Recent Developments in LC-MS-MS

for the Identification and Measurement of Nanoscale Amounts of Proteins and Peptides

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This article provides a current review of recent developments in LC-MS-MS technology for the analysis of biomolecules. In particular, the study focuses on an innovative approach called variable-flow/nanoscale capillary LC/electrospray MS for identifying and measuring complex mixtures of proteins and peptides at the low femtomole level.

The announcement last year of the successful sequencing of the human genome will clearly have a dramatic impact on the way pharmaceutical companies discover and develop new drugs. It will enable drug discovery programs to be based on the identification of the best targets for a particular disease by studying the products of gene expression — nucleic acids and proteins. Most of the early work in this area has involved profiling gene expression at the RNA level as a function of the cellular state (1). However, although this approach is very sensitive, it does not indicate changes in protein expression, which are essential for disease mechanisms to be fully defined at the molecular level (2). The identification and measurement of peptides and proteins (traditionally called proteomics), particularly at the nanoscale level, will therefore become a critical area of research because of their importance as potential targets for pharmacological research.

Most traditional approaches for the analysis of proteins have involved separation by one- or two-dimensional polyacrylamide gel electrophoresis (1D or 2D-PAGE). Even though this technique is an excellent quantitative tool, it is often inadequate for the separation of complex protein mixtures. In addition, it is very difficult to automate, is labor intensive, and requires a very experienced analyst to read the gel fingerprints. For these reasons, liquid chromatography (LC) coupled with mass spectrometric (MS) detection has become the technique of choice. It can either be applied to the analysis of the actual 2D spots to improve the resolution of the separation or be used for the direct analysis of the protein mixture. However, LC-MS is limited in its application because a single mass separation device doesn't have good selectivity and struggles to detect lower concentration proteins in the presence of more abundant ones (3). This limitation has led to the development of systems with tandem mass spectrometers (LC-MS-MS and electrospray MS-MS), in which multiple mass-resolving devices are used to identify complex mixtures. This approach, which is predominantly used as a qualitative tool, has significantly im-

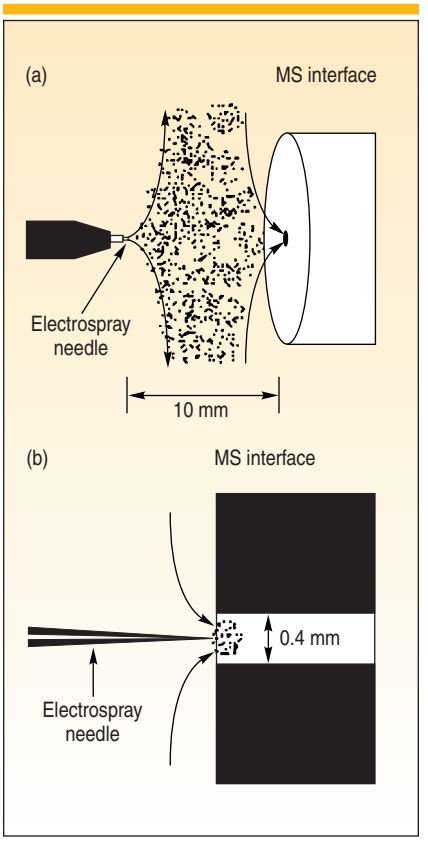

Figure 1. Schematics of (a) traditional and (b) low-flow electrospray designs, showing the difference in position of the needle relative to the mass spectrometer interface.

