Clinical Chemistry 62:1
30–36 (2016)

Q&A

A Spectrum of Views on Clinical Mass Spectrometry

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The June 2009 issue of *Clinical Chemistry* contained our very first Q&A, which has since become a monthly feature in the journal. In that Q&A we asked 5 experts about mass spectrometry (MS)[9] in the clinical laboratory. We wanted to find out where we stood and where we needed to be.

Not only has it been nearly 7 years since we first asked about clinical MS, but we have devoted the entire January 2016 issue of *Clinical Chemistry* to this important technology. In this Q&A we ask 6 experts representing instrument design, research, and the clinical laboratory for their perspectives on where we stand in 2016. We were particularly interested in the challenges instrument manufacturers face in meeting the needs of customers and regulatory agencies, the potential of MS moving toward point-of-care (POC) testing, whether there was a next "big thing" in MS on the horizon, and whether MS had matured to the point that it was becoming a true clinical instrument.

As scientists involved in instrument development, what demands are manufacturers facing with new applications or instrument designs? How about regulatory hurdles?

Reza Javahery: Increased analytical sensitivity, reproducibility, durability (uptime), and ease of use all continue to be features demanded by users. Thus, we cannot focus on just one of these areas. Serviceability is also a major concern. As far as regulatory hurdles, we are still in an environment where there are no clear guidelines.

Bradley Hart: As manufacturers, we are tasked with challenges that include improving ease of use and connectivity to automation and laboratory information systems/laboratory information management systems (LIS/LIMS), handling smaller sample sizes and spot samples, improving sensitivity for challenging applications, translating and enabling clinical omics assessment panels, and ultimately providing solutions that enable customers to deliver personalized and precision medicine.

In addition, manufacturers of equipment used as components of laboratory-developed tests must employ quality management systems to ensure the equipment is satisfactory for the measurement of patient samples per the requirements of applicable regulatory agencies. As such, manufacturers have invested in quality systems that meet the ISO 13485 standard and related requirements. Additional investment is also required to register products around the world. Those investments require continued funding to maintain system effectiveness and to rigorously monitor and resolve product quality or performance issues.

As MS moves into "intended use" or "closed" diagnostics platforms, further regulatory requirements are imposed. Provision of associated collection devices, reagent kits, and methods, and the requirement to assure clinical efficacy in addition to analytical performance, leads to additional process control and investment.

The time needed to meet regulatory requirements may increase uncertainty in terms of time to market. Some risks can be minimized by assembling solutions across companies who have broad clinical capabilities,

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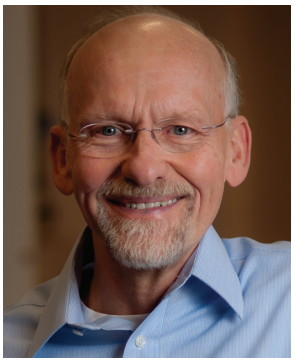
Received October 2, 2015; accepted October 15, 2015.

[9] Nonstandard abbreviations: MS, mass spectrometry; POC, point of care; LIS, laboratory information system; LIMS, laboratory information management system; ESI, electrospray ionization; LC, liquid chromatography; FTICR, Fourier transform ion cyclotron resonance; IMS, ion mobility separations/spectrometry; FDA, US Food and Drug Administration.

and through corporate partnering. Regulatory investment can also siphon funds away from research, so critical strategic business decisions must be made to drive successful ventures that advance science and meet all regulatory requirements.

Do you see a near-future push toward POC, miniaturized, or "sold for purpose" instruments? If so, what is going to get us there, and how long it is going to take us?



Richard Smith: I believe this will become an important area, with both decreasing cost of such platforms as well as increasing effectiveness. Much of this will be driven by advances in the 'front end' portions of such platforms, and to a lesser extent by the MS itself. The big challenges for the short term involve decreasing platform cost, while also continuing to advance performance, enhance throughput, improve robustness of operation, and implementing effective automation. I think we need some substantial departures from present platforms to really address the combination of these challenges.

