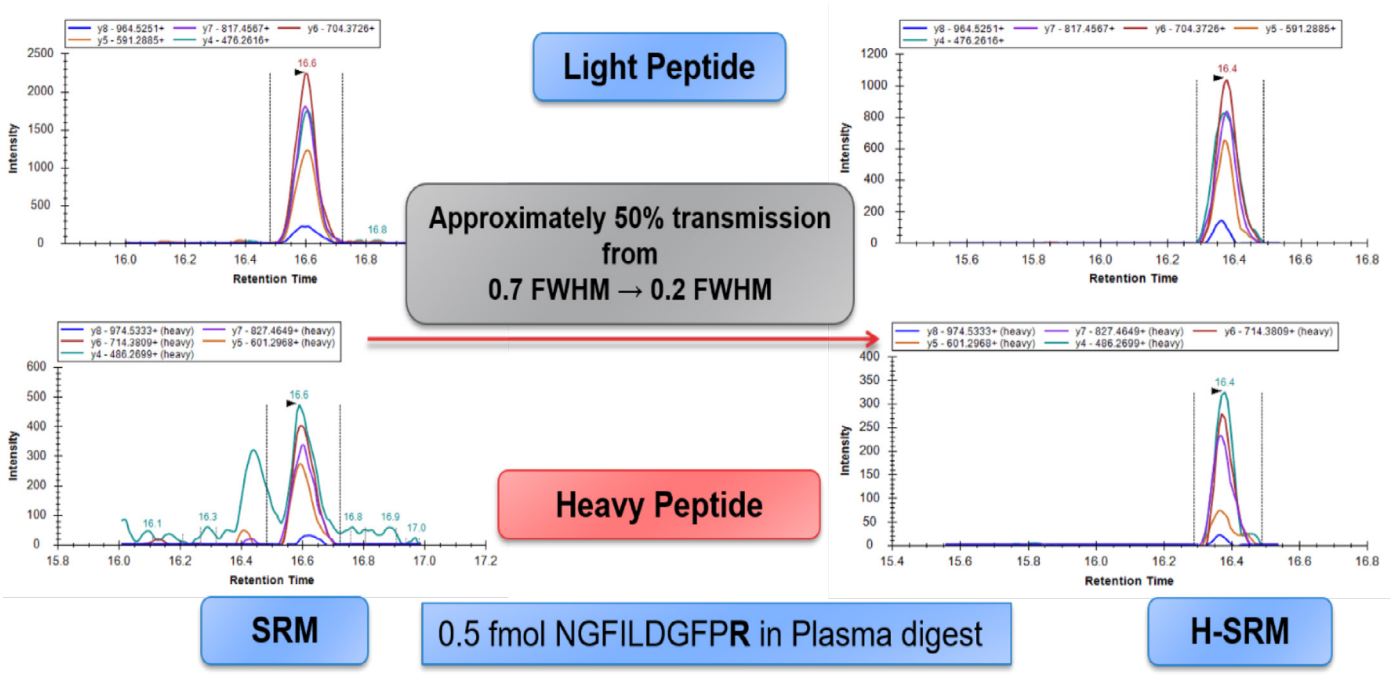

Figure 5. Comparison of SRM and H-SRM based workflows for quantitation of mAbs in human plasma.

acquisition. While the SRM mode showed a lot of matrix interference for the heavy chain of the digested mAb, H-SRM offered a much cleaner matrix, resulting in ease of quantitation with increased sensitivity, reliability, and reproducibility.

However, one must remember that success in peptide/protein quantitation was not necessarily obtained by better MS only. It is a result of an optimal workflow, which employs the digestion of the protein resulting in identification of the signature peptide (tryptic digestion is one of the most common processes), careful process of sample clean-up (SPE or immunoassay-based technologies), and optimization of the LC method followed by development of the most efficient MS method. In addition, major developments in software offer several functionalities within the software, otself, that have enabled rapid growth in adoption of LC-MS technology for peptide and protein assays.

Conclusions

LC-MS technology in the clinical world, with a combination of HRAM and QqQs, has exhibited incredible success and growth for analysis of a wide range of molecule types and matrices. MS finds many applications in the clinical laboratory, and the above-mentioned topics are far from comprehensive.

Most analytical laboratories using LC-MS for clinical assays started with biochemical genetics/newborn screening and drug/toxicology testing, and many have now expanded into more complicated tests, such as endocrine testing, including steroid analysis, biogenic amine testing, and other assays that demand a high level of sensitivity, specificity, selectivity, and robustness.

More complicated analytes, such as peptides and proteins are now being tried in the clinical research domain with signs of exceptional success. Successes in clinical translational research from biomarker characterization to quantitation add more value to the argument that LC-MS should be an omnipresent technology in every clinical laboratory. One can expect, if not predict, that the scope and capabilities of LC-MS technology in clinical tests will grow exponentially.

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