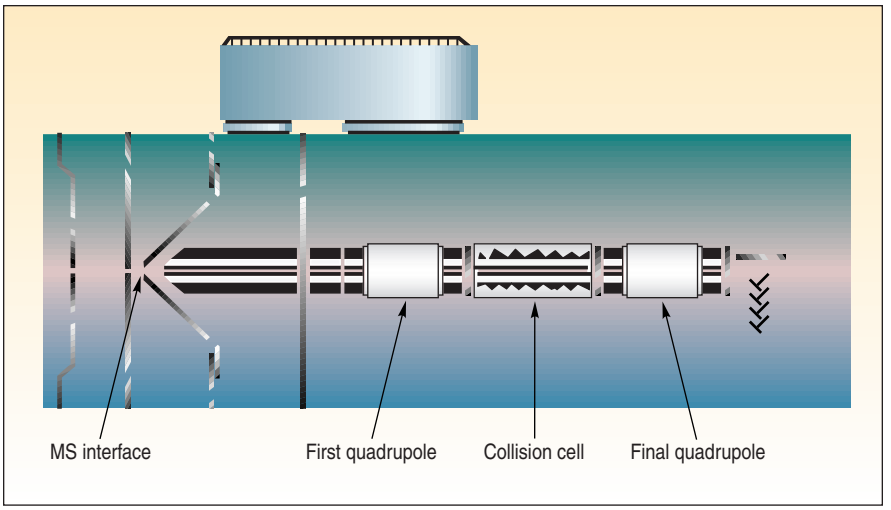

Figure 2. A typical triple-quadrupole MS-MS system (courtesy of PE Sciex).

proved both the selectivity and sensitivity for complex biomolecules; but it still comes up short in pharmacological applications, where very small amounts of samples are encountered. For these reasons there have been a number of recent developments in electrospray sample introduction, mass separation devices, nanoscale capillary LC, and peak interrogation techniques. These developments have allowed proteomics studies to be routinely performed at the femtomole level. Let's take a look at some of these developments in greater detail.

ELECTROSPRAY SAMPLE INTRODUCTION

Traditional electrospray technology generates an ionic cloud that is very large in relation to the opening of the mass spectrometer. However, because of the distance of the spray needle from the interface, only a small portion of the sample actually enters the mass spectrometer. Wilm and Mann were the first to show that positioning a very small electrospray needle close to the MS interface enables the introduction of 100% of the sample into the mass spectrometer, operating at flow rates of <50 nL/min (4). Figure 1 shows a simplified comparison of the traditional and low-flow electrospray designs.

In Wilm and Mann's design an electrical potential of 500–1000 V is applied to a metal-coated, fused-silica needle tip to produce an electroosmotic flow of the analyte solution and generate the electrospray (4). Unfortunately it had two major limitations. First, the needles were very fragile and could not be used reliably and routinely to perform multiple analyses. Second, the analyte flow and spray could not be decoupled from each other, which made it very difficult to precisely control the flow rate. These two limitations made it less than ideal for operation in an automated fashion, especially for use with LC. For this reason there have been a number of modifications and changes to their designs with the goal of making it more rugged and routine.

One such design, developed by Moseley for proteome studies, incorporates the use of a coaxial tip, whereby the analyte solution flows through a central tube while a make-up or sheath liquid flows coaxially through an outer tube around the analyte (5). Electrical contact is made between the source and the tip via the make-up liquid. Therefore this approach is far more rugged because it precludes the need to use metal-coated tips. The pneumatic-type design is particularly useful for coupling capillary LC to MS. Some of its benefits for this type of work include the ability to perform on-line modifications to the chromatographic chemistry, introduction of organic modifiers to stabilize the ion beam, addition of mass reference calibration standards, and the ability to operate the LC system over a broad range of flow rates with good stability.

An alternative approach, described by Davis and co-workers, makes the electrospray system more routine and rugged for LC separation work without using a sheath liquid (6). In this design the needle assembly is connected to the LC transfer line via a commercially available stainless steel union. The electrical contact is made at the union by a platinum sleeve transfer line, which eliminates the need to use coated needle tips. This design is capable of gradient flow rates as low as 100 nL/min with the added benefit of using uncoated tips that are rugged, simple, and relatively inexpensive to construct.

Today many excellent electrospray devices, needles, tips, unions, and transfer lines on the market are capable of producing stable sprays at flow rates down to 20 nL/min. Some of the more intricate designs even include charge-coupled device (CCD) cameras to optimize the spray and to troubleshoot any potential problems. The choice of which type or what components to use will depend on the nature of the application. If proteome studies at the nanoscale level are being performed, some of the questions that should be asked include

- What type of sample is being analyzed?
- How much sample is available?
- How many analytes are required?
- Is the device being used to deliver the sample directly into the mass spectrometer?
- Is it being used with an LC separation device?
- If it is being used for low-flow capillary LC gradient work, what is the lowest flow required?

It is very important to fully understand the demands of the analysis, particularly the flow requirements, to select the optimum electrospray device and components.