Bradley Hart: Yes, but with caveats. A primary value of MS is that it can easily measure multiple analytes with very high sensitivity and selectivity. If you ask a mass spectrometer to perform a single measurement, it devalues the core capability that a mass spectrometer provides. Assuming that the "for purpose" use includes multivariate analysis such as large toxicology panels or measurement of multiple proteins and their isoforms and modifications, it's easy to imagine that MS will be optimized towards "for purpose" systems.

Miniaturization of MS has been achieved on a variety of fronts, specifically ion traps, but innovations in manufacturing technology will continue to drive miniaturization of systems in all aspects, including sample prep and vacuum systems. It will take the combination of a specific use that fits the performance characteristics of a miniaturized system and a clear business opportunity for these types of systems to reach the routine market.

If a market develops that drives a "for purpose, killer application," and miniaturization and technology development continues at its current pace, then it's not a big step for MS to be employed as a POC technology. MS could be employed for a system today as a POC analyzer, but the industry still lacks a compelling application and business opportunity to drive investment.

LC-MS lends itself to a wide variety of clinical applications, including large and small molecules. A nexus of activities by the industry and manufacturers could deliver an LC-MS–based "clinical analyzer" with a wide menu of intended use tests. If those efforts are successful, then we could reasonably expect POC systems to enter the market in 5–10 years.

Reza Javahery: Yes, depending on requirements for POC. We are almost in a position of replacing mass spectrometers in central hospital laboratories, similar to how x-ray and other scanning equipment is going to the patient rather than the patient coming to the equipment. Sample preparation is a hurdle in this respect, but there are many other industries heavily moving forward by producing robotics so that sample preparation can be done in real time.

Are the improvements in MS instrument sensitivity going to be in the ionization, ion transfer, or ion detection stage?

Richard Smith: The answer depends on the platform and the manner in which it is applied. In many cases the key challenge is having the sensitivity needed to routinely detect and quantify low-concentration analytes, and here all 3 of these aspects are important and somewhat intertwined.

The ion transfer challenge has been essentially solved using well-implemented ion inlets and ion funnel designs with electrospray ionization (ESI) sources, since it is possible to achieve almost perfect ion focusing and transfer. Improved interface designs are still needed that can handle much larger ion currents from more intense ESI sources without the need for expensive pumping arrangements, but in general the source of the issues and how to address them is understood, and the solutions are beginning to become broadly available.

The ionization step is actually the greatest challenge, particularly using on-line liquid chromatography (LC)-ESI-MS, which has become the real workhorse of modern MS. The efficiency of ESI increases as the flow rate from the LC decreases (and where the electrosprayed droplet size decreases and the charge available per analyte molecule increases) and for some species can approach 100% at very low flow rates $<$10–50 nL/min. Effective LC separations at such low flow rates presently require the use of nanobore LC columns that are both more difficult to prepare and use, and also readily plugged. The use of these columns also creates significant challenges in maintaining stable LC flow rates, reproducible mobile phase gradients, and ESI performance. At present I would not want to risk valuable samples to achieve the most sensitive measurements possible, which might require, for example, the use of 15-micron inner diameter

packed columns to achieve very low flow rates and optimal ESI efficiency. Rather, I would probably use 50-micron- or even 75-micron-diameter columns, which work best with somewhat higher flow rates and provide much more robust performance, but result in somewhat reduced sensitivities. But this might not make any significant difference, depending on the platform and the specific application. For example, an orbitrap or Fourier transform ion cyclotron resonance (FTICR) analyzer is constrained by both the charge capacity of the trap and the time it takes to make a measurement, and thus benefits of nanoflow LC would generally only be significant when sample sizes are very small. For other MS platforms (e.g., TOF or triple quadrupole) that do not have such constraints, much greater gains can be achieved.

