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*Review Article*

# Mass Spectrometry Innovations in Drug Discovery and Development

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This review highlights the many roles mass spectrometry plays in the discovery and development of new therapeutics by both the pharmaceutical and the biotechnology industries. Innovations in mass spectrometer source design, improvements to mass accuracy, and implementation of computer-controlled automation have accelerated the purification and characterization of compounds derived from combinatorial libraries, as well as the throughput of pharmacokinetics studies. The use of accelerator mass spectrometry, chemical reaction interface-mass spectrometry and continuous flow-isotope ratio mass spectrometry are promising alternatives for conducting mass balance studies in man. To meet the technical challenges of proteomics, discovery groups in biotechnology companies have led the way to development of instruments with greater sensitivity and mass accuracy (e.g., MALDI-TOF, ESI-Q-TOF, Ion Trap), the miniaturization of separation techniques and ion sources (e.g., capillary HPLC and nanospray), and the utilization of bioinformatics. Affinity-based methods coupled to mass spectrometry are allowing rapid and selective identification of both synthetic and biological molecules. With decreasing instrument cost and size and increasing reliability, mass spectrometers are penetrating both the manufacturing and the quality control arenas. The next generation of technologies to simplify the investigation of the complex fate of novel pharmaceutical entities *in vitro* and *in vivo* will be chip-based approaches coupled with mass spectrometry.

**KEY WORDS:** mass spectrometry; drug development; high throughput screening; pharmacokinetics; metabolite identification; mass balance; biomolecule characterization.

## INTRODUCTION

In the past thirty years, there has been an evolution in mass spectrometry from an esoteric technology practiced by a few specialists to a broadly used detector found in all sectors of the pharmaceutical industry. The race by pharmaceutical companies to market new chemical entities (NCEs) has fueled the high-throughput approach to drug development (1). This in turn has led to the implementation of many of the recent technological and methodological advances in mass spectrometry. This review is not intended as a discussion of the basic theory or historical developments of mass spectrometry, rather it is an examination of the emerging technologies that have and will continue to revolutionize the drug discovery and development process. In this review, "development phase" refers to the clinical investigation phase, while "discovery phase" refers to all studies leading to the selection of a clinical candidate. The focus will be on small synthetic molecules and biotechnology-derived proteins, and the review will not address applications to nucleotide- or oligosaccharide-based therapeutics.

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## WORKING CONCEPTS

One feature common to all mass spectrometers is that they separate molecules according to their mass-to-charge ratio. A second common feature is the requirement that the molecules must first be ionized. Initially, the majority of molecules amenable to mass spectrometric analysis were volatile and stable to heat, which significantly limited the range of molecules that could be analyzed without derivatization. This early constraint has been lifted with the introduction of both electrospray ionization (ESI (2)), and matrix-assisted laser desorption and ionization (MALDI (3)).

## ADVANCES IN DISCOVERY AND DEVELOPMENT

### Combinatorial Chemistry

The advent of combinatorial chemistry has led to an explosion in the numbers of compounds that can be synthesized and subsequently screened for biological activity. To keep up with the accelerated pace of NCE generation, high-throughput methods to purify NCEs, verify their integrity, and screen them for biological activity are requisite. Mass spectrometry offers a sensitive and rapid approach capable of confirming both the identity and purity of NCEs as well as the ability to ascertain biological activity.

#### *Characterization*

To keep pace with NCE generation, some changes to standard approaches of mass spectrometric analysis of struc-

ture and purity have been implemented to shorten analysis time. These approaches include flow injection analysis, parallel multi-column analysis, ultrafast separations, and supercritical fluid chromatography (SFC). Flow injection analysis combined with mass spectrometry (FIA/MS) is the fastest approach. During FIA/MS, the sample is injected into a stream of mobile phase that is directed into either an ESI source or an atmospheric pressure chemical ionization (APCI) source, bypassing any high-performance liquid chromatography (HPLC) columns (4). The speed of sample injection determines the number of samples that can be analyzed in a given period of time. One innovative solution to increase the speed is to incorporate a multi-injection system, such as the Gilson multiple probe 215 injector. This device is equipped with up to eight Rheodyne injection valves, which allow some valves to wash, while others load or inject. Kassel and coworkers have demonstrated that 96 samples can be analyzed in less than seven minutes by using a multiple probe injector with FIA/MS (5).

Where FIA/MS derives its speed (i.e., the lack of chromatographic separation) becomes its major drawback. Without chromatographic separation, little can be said regarding purity. Furthermore, competition for ionization from the solvent and contaminants can prevent detection of the desired NCE. Several recent approaches minimize the delay due to chromatographic separation. The first approach uses two or more columns in parallel with multiple UV detectors that operate prior to mass spectrometric analysis. With two columns and a dual ESI interface, separation and mass spectrometric analyses of up to 20 samples per hour can be achieved (6); however, this is still 40 times slower than FIA/MS. By increasing the number of sprayers on the ESI interface to four, 96 samples can be analyzed in one hour (7).

Another way to hasten analysis time is to run ultrafast separations, which are defined as HPLC gradient separations that occurs in under one minute. Quadrupole instruments, which are the most frequently used mass analyzers for small molecules, can scan the desired mass range of 80 m/z to 700 m/z in 1 a.m.u. steps in about 0.5 sec. If the total chromatographic separation occurs within 30 seconds, data collection every 0.5 sec is insufficient to define the chromatographic peaks. In comparison, time-of-flight (TOF) mass spectrometers, with their capability to acquire spectra every 10-100 μsec, can better define chromatographic peaks from ultrafast separations of compounds from combinatorial libraries (8). Using a similar approach, an entire 96-well plate has been analyzed in less than one hour (9). Additional advantages of TOF analyzers are 10-100 fold lower limits of detection and greater mass accuracy (<10 ppm). An alternative to ultrafast HPLC separations is supercritical fluid chromatography. The primary advantage of SFC is that greater resolution can be achieved with similar run times, thereby enabling a more accurate assessment of purity. The greater resolution is achieved because SFC uses a compressible gas, which imparts greater diffusivity and lower viscosity than a liquid mobile phase (*e.g.* acetonitrile) (10,11). These physical characteristics of a supercritical fluid allow higher flow rates and use of longer columns without increased back-pressure. Another advantage of SFC is a reduction in organic waste, which is particularly useful for large-scale operations.