MASS SPECTROMETRIC DETECTION

The major breakthrough in mass spectrometric detection of proteins and peptides is the ability to identify and confirm a particular molecular ion by the generation of its daughter species using collisionally activated dissociation or fragmentation (7). This technique, traditionally known as tandem mass spectrometry or triple-quad MS-MS, consists of an evacuated chamber containing two resolving quadrupole mass filters separated by another quadrupole (used in the rf-only mode), which is slightly pressurized (but still under vacuum) by the introduction of a collision gas. (In some systems the rf-only quadrupole is replaced by a hexapole or octapole). In the MS-MS mode the first quadrupole is used in a mass-resolving mode to select the precursor ion. The second quadrupole (or pressurized multipole collision cell) is used to

produce fragmentation of the precursor or parent ion. The final quadrupole is used in a mass-resolving mode to provide mass analysis of the resulting fragmented or daughter ions. These species are then compared with reference spectra or data to produce unambiguous identification of the biomolecules of interest. Figure 2 shows a typical triple-quadrupole tandem mass spectrometer.

The benefit of the MS-MS design is that it can also be used in MS mode. In this mode the first quadrupole is used in the rf-only mode as a wide bandpass filter to transmit molecular ions of a wide mass range. The collision cell is also used in the rf-only mode, but this time no collision gas is flowing, so the cell is just used to transmit ions to the final quadrupole, which is used in the mass resolving mode. When nanoscale amounts of sample are being analyzed, the ability to switch between both modes is very important for maximizing the amount of data being generated. There is no question that the higher number of daughter or fragmented ions that are being identified will lead to better confirmation of the parent molecules. This is especially important in analyzing data from an LC separation. Peptides, for example, generally are eluted from the column over a period of 10–30 s. For this reason, it is absolutely critical to be able to switch very rapidly between MS and MS-MS under computer control to collect both the molecular and fragment information for positive and unambiguous identification of these species.

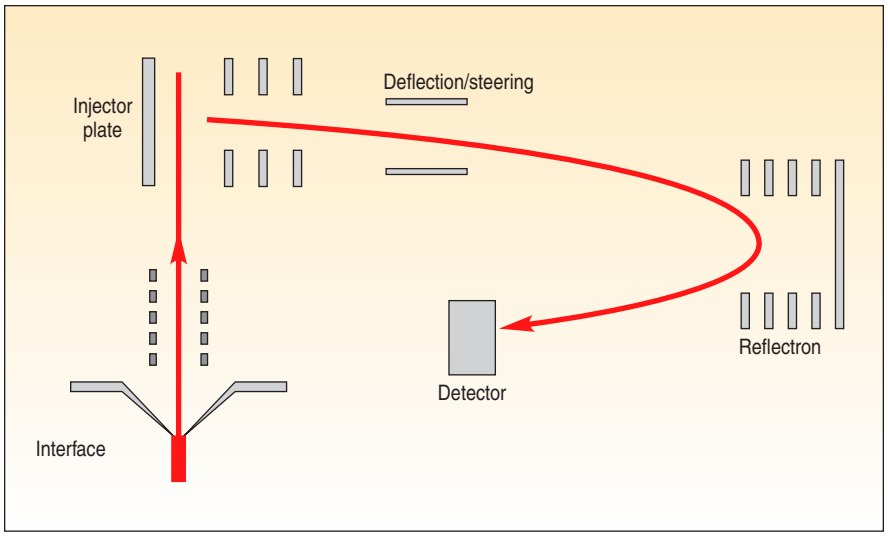

Figure 3. Schematic of an orthogonal TOF mass spectrometer.

Triple-quad mass spectrometers have been a major innovation in the identification and measurement of biomolecules. However, for some applications, quadrupoles have certain limitations that can be restrictive when they are used as the final mass resolving analyzer in the MS-MS mode. In the process of fragmentation of the parent ion, many daughter ions are produced by interaction of the pressurized collision gas with the parent molecule. These ion-molecule reactions between the collision gas and the biomolecule can take place by a number of mechanisms, including atomic/molecular association, dissociation, gas fragmentation, charge transfer, and proton transfer. The products of these collisions and reactions can show up in many different forms, including brand new molecular ions, ions displaced by the molecular weight of the collision gas, and even doubly, triply, or quadruply charged species of the parent ion. This often renders the resulting mass spectra very complex. The ability to resolve these species is critical in the positive confirmation of certain protein and peptide-based molecules.

Quadrupoles typically operate at a resolution of approximately 1 mass unit (dalton), but by changing the voltage on the dc rods the resolution can be adjusted to achieve 0.4–4 mass units, depending on the type of sample being analyzed. However, when resolution is increased, sensitivity decreases. This is not a problem with a very healthy signal, but can be potentially serious when one tries to identify weaker daughter species in complex mass spectra. If the quadrupole must be operated in its highest resolving mode, many of these daughter ions will not be detected.

