

LC/MS Solidifying Key Role in Clinical Labs

Novel Realms Are Being Explored to Expand the Technique's Usage over the Next Decade



K. John Morrow Jr., Ph.D.

Gas or liquid chromatography combined with mass spectrometry is widely recognized as a versatile and powerful technique for the investigation of complex chemical mixtures. A state-of-the-art accounting of progress in this area took place recently in Hong Kong at the conference on “New Horizons in Clinical Chemistry.” The meeting, organized by C. S. Ho, M.D., of the Prince of Wales Hospital, Hong Kong, had a strong focus on clinical applications and explored new advances in liquid chromatography technology coupled to mass spectrometry.

“Mass spectrometers as clinical tools are being increasingly adopted for a host of clinical applications by the global clinical laboratory community,” said Michael Morris, Ph.D., head of clinical business operations for Waters (www.waters.com). According to Dr. Morris, current clinical assays harness only a fractional percentage of the power of the instrumentation so there is a large effort in place toward the development of new MS-based biomarker assays.

From the mid '90s, with the analysis of acylcarnitines and amino acids from neonatal blood spots, high-throughput applications based on mass spec have been widely adopted. Since that time, this technology has played an increasingly important role in clinical analysis, to the point where it is common to find it in laboratories with applications for endocrinology, toxicology, and therapeutic drug monitoring.

“Now, new realms are being explored, and while they are still in the discovery phase, they promise to yield exciting new

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applications in the next decade,” Dr. Morris explained.

The rise of automation, combined with ease of clinical operations and the growing use of these systems in a nonspecialist environment have become motivating forces for companies engineering new, more user-friendly instruments. The concept of analyzing hundreds of samples per day on a single system was met with incredulity a mere decade ago, whereas, that is a minimum expectation today. As the needs of clinical laboratories continue to evolve, companies are looking to mass spec for new applications.

Classically, 95% of LC/MS technology has been applied to characterizing compounds with a molecular weight of less than 1,300 daltons. However, recent developments in biomarker discovery using more advanced types of mass spectrometers are generating data pertaining to multiplexed indicators of disease.

“With the emergence of protein biomarker analysis by mass spec, some significant sample preparation may be required,” Dr. Morris said. “This is due to the fact that the target compounds need to be broken down into their constituent peptides for accurate and precise quantification, and interfering contaminants need to be eliminated from the sample to ensure analytical integrity.”

Alan Wu, Ph.D., is chief of the clinical chemistry and toxicology laboratories at San Francisco General Hospital. At the meeting, he discussed the transitioning of his toxicology lab from gas to LC/MS, as well as the role of immunoassays and time-of-flight (TOF) mass spec in compound identification.

Most toxicology testing performed in hospitals is immunoassay based, an approach which is less than ideal for a number of reasons. There are many more drugs than there are immunoassays, and many compounds have similar structures. Highly specific antibodies can be raised to individual drugs but

this increases the number of assays by an order of magnitude, and companies have not stepped up to improve testing accuracy.

According to Dr. Wu, immunoassays won't become obsolete as they cannot be replaced. Immunoassays are very fast, 20 minutes at most, and they require no sample preparation. Incremental costs are quite low, under one dollar per assay. On the other hand, LC/MS requires at least an hour depending on whether there is on-line extraction, but this contrasts with GC/MS, which requires three to four hours.

Various mass spec tools provide a range of options for compound identification. Because LC/MS is based on the use of a polar mobile phase, many preliminary processing steps can be eliminated. But the advantages must be weighed against the higher instrument costs and the caveat that GC/MS provides more resolution than liquid chromatography. Another option is the addition of tandem mass spec to the platform because the mass spectrum can differentiate between co-eluting compounds utilizing parent-daughter transition ions.

According to Dr. Wu, TOF is the next emerging technology; it offers interesting advantages over both GC and LC. These latter technologies rely on fragmentation patterns, retention times, and library searches for identification, so they require preexisting knowledge. TOF can identify complete unknowns since it gives exact molecular weight to parts per million accuracy.

“You have to know where these things come off,” Dr. Wu continued. “We're not interested in introducing fragments. TOF changes the paradigm because it gives precise molecular weight to 3–4 decimal points. You can determine the exact molecular formula of a target compound so that

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compound can now be found without knowledge of its retention time.”

Among the most significant advances in late 20th century medical science are PCR and LC/MS, according to Paul Taylor, senior scientist in the department of clinical pharmacology at Princess Alexandra Hospital, Queensland, Australia. “Mass spectrometry is over 100 years old, yet it continues to exert a profound influence over the field of clinical diagnosis,” Taylor stated.

Mass spec with its speed, sensitivity, and specificity is an ideal tool to aid clinical

diagnosis. With recent improvements in the technology, it is now accessible to clinical laboratories that lack a mass spec specialist. Historically limited to gas chromatography, the technology was confined to compounds that could be easily volatilized, so as to be amenable to ionization.