Bradley Hart: Ionization, or more specifically the generation of ions and efficient transfer from atmospheric pressure to the first vacuum region, is still a major challenge for manufacturers. This area is fraught with intellectual property challenges and is one of the major areas of differential performance amongst the vendors. Today, each vendor's design may have preferential performance towards specific analytes, but vendors are working on designs to ionize the widest variety of compounds with minimal ion suppression effects. It is possible with "for purpose" systems that the ion source designs would be simplified and optimized for specific analytes and specific sample introduction flow rates or techniques.

There are continuous incremental improvements in ion transfer efficiencies, and these again may be specific to certain types of mass analyzers, including relatively new mass analyzers like the orbitraps. There are also specific transmission techniques that are related to transmission of large molecules vs small molecules. Efficient transmission of large proteins or antibodies requires specific settings that may be invoked as "modes" of operation or specifically optimized in "for purpose" designs.

Ion detection technologies also continue to improve incrementally, but new digital technologies offer promise for substantive leaps in sensitivity, selectivity, and unique ways to detect ions.

Reza Javahery: Sample extraction from a complex mixture is most challenging. If the ionization process occurs at atmosphere pressure, then ion introduction into the mass spectrometer remains the critical issue that needs to be addressed. It is highly important to be able to transfer most, if not all, ions of interest to the mass analyzer. In modern MS, ion transportation has become a key factor for the production of high-sensitivity instrumentation.

We are seeing an increasing number of reports on new protein biomarkers detected by MS, but few have made it into routine analysis. What are the bottlenecks? What will it take for MS protein marker assays to be ready for regular use?

Samir Hanash: There are several major bottlenecks. A consequence of an increasing number of reports on new biomarkers is a tendency to confuse which report(s) and which biomarker(s) to pursue further by, say, a diagnostics company, particularly when the evidence is relatively weak with respect to the extent to which the discovery and initial validation are relevant to the intended clinical application. Assuming that the evidence is strong for particular MS-based marker(s), the next challenge stems from whether markers that result from other discovery strategies for the same application(s), e.g., nucleic acid–based markers instead of proteins, may be equally strong and may be more amenable to clinical grade assays. Assuming the latter is not the case, the next decision to be made is likely going to be the nature of the assay to be developed, the standard approach being an immunoassay. For MS marker assays to be ready for clinical use requires a substantial investment on the part of MS instrumentation companies to develop suitable front-end sample preparation methodologies with the necessary analytical reproducibility together with MS instrumentation that yields the prerequisite sensitivity (typically below ng/mL) for assays of biospecimens like plasma and urine. Clearly much progress has been made and it is likely that in the near future, based on informal feedback from reliable industry sources, effort in this direction will intensify.

Ravinder Singh: I agree that few biomarkers have made it into routine use. MS has helped in the detection of new protein biomarkers in well-controlled studies; however, not many biomarkers have proven to be clinically useful in confirmation studies and used in large clinical studies. Biomarker detection either by MS or any other technology is essential in generating a proper hypothesis, but ultimately confirming the biomarker in circulation for improving patient care is what makes the final product for patients and clinicians. The identification and confirmation of the biomarker, as well as elucidating its pathophysiology, are critical before

assigning an analyte as novel biomarker. Because of financial constraints, where dollars are limited, various clinical procedures and tests are already being questioned in the medical industry. For example, the clinical use of prostate-specific antigen, which was a well-established biomarker at one time, is being questioned. Currently cancer diagnostics testing is mainly confirmed postsurgically by anatomic pathologists using anatomical and histochemical tools. It is critical for the development and validation of novel biomarkers that clinical chemists collaborate with clinicians and anatomic pathologists. Clinicians are aware of current diagnostic limitations, and thus can help identify new biomarkers that can improve clinical practice or patient care.

MS users complain of frequent instrument downtimes, especially when they use nanoflow vs microflow liquid chromatography. What is your view of this technology becoming more robust, and how?