This limitation in quadrupole technology has opened the door to the use of other types of higher resolving separation devices for proteomics studies, including

Table I. Preconcentration/desalting stage parameters.

Parameter	Specification
Protein digest sample volume	20–50 µL
Sample loading	50 µL/min 0.1% formic acid/H_2O for 3 min
Preconcentration/matrix removal column	300 µm × 1 mm/PepMap C18
Analytical column	75 µm ID × 15 cm/PepMap C18 (3 µm)
Gradient flow for separation (see Table II)	200 nL/min
Gradient flow for peak elution	25 nL/min

Table II. Gradient flow conditions.

Gradient flow times	Mobile phase
Initial	1% Acetonitrile (both with 0.1% formic acid)
1 min	5% Acetonitrile
30 min	40% Acetonitrile
31 min	80% Acetonitrile (column flushing)
36 min	80% Acetonitrile
37 min	1% Acetonitrile (reequilibration)
50 min	1% Acetonitrile

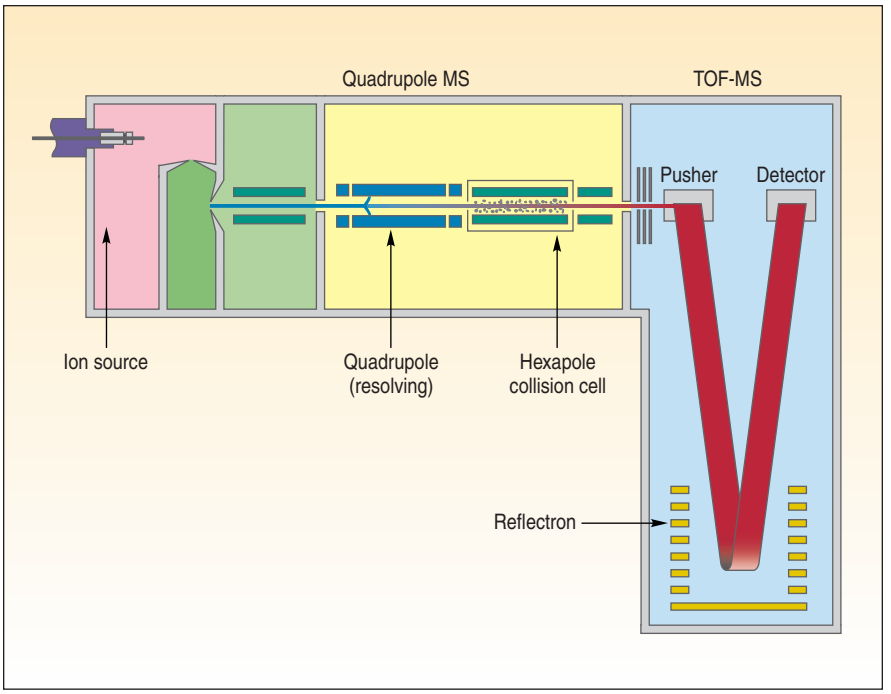

Figure 4. A typical quadrupole TOF-MS-MS system (courtesy of Micromass).