Though FIA/MS is the fastest method of the listed approaches, it is the least able to determine purity. However, the other approaches may not be any better for the estimation of purity because of non-equimolar ionization in the electrospray source. Figure 1 illustrates this for a mixture of analytes that differed only in their pKa. Electrospray ionization underestimated the proportion of the acidic compound when analysis was performed in the positive-ion mode. One solution to this limitation is to use evaporative light scattering (ELS) detection in parallel with MS detection (12). The principle behind ELS detection, and the relationship between analyte mass and detector response, has been reviewed elsewhere (13). As can be seen in the lower panel of Figure 1, the ELS detector more accurately reflects the molar proportions of the analytes present. Evaporative light scattering detection in parallel with mass spectrometric detection is gaining popularity for assessing the purity of combinatorial libraries (12–15).

### *Purification*

For efficient purification of NCEs, methodologies must be rapid, automated, and reliable. One approach has been to fractionate the HPLC effluent from four simultaneously run columns using a Biotage Parallex preparative HPLC (16). The fractionation is guided by UV detection, and the pooled fractions are subsequently analyzed by FIA/MS for identification and ELS detection for quantification. Even though 22 samples can be fractionated in one hour, full processing and MS analysis take much longer with this approach. A second approach, which utilizes m/z data-dependent fraction collection, can fully process four samples in one hour (6). This approach relies on two simultaneously run columns, a dual sprayer in the ion source, and two fraction collectors. Neither approach is fast enough to meet the rate with which compounds are synthesized in combinatorial libraries; thus, considerable improvements remain to be made.

### *High-Throughput Screening*

Combinatorial chemistry has also created a need for rapid and predictive methods for screening thousands of compounds for receptor agonist or antagonist activity to identify lead compounds. This process has led to a new term, "high-throughput screening" or HTS (17). HTS often utilizes spectroscopic detection in 96 and 384 well formats. In contrast to optical-based methods, the mass spectrometric methods offer the ability to screen mixtures, even complete libraries, while simultaneously providing structural information. Most of the mass spectrometric-based methods have been recently developed in academic laboratories, and only some of these technologies are being investigated for drug discovery in the pharmaceutical industry.

The first of these methods is called affinity capillary electrophoresis-mass spectrometry (ACE–MS) (18). Binding constants of multiple ligands for a receptor can be simultaneously measured in solution based upon mobility shifts of the ligand during a capillary electrophoretic separation. In the original ACE-MS method, the running buffer containing the receptor and a mixture of ligands is injected onto the capillary (18). Each ligand's electrophoretic mobility is then altered by complexation with the receptor, and it is then identified by either ESI-MS or ESI-tandem mass spectrometry (MS/MS). The advantage of this technique is the ability to screen 100-200 fem-

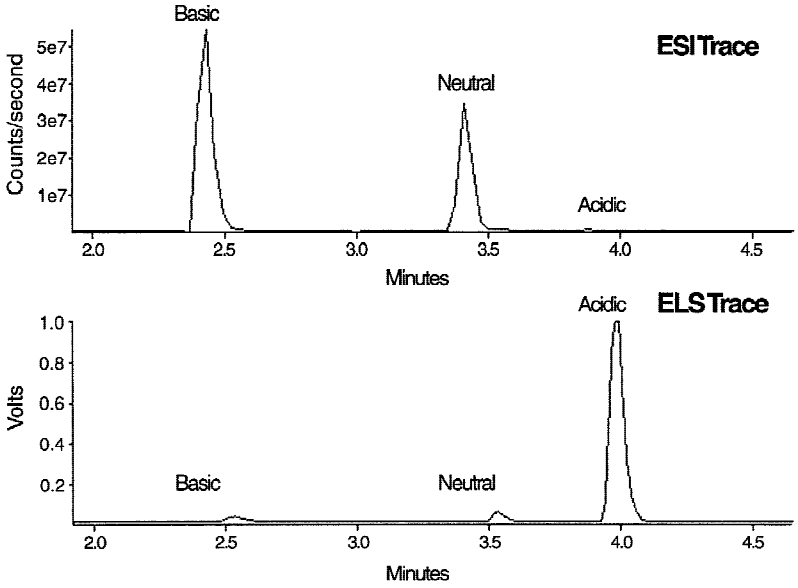


**Fig. 1.** Analysis of a mixture of three compounds by positive-ion electrospray (upper panel) and evaporative light scattering detection (lower panel). The compounds (a basic amine, a neutral amide, and an acidic carboxylic acid) were injected onto a column in the following proportions (basic—0165 μg; neutral—0.165 μg and acidic—1.33 μg). The effluent from the column was split with 0.48 mL/min going into the evaporative light scattering detector and 0.12 mL/min going into th electrospray ion source. This figure shows that the ELS detector better represents the proportion of each compound in a mixture.

tomole amounts of ligands within mixtures in only minutes. Additionally, the receptor need not be totally pure and the ligand concentration does not need to be known. The first demonstration of ACE-MS for screening combinatorial libraries was the screening of a peptide library containing 100 peptides for binding to vancomycin (18). Screening of larger peptide libraries (1000 peptides) by this approach requires initial pre-concentration and enrichment by affinity selection due to the limited solubility of a mixture of ligands. The current disadvantage of ACE-MS is the need for a rapid ligand off-rate relative to the length of the experiment, and for the receptor to be soluble and available. Presently, this excludes all G-protein coupled receptors due to their limited solubility, although this limitation may be overcome with the use of micellar electrokinetic chromatography coupled to ESI-MS (19). A second model system, using the Src SH2 domain and several phosphorylated peptides has presented further refinements to the ACE-MS method (20). In this system, capillary isoelectric focussing (cIEF) replaces capillary electrophoresis as the separation method, thereby allowing concentration of the sample (~100X) and lowering the limit of detection. In contrast to the CE separation, the cIEF-ACE-MS method requires that the ligand has a slow off-rate, slower than the experimental run time. Another difference between the CE and cIEF methods is that the receptor and ligand are eluted separately from the capillary in CE, but remain bound and must be dissociated in the source prior to mass spectrometric detection in cIEF.