Lorin Bachmann: Our laboratory does not employ nanoflow or microflow LC techniques. However, we have experienced problems with LC robustness even at the more standard flow rates commonly used for measurement of small molecules. The quality of the LC data depends on technical factors such as how evenly the LC tubing is cut, how precisely the frits are set, and how carefully system pressures are maintained. Developing proficiency for these sorts of tasks requires intensive training and highly committed personnel. The complexity of current-generation LC systems has created a barrier to adoption of MS assays in our laboratory, which is resource limited and unable to hire personnel with expertise to maintain the systems.

Improvements in robustness and ease of the separation step are needed for widespread acceptance of LC-MS systems into routine laboratories. Increased robustness can be accomplished by reengineering of the LC system design with the goal of decreasing system complexity. A reduction in the number of moving parts and individual junction points, a more seamless continuity in flow path, and a reduction in the number of interventions needed to achieve optimal performance would go a long way toward decreasing the number of failure points and improving the laboratory's ability to reliably generate high-quality chromatographic peaks.

Richard Smith: This is not a newly recognized problem; a lot of us have been dealing with this challenge for well over a decade by making the needed tradeoff to achieve more robust performance. As you can see from my response to an earlier question, I am personally not optimistic that we will have robust nanoflow LC-based platforms that achieve truly optimized sensitivity anytime in the near future. The ideal system needed would have robust performance, high resolution and perfectly reproducible fast separations, and highly efficient coupling with MS, along with this extremely high sensitivity. I believe that the key is to move away from the use of nanoflow LC.

Bradley Hart: Input from high-throughput laboratories indicates that the majority of down time in LC-MS is due to the LC system. The mass analyzer itself has very high uptime usually exceeding 95%.

Nanoflow is a particular challenge for routine operation, and while improvements have been made to lessen the level of "art" in this technique, the historical need to maximize sensitivity for the mass spectrometer has been primary. More solvent (higher flow rates) reduces sensitivity due to thermodynamics, desolvation inefficiencies, and neutral interferences. It is less expensive to run nanoflow LC vs building huge vacuum pumping systems.

In the discovery phase or work flow, where low-abundance proteins and modifications were of interest, sample amounts were often very limited, and using nanobore–nanoflow didn't cause an issue with sample capacity. If a marker was "discovered" in nanoflow mode, people were often afraid or resistant to change since it was just too much work to revalidate.

Mass spectrometers are gaining 5–10-fold sensitivity every 2–4 years. Laboratories now have baseline data indicating that capillary flow rates or higher could be used in lieu of nanoflow with older systems. As the discovery phase wanes and we focus on specific proteins and pathways, we can often collect a larger sample amount, thus reducing instrument sensitivity requirements. As people target specific proteins for quantification, they will rapidly move away from nanoflow. LCs and columns are being designed more and more with MS applications in mind (e.g., reduced dead volumes, improved mixing, appropriate materials, speed), allowing for better results when running the systems (e.g., optimizing peak heights, loading). Lower flow and capillary systems now work better and provide cleaner baselines and sharper and taller peaks, again reducing the mass spectrometer sensitivity requirements. Mass produced systems and columns for lower flows and capillary will reduce the art required in nanoflow, and will improve reproducibility vs nanoflow setups for routine operation.

Looking into your imaginary crystal ball, in 5 years where do you see MS making new contributions in the

diagnosis and treatment of diseases? Do you see a next "big thing" in MS?

Lorin Bachmann: We will likely see increasing use of MS technology for measurement of clinically important peptides and protein biomarkers. With the increasing focus on the benefits of personalized medicine, the next "big thing" might be a rapid expansion in the number of MS applications for evaluation of protein-based therapeutics. One current example is use of MS to measure infliximab for the purposes of establishing individualized dosing strategies, evaluating drug efficacy, and investigating loss of therapeutic response.

For meaningful implementation of MS protein-based or small-molecule testing in routine clinical laboratories, probably the important advancement in technology needed is massively increased processing parallelism. A typical routine immunoassay analyzer can perform more than 50 reactions almost concurrently. Although the mass spectrometer itself is capable of making measurements with a high degree of speed, the separation step requires that samples be processed in a linear manner, where one sample must be separated before the next is loaded onto the chromatography column. Even with the advent of multiplexing systems that can accommodate multiple columns with elutions that are each offset in time, LC still remains the major rate-limiting step to throughput. MS systems will likely not be able to compete with standard immunoassay analyzers in terms of throughput and turnaround time until analyses can be performed in a nearly concurrent manner without substantially increasing the number of system failure points.