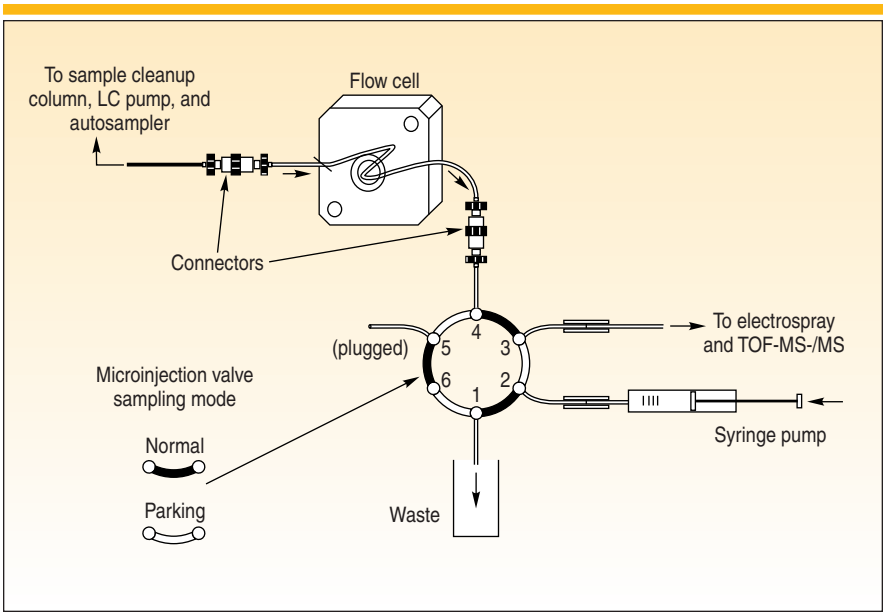

Figure 5. Schematic of instrumentation used for the variable-flow, peak-parking studies.

double focusing magnetic sector, ion trap, and time-of-flight (TOF) mass spectrometers. Magnetic sector systems offer the highest resolving power but scan very slowly. This makes them less than ideal for handling transient peaks being eluted off an LC column. Ion traps don't offer such high resolution but are well suited to transient peaks because of the simultaneous nature of trapping and measuring the ion. However, for the unique demands of MS-MS studies, TOF mass spectrometers are becoming the most attractive alternative to quadrupoles as the final mass-resolving device. Figure 3 shows the principles of this technique. Basically, in a TOF mass analyzer all ions that contribute to the mass spectrum are sampled and accelerated into either an axial (on axis to the ion beam) or orthogonal flight tube (right angles to the ion beam) at exactly the same time.

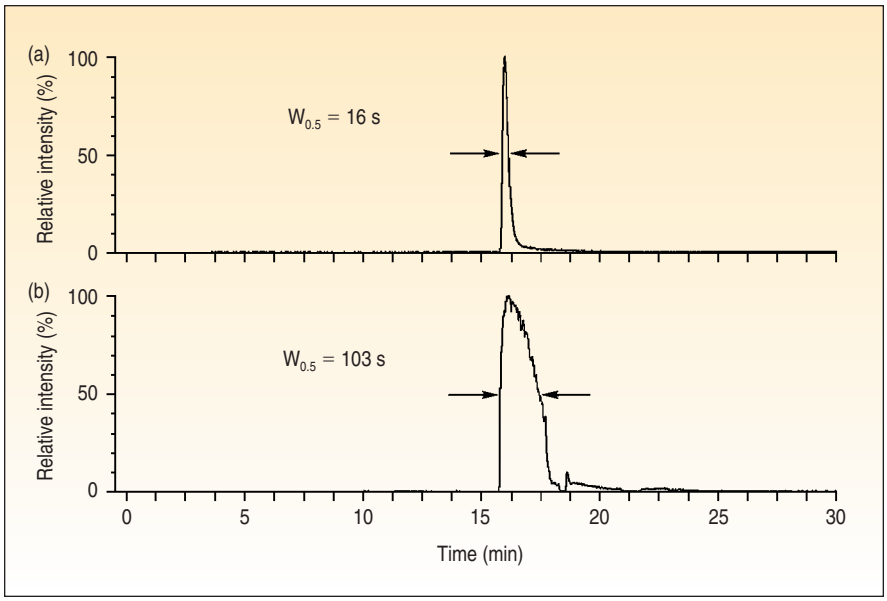

Figure 6. Mass chromatogram of neurotensin in a standard peptide mixture using (a) normal flow (200 nL/min) and (b) low flow (25 nL/min).

Because all the ions have the same kinetic energy, the time taken to reach the detector is related to their mass (8). Unlike a quadrupole device, the resolving power of the TOF system is related to the applied accelerating voltage and the length of the flight tube. In practice, this translates to a resolution of 0.01–0.1 mass units, which is typically one to two orders of magnitude better than a quadrupole. This improvement in resolution makes it ideal to handle the complex daughter spectra generated by a collision cell mass spectrometer without having to sacrifice sensitivity. In addition, the simultaneous nature of sampling the ions makes TOF a much better detection system than a quadrupole to handle the transient peaks generated by a liquid chromatograph (9). In fact, because quadrupoles are scanning devices, they are restricted in the amount of spectral information they can handle in a fast transient event. For the identification and measurement of very low concentrations of protein and peptide mixtures by LC-MS-MS, TOF-MS coupled with a quadrupole and a collision cell probably represents the best approach. A typical TOF-MS-MS system using a quadrupole mass filter and a hexapole collision cell is shown in Figure 4.