A second approach, which also involves solution-phase binding between multiple ligands and a receptor monitored by mass spectrometry, is pulsed ultrafiltration mass spectrometry (PUF-MS) (21). In PUF-MS, several ligands and a candidate receptor are mixed in an ultrafiltration chamber fitted with the appropriately sized molecular-weight cutoff filter. The cutoff filter prevents the receptor or enzyme from passing through the reaction chamber and into the mass spectrometer, allowing only the ligands to pass into the instrument for detection. After introduction of the ligand and receptor into the reaction chamber, the mixture is first washed with water to remove unbound ligands, and the bound ligands are then eluted into the mass spectrometer with a solvent-containing mobile phase. An advantage of this method over ACE-MS is the ability to reuse the receptor. This advantage is receptor-specific and dependent upon the denaturant used to dissociate the complex. Similar to cIEF-ACE-MS experiments, PUF-MS can concentrate ligands from dilute solutions. Two disadvantages of this approach are that both the ligands and the receptor must be soluble, and that fast on-off interactions are not detected.

A third approach, library affinity selection mass spectrometry (LAS-MS), relies on the affinity of ligands for target molecules (22–24). LAS-MS entails covalent coupling of a receptor, antibody, or enzyme to a stationary support in order to selectively bind and remove compounds from a library (22–23). The bound ligands are then released, usually by lowering the pH. In an alternate version of this approach, analogous to PUF-MS, the receptor or target molecule interacts with the compound library in solution and the bound compounds are separated from the unbound using size-exclusion chromatography (24). Following size-exclusion, the receptor-ligand complex is desalted by reverse-phase chromatography. Under these conditions, dissociation of the receptor-ligand complex occurs. In either version, the eluted ligands are subsequently identified and characterized structurally by ESI-MS and ESI-MS/MS. The primary advantage of this approach is the ability to automate the screening by combining a column

switching system with the affinity column containing the target molecule. Libraries as large as 576 peptoids (24) and 361 peptides (22) have been successfully screened with the off-line approach, whereas the automatable on-line approach has only been demonstrated with small libraries of 20 or less components (23). LAS-MS assumes that the native conformation of the surface-immobilized receptor is maintained and that the target is stable to repeated solvent exposure or pH changes. This assumption is target specific, thus requiring significant development time. Furthermore, rapid on-off interactions are not detected.

Additional limitations to all of these approaches are their inability to differentiate nonspecific binding from allosteric binding and complications associated with determining binding affinities. A complication in ranking by ACE-MS is the possibility that the ligands migrate in both directions, which can give overlap of high affinity with very low affinity ligands. With cIEF-ACE-MS, PUF-MS and LAS-MS, on the other hand, ranking can be difficult since all assume equimolar ionization in the mass spectrometer. As a result of these limitations, the methods will remain a first-pass screen to subsequently enable structural characterization of the most interesting analytes by MS/MS. Of these three approaches, ACE-MS may be most quickly assimilated into the pharmaceutical industry, as evidenced by the recent formation of a contract research organization, Cetek Corporation, that is based upon the ACE-MS technology (25).

## ADME

Inadequate ADME properties constitute the reason that ~25% of clinical candidates do not reach the market (26). It is more desirable that compounds fail cheaply early in the discovery phase than during the more costly development phase. Hence, over the past decade, ADME studies have shifted to earlier in the discovery phase, and have been a bottleneck due to the large number of compounds. This increased demand for ADME resources has led to the development of more efficient and higher throughput techniques in which mass spectrometry has played a major role. Mass spectrometry has accomplished this task by providing additional selectivity and sensitivity that is frequently unavailable with UV, fluorescence, or radioactivity detection.

### *Pharmacokinetics*

In this review, pharmacokinetics includes both the *in vivo* and the *in vitro* studies that describe the extent and time course of drug absorption, distribution, biotransformation, and elimination. The most commonly used technique for obtaining this quantitative information is HPLC coupled with atmospheric-pressure ionization-tandem mass spectrometry (API-MS/MS) using a triple quadrupole mass analyzer. Atmospheric pressure ionization encompasses two ionization techniques: atmospheric-pressure chemical ionization, APCI, and electrospray ionization, ESI (27). APCI ionizes the molecules by a chemical ionization process after the molecules are already in the gas phase. In contrast, ESI ionizes the molecules in the solution phase prior to getting the ions into the gas phase. The best choice of ionization source depends upon the molecules being analyzed, but nonpolar molecules that do not contain acidic or basic sites frequently work better with APCI. After ions are formed, they are typically detected by the MS/MS technique of selected-reaction monitoring (SRM). SRM entails selecting the parent ion with the first quadrupole and fragmenting it in the second quadrupole by collision with an inert gas (e.g., $N_2$, Ar). The third quadrupole then monitors a specific product ion, thereby quantifying with maximum signal-to-noise (27). A significant advantage of mass spectrometers over other forms of detection (e.g., UV, radioactivity) is the ability to separate molecules by m/z. This dimension of separation allows abbreviated chromatographic separations without a need for baseline resolution, thus increasing throughput. The selectivity afforded by mass spectrometry, along with improvements in automated sample preparation, a greater reliance on *in vitro* methods, and the use of cassette dosing (also called N-in-one dosing) have all enabled the bioanalytical chemists to keep pace with the high-volume studies of the *in vivo* fate of compounds.