Samir Hanash: The next big thing is a "turn-key" solution to MS-based assays with automated sample preparation, fractionation or capture of desired protein(s) for assay followed by digestion and quantitative analysis.

Bradley Hart: The next "big thing" in the application of MS to clinical research is integration of genomics, MS omics, and exogenous drug measurements to enable our customers to "measure anything." MS will provide more precise knowledge of a patient's disease that allows for targeted treatment approaches, reintroduction of drugs that are specific to certain patient populations, and rapid validation of new drugs and will drive significant healthcare industry cost savings.

Reza Javahery: MS will continue to provide more and more valuable information that is complementary to many current diagnostics tools because of its specificity and speed. The analytical sensitivity of MS plays a major rule in many scientific areas, specifically in diagnostics, providing necessary information that we could not obtain with other techniques. I hope that many potential advances in MS become reality since MS technology is relatively young and has great future potential.

Ravinder Singh: The paradigm will shift from the use of a single biomarker for confirmation of a single disease. MS has the potential of reporting multiple biomarkers, and bioinformatics will help in simplifying complex reports for clinicians. The validation of multiple biomarkers in biological matrices like serum, urine, and saliva for small molecules and proteins will be the need of the time. The reporting of analyte modifications by MS, e.g., drug and steroid metabolites and glycosylation and phosphorylation of proteins during the processing of the signal and secretion, will add clinical value to the existing and novel biomarkers.

Richard Smith: Overall I think detection capabilities of what we think of as being modern MS platforms are increasingly mature, and while there will certainly be advances, I think they are going to be quite incremental and modest impacts. Having said that, I believe the combination of ion mobility separations/spectrometry (IMS) with MS is going to be disruptive in broad areas of application. IMS can be orders of magnitude faster than LC, is practical with much smaller sample sizes than LC, and since it does not involve a stationary phase it avoids all the issues associated with surface interactions. It is also incredibly robust and reproducible.

While most major MS vendors now offer instruments adding IMS to the front end based on one approach or another, they fall short in terms of either their resolution or sensitivity achieved, and very often both. Yet, I see IMS as the next big thing for MS, and something that will enhance its performance and robustness for the broad scope of all its possible contributions in the diagnosis and treatment of diseases. My bet is that in 5 to 10 years from now you will see IMS coupled to most MS instruments.

Further down the road, I also see major changes coming from massively parallel MS platforms; i.e., platforms in which substantial numbers of analyzers work in parallel, resulting in proportionate gains in throughput, data generation, etc. But I think this is also likely a decade away.

Both research and clinical applications have heavily relied upon immunoassay (e.g., ELISA) methods, but there is now competition between immunoassay and MS for protein analysis. Is there any evidence that MS is now winning on some fronts?

Ravinder Singh: Not really. Both technologies will remain in use in clinical laboratories since both have numerous advantages. MS has the potential of discovering and characterizing novel biomarkers but ELISA may be the best for the performance characteristics, like throughput and sensitivity. Automating an ELISA and making

sensitive sandwich assays for proteins is very convenient for manufacturers as a way to get US Food and Drug Administration (FDA) approval. Automated assays are more likely to be used in the laboratories.

Lorin Bachmann: Laboratory scientists recognize that MS can provide improved accuracy compared to other laboratory techniques. However, our ordering physicians are now beginning to appreciate the analytical performance benefits afforded by MS and are specifically requesting that measurements be made using MS technology. For example, our endocrinologists have recently begun requesting thyroglobulin testing by LC-MS to avoid longstanding antithyroglobulin antibody inferences that are problematic when using immunoassay for measurement of thyroglobulin. We have also used LC-MS to resolve several cases of rare hemoglobin variants that were not definitively identified by chromatographic or electrophoretic techniques.