NANOSCALE CAPILLARY LC

LC systems typically operate at flow rates of 50–1000 µL/min using 1–5 mm i.d. columns. This makes them less than ideal to be coupled to a mass spectrometer via an electrospray sample-introduction system that runs at 1–10 µL/min. With the first commercially available LC-MS systems, the electrospray device struggled to efficiently handle the high sample flow rates coming off the LC column, thus making them unsuitable for extremely low-level biomolecule studies. This weakness drove the development of pneumatic-based electrospray devices (described earlier), which had the ability to control the sample flow rate far more precisely.

All the drawbacks associated with connecting an LC system to a mass spectrometer led to the coupling of nanoflow electrospray directly to the MS system. Unfortunately this also had its drawbacks because, even though it worked very well with sample volumes of a few microliters, sample loading, matrix separation, and analyte preconcentration were manual and labor-intensive. It was also limited to simple mixtures because all the separation was taking place inside the mass spectrometer. These limitations made the direct coupling of electrospray to an MS practical for only the low throughput of noncomplex mixtures.

The need to detect lower concentrations of analytes in complex protein mixtures with a high degree of automation has driven the demand to develop ultra-low-flow capillary LC to take advantage of low-flow electrospray technology. As a re-

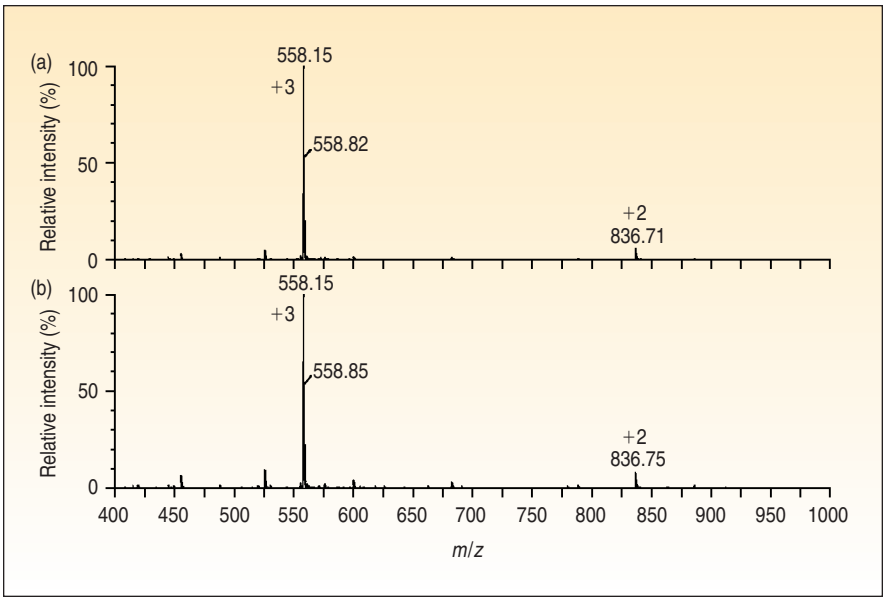

Figure 7. Spectra obtained for neurotensin in a standard peptide mixture using (a) normal (200 nL/min) and (b) low (25 nL/min) flow rates. Ion counts: (a) 3300, (b) 25,200.

sult of this demand, a number of commercial suppliers now provide <50 μm i.d. microtubing, 50–200 μm i.d. columns, and zero-displacement microvalves, which are capable of handling flow rates of 20–200 nL/min. This has meant that the recognized benefits of nanoscale LC, such as low femtomole sensitivity, fully automated sample handling and preparation (including procedures such as desalting and preconcentration), high separation efficiency, and variable nanoliter gradient flows, can now be combined with all the advantages of nanoflow electrospray.