Any improvements that reduce sample preparation time have a significant positive impact on throughput of PK studies. Historically, compounds in biological matrices (e.g., microsomes, plasma, urine, etc.) were processed by liquid-liquid extraction to remove the interfering materials and to concentrate the analyte prior to analysis. The availability of robotic liquid-handling systems has now resulted in the automation of liquid-liquid extractions in 96-well plates, thereby reducing the sample preparation times by ~3-fold (28). Similar gains in speed have been observed when robotic liquid handling systems are combined with the 96-well solid-phase extraction (SPE) plates (28). The primary advantage of the 96-well SPE formats that use disk, instead of column, cartridges is the reduction in elution volume and the compatibility of the 96-well format with robotic liquid handlers and autosamplers (29). During lead optimization and in the development phase, automated SPE combined with column switching techniques have enabled clinical sample analyses with <10 min analysis times, while simultaneously maintaining ruggedness and low variance (<15%) (30–32). Column switching and pre-column setups have also made direct plasma and urine analyses feasible (33).

*In vitro* methods offer the pharmaceutical industry a rapid means to screen thousands of NCEs for potential PK problems before a compound is elevated to lead candidate status. These screens include intestinal absorption, metabolic stability, and plasma protein binding, all of which are facilitated by LC-MS. Typically, selected-ion monitoring or a full-scan is used to detect the intact compound. Therefore, a single quadrupole or TOF instrument is sufficient for these *in vitro* methods.

Intestinal absorption potential is commonly estimated by measuring the permeability of NCEs across Caco-2 cell monolayers or intestinal tissues in an Ussing chamber. Mass spectrometry offers the advantage of measuring mixtures of compounds, thereby significantly increasing sample throughput in these models. ESI-MS has successfully allowed monitoring Caco-2 permeability of 150 separate mixtures containing 2500 tri-peptides per mixture (34).

Metabolic stability of an NCE is most frequently evaluated *in vitro* using liver microsomes. Once again, the selectivity of mass spectrometers increases throughput by allowing the use of fast gradients (<5 minutes) with short columns as well as generic methods to quantify analyte concentration. By incorporating computer control and data analysis into this

process, the analysis of samples from metabolic stability studies can be further expedited. An automated mass spectrometric approach that encompasses these ideas has been developed and shown to be capable of determining the metabolic stability of 300 NCEs per month (35).

*In vitro* screens have been developed to determine the extent of plasma-protein binding which could affect a compound's pharmacokinetics. Albumin is the most abundant protein in plasma and the primary species to which NCEs bind. Typically, plasma protein binding experiments are conducted one compound at a time by equilibrium dialysis or ultrafiltration. Recently, PUF-MS has ranked the affinity of multiple compounds for binding to human albumin (36). Also, as a result of the sensitivity of MS techniques, binding to other blood components as well as stability in biological matrices can be assessed at relevant *in vivo* concentrations.

The same LC-MS conditions developed to assess *in vitro* PK parameters have been extended to screen the pharmaceutical properties and identify potential degradation hotspots of NCEs. For example, the hydrophobicity index (partition coefficients), as well as the sites of degradation under conditions of pH, thermal, and oxidative stress, have all been rapidly assessed by ESI-MS (37). Furthermore, the extent of degradation upon storage has been determined with abbreviated methods using simultaneous parent and product ion monitoring without the need to optimize the HPLC separation method (37).

The unique MS/MS spectra of compounds from different structural classes provide the selectivity necessary to analyze multiple compounds in mixtures without the need for chromatographic separation. Hence, a strategy used to increase throughput of *in vivo* PK sample analysis has been to dose and analyze multiple compounds simultaneously (cassette dosing) (32,38). The largest number of samples analyzed simultaneously is 11 NCEs in two days, increasing the throughput of analysis the in-life portion by 4-fold and 19-fold, respectively, compared to the single-compound screening mode (39). A limitation of this approach is the potential for drug-drug interactions (e.g., inhibition of metabolic enzymes). This limitation can be overcome by pooling biological samples after individually dosing the compounds (40–41). Post-dose sample pooling requires more animal resources than cassette dosing, but the analysis time is still significantly shorter than individual dosing and analysis. A second limitation of both cassette dosing and post-dose sample pooling, which can lead to errors in quantification, is the possibility that the metabolite of one compound is identical or isobaric to another compound present in the dosed mixture. This problem can be minimized by careful selection of compounds that are co-administered or pooled (38). With either approach, the larger the number of compounds in a mixture, the greater the likelihood of co-elution of some analytes. This could potentially result in competition for ionization, leading to inaccurate quantification.

Mass spectrometric technologies have impacted not only the discovery phase, but also the development phase in which thousands of clinical samples must be analyzed within a few months. The selectivity offered by mass spectrometers provides a unique opportunity to determine bioavailability of a compound by simultaneously dosing different isotopic forms via two or more routes in the same subject. Because the isotopic forms of a compound have different molecular weights, they can be monitored simultaneously by HPLC-API-MS/MS. This approach has been used successfully to determine the oral and ophthalmic bioavailability of timolol in dogs (42). More recently this approach has been extended to determine the bioequivalence of an oral solution of moricizine compared to film-coated tablets in humans (43). The primary advantage is the elimination of intra-subject variability, providing greater statistical power with reduced number of subjects. The savings in time and money provided by this approach offset the additional expense and time required for the synthesis of the stable isotope analogs.