In addition to proteins, our interest in small molecules has increased. Almost half of our laboratory orders for testosterone testing represent specimens collected from female and pediatric patients, and a large percentage of the remaining orders are from hypogondal males. Due to nonspecificity of testosterone immunoassays in these patient populations, our physicians have requested that testosterone testing be performed using MS. We also rely on MS techniques to resolve diagnostic dilemmas caused by suspicious free thyroid hormone immunoassay results and to routinely monitor chemotherapeutic, antifungal, and antiretroviral therapies.

Samir Hanash: I would not say winning as yet, but we are all aware of the challenges in developing antibodies or antibody pairs with the necessary affinity and specificity. We have many candidate markers that are sitting on the shelf for which success in coming up with a good antibody pair has eluded us. It would be more straightforward to develop an MS-based assay if we could achieve the prerequisite sensitivity and throughput with the MS approach.

Users often forget that MS analyses often involve upfront chromatographic separation. Are there improvements or new chromatographic approaches that you have used to improve MS analysis?

Ravinder Singh: Chromatography is definitely important for low-concentration endogenous biomarkers. Unfortunately, there has not been much improvement. For inborn errors of metabolism and toxicology methods where the concentrations of analytes are much higher than the matrix background, there is minimal sample extraction and purification required and rather dilute and shoot is commonly used. The use of online extraction has picked up but the extent of complexity and plumbing has not been cost effective for low volume laboratories. MALDI is becoming attractive again in the clinical laboratories for qualitative detection of high-abundance proteins in serum and tissues. MALDI-TOF has revolutionized the work flow of microbiology laboratories.

Lorin Bachmann: Although our laboratory would benefit from increased chromatographic quality and throughput, we have done little to change our current methods because we lack personnel with adequate LC technical expertise. The requirement for substantial expertise for development, maintenance, and improvement of chromatographic methods is the most challenging issue we face for implementation of additional MS testing. We would develop a larger number of MS assays if currently available separation approaches could be simplified.

What changes do you want to see in MS to call it a true clinical instrument?

Lorin Bachmann: Historically, MS instrumentation was developed for basic science and pharmaceutical applications. Clinical laboratories adapted their work flow around limitations of existing systems, rather than benefiting from technology specifically engineered for clinical laboratories. For widespread acceptance of MS in routine laboratories, a major shift in approach regarding equipment and testing process design is needed.

Current MS systems lack seamless integration of instrument components, sample processing procedures, reagent handling, data management, and application of quality assurance metrics. For MS to serve the needs of the clinical laboratory, all components and processing steps need to be automated and fully integrated. The current model of piecing together fundamentally standalone components results in robustness problems and process inefficiencies. Ideally, sample loading should use a random access format and no manual manipulation of any process should be required. Complete automation of sample identity tracking, reagent handling, inventory tracking, and dilution and repeat functions will greatly improve efficiency. Bidirectional communication must be established among instrument components such that error feedback from any point in the testing process will activate a system-wide error response. Also, automated quality assurance metrics should be applied to each result and alert mechanisms displayed in real time. These types of hardware and software changes would enable movement of MS instrumentation out of specialty laboratories staffed by specifically trained technologists and into the highly automated, routine clinical laboratory.

Ravinder Singh: Not many MS methods for clinical laboratories are fully automated and are FDA approved.

It is very challenging to have smooth communication between the liquid chromatograph, auto sampler, MS, and laboratory information systems. To run LC-MS methods, equipment is very complex and requires expensive hardware from various vendors and for multiple software applications to be used. Until all these are fixed no vendor will be able to get an FDA approval for the final product to be useful and comparable to the automated immunoassay instrument.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:

Employment or Leadership: T.M. Annesley, *Clinical Chemistry*, AACC; E.P. Diamandis, *Clinical Chemistry*, AACC.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: None declared.
Expert Testimony: None declared.
Patents: None declared.

Previously published online at DOI: 10.1373/clinchem.2015.250258