VARIABLE-FLOW LC

The ability to vary the flow in nanoscale LC separation offers some unique benefits for the handling of minute quantities of sample, which cannot be realized with a fixed-flow system. This technique, known as variable-flow nanoscale capillary LC, combines the recognized benefits of long sampling times characteristic of nanospray technology with all the attributes of nanoflow capillary LC. By reducing the flow rate on the fly, eluted peaks can be slowed down and for all intents and purposes stopped to allow for much longer interrogation of the spectral data. This technique, commonly known as *peak parking,* was first described by

Lee and co-workers (10), who used it to analyze peptide mixture digests by LC-MS-MS. They reported that by reducing the flow rate from 200 nL/min to <40 nL/min, whenever spectra for one of the major peptide components appeared, they significantly increased the acquisition time, resulting in a factor of five improvement in the number of spectral scans (50 compared with 10) (11). This work has since been refined by Moseley and coworkers, who reported more than a sixfold increase in acquisition time, based on a reduction of flow rate from 200 nL/min to 25 nL/min. In this work they took a digested protein sample and loaded it via an autosampler onto a micro precolumn, which served to both preconcentrate the analytes and remove the matrix components (desalting). The analytes were then backflushed onto the analytical column at 200 nL/min by gradient flow. After separation the eluent was aspirated into a TOF-MS-MS system via a nanoflow electrospray device fitted with a PicoTip electrospray tip (New Objective Inc., Cambridge, MA). Data were collected initially at a flow rate of 200 nL/min. The flow rate was then reduced to 25 nL/min to perform the peak-parking measurement. Tables I and II provide full details of the LC separation methodology.

A schematic of the setup is shown in Figure 5. The microcolumns, autosampler, and pumps were supplied by LC Packings (San Francisco, CA); the microinjection valve was supplied by Upchurch Scientific (Oak Harbor, WA); the PicoTips for the electrospray unit were supplied by New Objective Inc., and the TOF-MS-MS system (Q-TOF) was supplied by Micromass Ltd. (Manchester, UK).

It is worth noting that in nanoscale separation work of this kind it is critical to use components that are optimized for injecting ultrasmall sample volumes. For this reason it is important to use microinjection valves that have extremely low internal displacement to reduce peak tailing and minimize delay volume from the system. It is also very important to use electrospray tips and contacts that have been designed for postcolumn LC separation, as opposed to ones that are used for precolumn work or designed for use with flow injection sample introduction.

RESULTS OF PEAK-PARKING STUDIES

Some preliminary data from the peak-parking studies can be seen in Figure 6, which shows the separation of neurotensin from a standard peptide mixture (11). Figure 6 shows that by reducing the flow rate from 200 nL/min to 25 nL/min during peak elution into the mass spectrometer, the temporal resolution has been improved from 16 s to 103 s, a 6.4-fold increase in the time available to interrogate the mass spectra.

The increase in interrogation time is relevant, because if the neurotensin spectra are summed across the chromatographic peak at low flow and compared with the normal flow approach, a 7.6-fold increase is realized in the total number of ions counted. This can be seen in Figure 7, which shows a total ion count of 25,200 at 25 nL/min compared with 3300 at 200 nL/min. In addition to neurotensin, other peptides in the mixture, including bradykinin and Lys-bradykinin, showed similar separation performance. This is an exciting breakthrough, particularly for MS-MS studies, because it means a significant increase in the number of precursor peaks than can be used for the positive identification and quantitation of biomolecules of interest.

In fact, in a similar study using a complex protein digest, the peak-parking approach generated almost 4500 MS-MS spectra compared with 770 using normal flow, representing almost a sixfold increase. When an internal quality control threshold was applied to the data to compensate for noise and inconclusive spectral data, peak parking accounted for almost 1200 *real* MS-MS spectra compared with 550 at the normal flow. In other words, under normal flow conditions nearly 650 peaks would have been missed and therefore not available for identification. Besides its analytical advantages, the added benefit of this approach is that once the instrumentation is set up it can be very easily automated and run on a routine basis.

CONCLUSION

There are many excellent techniques available for analyzing proteins and peptide mixtures, including gel electrophoresis, nanoflow electrospray-MS-MS, LC-MS-MS, and nanoscale capillary

LC-MS-MS. However, the unique requirements of pharmaceutical companies have put enormous demands on these analytical techniques, particularly in the area of drug discovery. To understand the importance of proteins as potential targets for pharmacological research, detection at the low femtomole level is absolutely critical. Until now the most attractive approach to meet this challenge was liquid chromatography coupled with a tandem mass spectrometer (LC-MS-MS). Its unique capabilities have significantly enhanced this area of research, which didn't seem at all possible 10 years ago. However, it is only fairly recently with innovations in nanospray delivery to the mass spectrometer and nanoscale LC components that handling such small sample volumes has become a reality. There is no question, based on current evidence, that the variable-flow, peak-parking approach represents the most exciting breakthrough for automated proteome studies at the low femtomole level.

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