LC-MS techniques have become a common tool for efficiently optimizing the ADME properties of drug candidates. The next wave in MS-technology advancement for ADME studies will rely on the vastly greater resolution and sensitivity of time-of-flight mass analyzers (44).

### *Metabolite Identification*

It is important to identify the metabolites of a lead candidate to obtain mass balance and to understand the source of potential toxicity. In the past, metabolite identification was accomplished by the synthesis of a radiolabeled compound followed by the laborious isolation of metabolites from urine or feces, and subsequent structural characterization. Over time, the benefits of identifying the metabolites earlier in the discovery phase were understood. The advantages of early metabolite identification include the ability to: (a) find metabolic hotspots responsible for shortened half-life or low oral bioavailability, (b) gain insight into reactive or toxic metabolites, and (c) find metabolites that are active at the target site. Metabolite identification now plays a pivotal role in generating better molecules, and ultimately in selecting potential clinical candidates.

HPLC-API-MS/MS using a triple quadrupole instrument is the standard technique used for metabolite identification. The power of this approach is the ability to perform both MS/MS scans (e.g., neutral loss and precursor ion scans) and full-scans that search for predicted molecular weight changes (45–46). Yet data interpretation is slow and several new approaches are now being implemented to overcome this bottleneck. These enhancements include (a) using intelligent software for data acquisition and interpretation, (b) performing multiple stages of mass spectrometry experiments (known as $MS^n$), and (c) increasing mass accuracy, as described below.

Improvements in software for data acquisition have led to significant reductions in both instrument and operator time. One such example is the development of an instrument control language for the TSQ 7000 LC-MS/MS, referred to as intelligent automated LC-MS/MS (INTAMS) (47). The procedure eliminates the operator's need to: (a) record the retention times of the chromatographic peaks, (b) identify the ion's mass-to-charge that is responsible for the chromatographic peak, and (c) subsequently acquire a product ion spectra for each chromatographic peak. Freed from these tasks, the operator can devote more time to data interpretation. A second improvement in data analysis software is the development of a pattern recognition program that uses correlation analysis to compare product ion spectra, thereby enabling simultaneous analysis of metabolites formed from mixtures of compounds (48). The correlation analysis software scores the similarity between the spectra of parent com-

pounds and those of the potential metabolites or endogenous materials. The higher the correlation value, the greater the similarity, thus helping to assign metabolite masses to their respective parent compound while eliminating masses from endogenous substances. This approach to the analysis of multiple metabolites in one sample is analogous to cassette dosing, and therefore reduces both instrument time and sample preparation efforts. As with cassette dosing, devising this type of experiment requires that the mixtures of compounds contain diverse structures so as to minimize the possibility of forming common metabolites.

The ion-trap mass spectrometer offers another means to acquire $MS^n$ spectra (where $n \geqslant 2$). The advantage of $MS^n$ is the ability to expedite the interpretation of collision-induced dissociation spectra. Fragments necessary for defining the site(s) of modification are frequently not formed in a two-staged (MS/MS) experiment. Additional stages of fragmentation and product ion formation provide information that increases the likelihood of elucidating the site(s) of modification (49). An additional advantage of the ion-trap mass spectrometer is its low limit of detection, which is attainable because a specific population of ions can be accumulated in the trap. By combining capillary chromatography with ion-trap $MS^n$ detection, structural information can be obtained with as little as 5 picograms of metabolite (50). This level of sensitivity allows the direct injection of urine without sample pretreatment, thereby saving sample preparation time.

Increased mass accuracy offers yet another means of hastening the structural elucidation of metabolites. For example, a mass accuracy of better than 5 ppm allows the determination of the product ion's elemental composition and affords greater confidence in the interpretation of MS/MS spectra. The tandem quadrupole time-of-flight mass spectrometer (Q-TOF) is capable of routinely providing a mass accuracy of <5 ppm for product ions from drug metabolism studies (51). Finally, automated screening for active metabolites can facilitate the rapid understanding of a drug's pharmacodynamics. Such automation has been achieved by combining 96-well format receptor-binding assays with mass spectrometric detection (52).

*Mass Balance*

Mass balance studies require that the majority of the administered drug and its associated metabolites be identified and accounted for in its elimination. The most frequently used approach is to administer the drug containing a radioactive isotope such as $^{14}C$ or $^{3}H$, and conduct either liquid scintillation counting of collected fractions or direct radioactive monitoring (RAM) of the HPLC effluent. There are several disadvantages to these approaches. First, there are environmental concerns around disposing of radiolabeled compound. Second, for drugs with long half-lives, the exposure may be too high to allow for the use of radioactivity. Lastly, these approaches are not approved for studies in women and children.

Several mass spectrometric approaches are on the horizon that promise to overcome these shortcomings. One of these is accelerator mass spectrometry (AMS) (53). In biomedical studies using AMS, the drug is first labeled with either $^{3}H$ or $^{13}C$. AMS then measures the ratios $^{14}C/^{13}C$ or $^{3}H/^{13}H$. Prior to analysis, the samples are converted into a solid form that is amenable to AMS. For $^{14}C$ measurements, the samples are converted to $^{14}CO_2$ and subsequently to graphite; for $^{3}H$ measurements, the samples are converted to metal hydrides. Although AMS requires the use of a radiolabeled compound, the radiological dose is negligible (<10 nCi) (53). AMS has demonstrated a 12-fold lower concentration limit of detection than liquid scintillation counting with better accuracy and precision (53–54). Recent sensitivities down to zeptomole ($1021^{-21}$ moles) levels for $^{14}C$ have been reported (55). This level of sensitivity could significantly impact the future of pharmaceutical development by enabling earlier investigation of the biotransformation or disposition of a lead compound in humans, thereby eliminating extensive toxicological studies. A practical limitation to the use of this powerful technology is low instrument availability due to its cost and size. The smallest instrument is approximately 4.5 × 6.0 meter (56). Additionally, no interface is currently available to direct the effluent from an HPLC into the AMS; samples must first be converted off-line.