All this changed in the 1980s when electrospray and atmospheric pressure chemical ionization enabled the coupling of HPLC and mass spectrometry. Now, a wide range of analytes, all the way from small molecules to proteins such as hemoglobin, can be subjected to analysis. This resulted in an explosion of publications applying electrospray to a variety of macromolecular characterization problems.

“With strong advances in instrumentation driven by the pharmaceutical industry, we now possess an exquisite clinical analytical instrument in the LC-tandem mass spectrometer,” Taylor said. Among the most notable applications is newborn screening for inborn errors of metabolism such as phenylketonuria. While these tests were classically performed using bacterial auxotrophs, “because the automated mass spectrometer recognizes other aminoacidemias simultaneously, it represents a useful development toward a broad-spectrum neonatal screening method.”

LC/MS-based screening for inherited metabolic disorders has grown into a major clinical segment, with four million infants screened in 2005. Today isotope-dilution mass spectrometry, which employs loop injection and allows the analysis of up to 1,000 samples per day, is favored. The technology makes possible the coverage of rare disorders, which was not possible in the past. Another important application of

LC/MS is therapeutic drug monitoring, especially immunosuppressive, antiepileptic, antipsychotic, and anti-AIDS drugs.

Yet another key clinical protocol is hormone measurement. Taylor discussed testosterone measurement by immunoassay, which has long been problematic due to the large range of concentrations in patients (e.g., children, females, and males) and because of issues of selectivity, accuracy, and sensitivity. LC/MS offers a viable alternative to measure this class of compounds.

Despite the merits of mass spec clinical diagnostic platforms, their acceptance has been limited. According to Taylor, a triple quadrupole instrument requires an initial capital investment of greater than \$150,000, whereas immunoassays are supplied by the manufacturer on a reagent-rental basis. Furthermore, the complexity of mass-spec technology has been a daunting barrier in the past, although this is gradually being overcome as more user-friendly instruments come on-line.

LC/MS is an important technology, clearly more versatile than GC/MS, still evolving but possessing the potential to greatly extend the analytical capabilities of the clinical laboratory. Taylor predicted that new MS-relevant biomarkers for disease will be discovered, which will extend the limited range of the current technology.

“A quantum leap is required to help place LC/MS in the same league as the chemistry auto-analyzer,” Taylor said, “and we may require the introduction of a radical alternative technology such as MALDI or the combination of antibody-based sample preparation with mass spectrometry to move the process forward.”



Eksigent's NanoLC systems employ the company's microfluidic flow control technology, which it says creates rapid, reproducible, low-flow-rate binary gradients.

Taylor concluded with a warning that there are many challenges facing the implementation of LC/MS in the clinical laboratory; the need for suitable internal standards, better sample preparation, improved automation, and the challenges of extensive interpatient variability. “But there is no doubt that this technology is making an impact on the diagnosis and treatment of patients now and will be increasingly relevant in the future,” he added.

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Ravinder J. Singh, Ph.D., co-director of the endocrine laboratory at the Mayo Clinic,

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related a complex story on the building of an accurate serum assay for human parathyroid hormone (PTH). This critical hormone increases the concentration of calcium in the blood by interacting with its receptor. "Its measurement is essential for the diagnosis of a number of pathological conditions, including hypercalcemia, hypoparathyroidism, hyperparathyroidism, and renal osteodystrophy," he stated.

Traditionally, parathyroid hormone levels are measured using immunoassays in which antibodies against the entire 84 amino acid molecule are employed. A truncated (7–84) peptide is an indicator of renal bone disease. Accurate quantitation of hormone levels is critical, given that elevated levels are frequently treated by surgery to remove the parathyroid.

Inaccurate measurement of parathyroid hormone levels has led to extremely serious consequences, according to Dr. Singh. In 2009, Quest Diagnostics was ordered to pay \$302 million to address accusations that Nichols Institute Diagnostics, its subsidiary, sold misbranded test kits.

The case, targeting Nichol's Advantage Chemiluminescence Intact Parathyroid Hormone Immunoassay, originated with a whistle-blower suit brought by Thomas Cantor. He noted that the test had an upward drift resulting in erroneously high values for serum parathyroid hormone levels, resulting in unnecessary parathyroidectomies and futile treatment with vitamin D analogues in misdiagnosed patients.

Dr. Singh and his colleagues have investigated the causes of the drift, and while there are numerous possible factors contributing to the failure of the assay, the most significant cause appears to be the reference calibrator, a

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human parathyroid hormone preparation from 1981. Today, none of the current PTH immunoassays use this calibrator.

With the help of LC/MS/MS to obtain accurate quantification of the 1–84 peptide, Dr. Singh's team investigated cleanup methods and the properties of the peptides from the digestion of the complete hormone molecule. They were able to obtain information on the interference of the various PTH peptides running the entire length of the molecule. The group performed a study comparing the LC/MS/MS methods with Roche's (www.roche.com) Cobas immunoassays.

"From these investigations we found that there is a need to harmonize immunoassays for quantifying intact PTH," Dr. Singh stated. For this reason we have developed a reference method to specifically quantify the bio-active form of the complete, biologically active parathyroid hormone." **GEN**

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