Two mass spectrometric approaches other than AMS use stable isotopes for mass balance studies. The two techniques are continuous flow-isotope ratio-mass spectrometry (CF-IRMS), which utilizes an elemental analyzer interface, and chemical reaction interface-mass spectrometry (CRIMS), as described below.

CF-IRMS is the only method of the three listed above which has successfully been used for mass balance studies(57–58). Browne and Szabo (58) recently analyzed $^{15}N^{13}C_2$-acetaminophen in human urine, feces, and plasma using an instrument (illustrated in Figure 2) commercially available from Europa Scientific (Franklin, OH). The samples are dried in tin cups before introduction into an elemental analyzer. The function of the elemental analyzer is to convert a solid or liquid sample into a gas. The combustion products, $^{13}CO_2$ and $^{13}N_2$, are then transferred via a continuous stream of helium to a gas chromatograph isotope ratio-mass spectrometer. Typically, this is a magnetic sector instrument equipped with Faraday cups for ion detection. The distinguishing features of an IRMS system are described by Brenna et al (59).

Although CF-IRMS is useful for determination of the total label in a sample for mass balance studies, several limitations must still be addressed before this technique sees widespread use. The major limitation is the lack of an interface that can accommodate the effluent directly from an HPLC. The mobile phase from the HPLC must be removed prior to analysis, or the natural abundance of the stable isotopes in the mobile phase will overwhelm the tracer in the drug. A second limitation is the instrument's modest sensitivity, which currently prevents the use of this technique for potent drugs whose concentration in urine, plasma, or feces is below 1 μg/mL.

The third mass spectrometric approach useful for mass balance studies is CRIMS. This method entails desolvating the effluent from an HPLC in a Universal interface and subsequently forming either $^{15}NO$ or $^{13}CO_2$ in a chemical reaction interface (60). The chemical reaction interface is microwave-induced helium plasma, which dissociates the molecules into individual atoms. These atoms are reacted with an excess of a reactant gas, typically $SO_2$, to form NO and $CO_2$ (60). Of the three methods listed above, CRIMS is the only one currently capable of on-line analysis of HPLC fractions. In two experiments comparing CRIMS to liquid scintillation count-

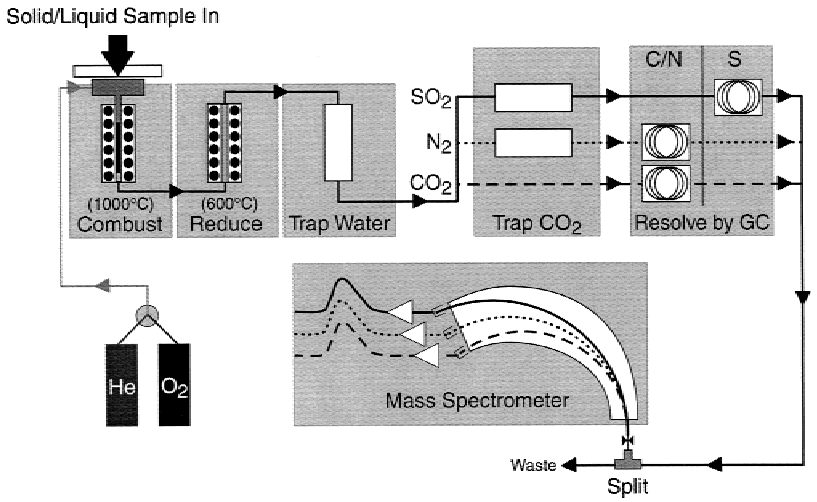


**Fig. 2.** Diagram of continuous flow-isotope ratio mass spectrometer (courtesy of Dr. Steve Brookes at PDZ Europa Limited).

ing and RAM detectors, CRIMS has demonstrated superior detection limits over RAM detectors for metabolite identification, as well as comparable accuracy and precision to liquid scintillation counting of fractions (61–62). Recently, an evaluation of CRIMS for mass balance studies was performed for AZT in urine and feces collected from rats (63). This study demonstrated that mass balance studies could potentially be conducted on NCEs administered at 1–2 mg/kg. This technique is not limited to small molecules, and has revealed the pharmacokinetics of $^{13}C$, $^{15}N$-labeled human growth hormone in rats (64). As with the other two technologies, CRIMS has not yet gained widespread use. Presently, no commercial instruments are available, although Scientific Instrument Services (Ringoes, NJ) plans to offer an instrument in the future. Another problem with CRIMS is the loss of volatile compounds in the interface, limiting the application of the technique to nonvolatile NCEs and their metabolites.

The attractiveness of conducting mass balance studies with these approaches is that only one ion is quantified for every metabolite; hence there is no differential ion formation or detection inefficiencies. Overall, AMS appears the most sensitive, only CRIMS has a liquid interface, and only CF-IRMS has demonstrated a mass balance study in humans. It will be interesting to see which technique will continue to move forward and be accepted by the pharmaceutical industry. The formation of a recent commercial venture, Centre for Biomedical Accelerator Mass Spectrometry Ltd., suggests that AMS will lead the way (55).

## Biomolecules

Mass spectrometry has become indispensable for the elucidation of biomolecular structure, both in the discovery of novel proteins and in the characterization of biotherapeutics for regulatory filings. The demands in discovery research to identify novel therapeutic targets have pushed scientists to identify the least abundant proteins in cells. The analytical challenges placed on identifying proteins expressed at less than 1000 copies per cell have spawned tremendous advances in mass spectrometric techniques. These advances include new combinations of mass analyzers, improved ion sources, more sophisticated computer algorithms, miniaturization of on-line sample introduction methods, and innovative sample handling techniques. Many of the advances pioneered in discovery research have been adopted by those in development who are responsible for the characterization of protein therapeutics. In contrast, the use of mass spectrometry (excluding gas analyzers) has been slow to enter the quality control and manufacturing segments of protein therapeutic development, as demonstrated by the paucity of articles in this realm.

### *Proteomics*

In the discovery arena, technologies for rapidly sequencing electrophoretically separated proteins from various organisms have revolutionized the path to disease target identification. The correlation of DNA databases (genomics) with disease states now relates the functional expression of protein products (proteomics) to stimuli in tissues or disease states. The common approach to proteome identification is to assign a protein sequence to a spot in a high resolution 2-dimensional electrophoretic gel (65–67). As shown in Figure 3, a tissue under one condition and its control under another condition are subjected to 2-dimensional gel electrophoresis. Computer-assisted image analysis matches the spots and compares the amount of protein in each spot. In the past, the spots showing differences in expression level between the two conditions were either electroblotted to a membrane (e.g., nitrocellulose or polyvinylidene difluoride) or were excised; the protein within the band was identified by amino acid analysis or protein microsequencing using Edman chemistry. The slow and expensive Edman sequencing approach, which is problematic for N-terminally blocked proteins, is now mostly replaced by the faster and more sensitive MS-based methods that are not limited by N-terminal blocking. Although the initial investment in mass spectrometry is significant, overall it is a more efficient and less expensive method for identifying proteins than Edman sequencing. As shown in Figure 3, the excised gel piece is first digested *in situ* by proteolytic enzymes, and the peptides are then analyzed by mass spectrometry. Additional sample handling steps can be implemented to deal with the heterogeneity introduced by post-translational

modifications such as glycosylation and phosphorylation (68–69).

Two common mass spectrometric-based approaches are used for proteome analysis. Peptide-mass fingerprinting is the first approach and entails determining the molecular weights of peptides generated from a specific proteolytic or chemical digestion (70). The generated peptides are analyzed either as a mixture directly by mass spectrometry (typically ESI-MS or MALDI-TOF), or are separated by HPLC or capillary-zone electrophoresis (CZE) prior to mass spectrometric detection. The resulting set of peptide masses produce a "fingerprint" of molecular weights unique to the protein of interest. To find the identity of the protein of interest, this experimental data set is then compared to a data set generated from theoretical enzymatic or chemical cleavage of all the entries in DNA and/or protein sequence databases (e.g., EST, GenBank, PIR, SWISS-PROT). Many computer algorithms using this approach have been developed to identify proteins isolated from 2-dimensional gels (71–73). The second approach used for proteome analysis combines MS/MS peptide sequencing strategies with still other computer search algorithms that utilize the data from tandem mass spectra (74–75). MS-based peptide sequencing is typically achieved on either an ESI-triple quadruple or an ion-trap mass analyzer with collision-induced dissociation. The latter mass analyzer has the advantage of greater MS/MS sensitivity in the direct injection mode because of the ability to collect ions in the trap prior to analysis (76). Peptide sequence information can also be generated by "post-source decay" analysis using a MALDI-TOF instrument equipped with a reflectron (77).

Several advances in instrumentation and source design have lowered the limits of detection and increased the mass accuracy of peptide analysis for proteomics. The miniaturization of the electrospray ion source resulted in the development of nanospray (NanoES) (78). NanoES has facilitated the identification of proteins that are present in gels at less than 10 ng and are visualized only by the more sensitive silver staining techniques. Advances in TOF mass analyzer design include the incorporation of both time-lag focussing (also known as "delayed extraction") and reflectrons. These advances have increased both mass resolution (> 10,000) and mass accuracy (<5 ppm) (79). The primary benefit of increased mass accuracy is the elimination of false positives (80). Although not readily interfaced with on-line separation techniques, MALDI-TOF is still a relatively high-throughput technique capable of being automated for unattended protein identification (81–82). In order to further minimize operator involvement, several attempts have been made to automate the MALDI-TOF analysis of chromatographic and electrophoretic separations. Off-line approaches have been developed for either collecting HPLC fractions directly onto polyethylene and Teflon membranes, or collecting CZE peaks directly onto the MALDI probe (83–84). On-line approaches have also been demonstrated allowing direct interfacing of LC separations with MALDI-TOF (85–86). The more recently introduced ESI-TOF instruments combine the advantages of easy interfacing with on-line separation along with increased mass resolution (> 4000) and mass accuracy (<10 ppm) that are advantageous for peptide-mass fingerprinting (87). Miniaturization of the chromatographic techniques have led to the interfacing of capillary columns with inner diameters of 50 to 100 μm to mass analyzers equipped with ESI sources. This size reduction increases the concentration of the eluting peptide peaks, thereby providing protein detection limits from gel slices down to 100 femtomole (88). The major disadvantage of capillaries this narrow is the potential for column blockage. Even lower limits of detection have been achieved by combining solid-phase extraction with CZE (SPE-CZE), where protein detection limits of 400 attomole have been demonstrated (88). Additionally, automated software routines such as "Triple Play" on the ion trap (LCQ system, Finnigan MAT) allow one-step determination of a peptide's mass, isotope profile, and sequence (76). A new combination of tandem mass analyzer is the tandem quadrupole time-of-flight analyzer (Q-TOF) fitted with an electrospray ionization source (89). This configuration provides not only peptide sequencing down to attomole levels, but also has mass accuracy of better than 0.1 Da (89).

The use of 2-dimensional gel electrophoresis for quantification of proteins prior to mass spectrometric identification is time consuming and difficult to automate. Recently, Aebersold and colleagues employed the stable isotope dilution technique to quantify changes in protein expression without the need for 2-dimensional gel electrophoresis (90). This new approach relies on a novel chemical reagent referred to as an isotope-coded affinity tag (ICAT). Biotinylated ICAT reacts covalently with reduced cysteines in all cysteine-containing proteins. The proteins from cells in two different states are labeled with the biotinylated tag. Cells in one state are treated with deuterated ICAT while cells in another state are treated

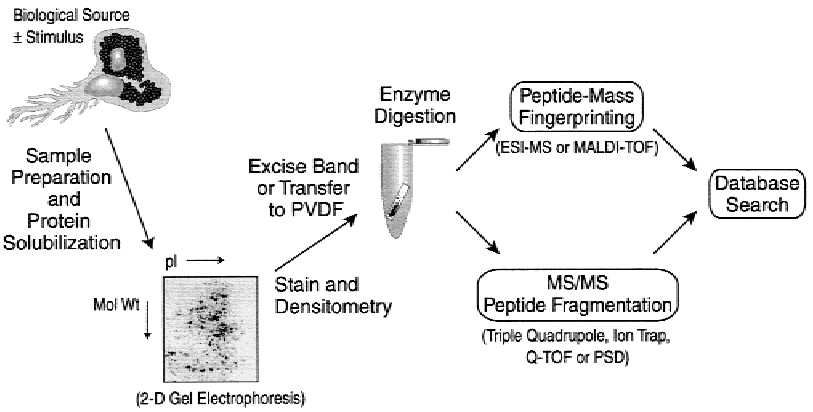


**Fig. 3.** Flow-scheme for mass spectrometric-based proteome discovery.

with the nonisotopically labeled ICAT. The labeled protein extracts are then combined, digested, and the tagged peptides are enriched by capture on an avidin column. Electrospray-MS analysis of the affinity-purified peptides provides the relative abundance in each cell state, with the deuterated samples serving a purpose similar to the internal standard used in the small molecule drug quantification. Aside from being quantitative, the additional advantages of this approach are reduced sample complexity, increased likelihood of detection of low abundance proteins, and reduced contamination from sample handling or from solubilizing agents (e.g., SDS, urea). The primary disadvantage of this approach is that not all proteins contain a free cysteine.

Overall the quest for novel therapeutic targets has triggered tremendous advances in mass spectrometry. The technologies initiated by the academic institutions are now further advanced by the pharmaceutical/biotechnology companies, in collaboration with the instrument manufacturers.

*Characterization*

In the development arena, the thorough characterization of a recombinant protein therapeutic and any associated impurities is requisite for FDA approval. Moreover, by demonstrating that a complex biotechnology-derived product is a "Well Characterized Biological" can accelerate the approval of new manufacturing processes or new production facilities (91). Mass spectrometry plays a significant role in this realm not only by confirming the mass of the intact purified protein, but also by characterizing the desired product impurities and variants such as post-translational modifications. Comprehensive characterization of the product is achieved by combining mass spectrometry with orthogonal separation techniques, such as HPLC and CE, to analyze the intact product and its proteolytic fragments (91–93).

There are several different types of mass analyzers used to determine the molecular weight of the intact protein product and its variants. Most of these mass analyzers require ESI to form multiply-charged species that fall within the instruments' mass/charge range. Both quadrupole and ion trap mass analyzers can achieve an upper mass/charge range of 4000 amu, and are capable of determining the molecular weight of electrosprayed-proteins as large as antibodies with 30 ppm mass accuracy. The newer ESI-TOF mass analyzers with greater resolving power and mass/charge range (~10,000) have the potential to detect the complete charge state envelope of glycoproteins, and therefore more accurately determine the molecular weight of an intact antibody and the relative amounts of the various glycoforms. Although MALDI-TOF generates predominately singly-charged molecular ions, it is still useful for previewing the mass of the intact protein because of the unlimited mass range of the TOF mass analyzer. An additional advantage of MALDI-TOF is its tolerance to moderate levels of salt or detergent present in the sample.

The complete structural characterization of a recombinant protein therapeutic entails enzymatic or chemical digestion followed by peptide mapping. Peptide mapping by mass spectrometry involves assigning a peptide from the digest to a chromatographic or electrophoretic peak based on its mass/charge. The identity of the peptide can be confirmed by MS/MS sequencing. As indicated previously, peptide identification is accomplished faster by mass spectrometry than by either Edman sequencing or amino acid analysis. Furthermore, the second dimension of separation provided by mass spectrometry usually allows identification of co-eluting peptides. Prior to peptide mapping, MALDI-TOF analysis of the whole digestion mixture can be used to evaluate the completeness of the enzymatic digestion. Following digestion, HPLC or CE peaks are characterized on-line by HPLC-ESI-MS (94-95), and off-line by MALDI-TOF (92–93). Since significant quantities of the protein are generally available, mass accuracy, not sensitivity, is the most important attribute of the mass spectrometer for peptide mapping. While the ESI-triple quadrupole instrument is the workhorse for characterizing the peptide map, other mass analyzers with better mass accuracy may eventually replace the triple quadrupole. Recent advances that have improved mass accuracy include the development and commercialization of ESI-TOF mass spectrometers, the re-introduction of delayed extraction and reflectrons for MALDI-TOF (77,79), and advances in ion-trap technology (e.g., ZoomScan) (95). With a mass accuracy of less than 5 ppm, differentiation of peptides that contain the isobaric amino acids glutamine or lysine can now be accomplished (96). Furthermore, with this type of mass accuracy, a one mass unit difference caused by deamidation can be unequivocally identified.

Accelerating the peptide mapping process is desirable and can be realized by several approaches. One approach is to use immobilized proteolytic enzymes (e.g., trypsin, endoproteinase Glu-C, etc.) combined with HPLC-ESI-MS to automate the procedure (97). A second approach to rapidly interpret peptide maps and identify sites of post-translational modifications enlists the use of computer algorithms first developed for peptide-mass fingerprinting (71–73).

A strength of mass spectrometry, not readily enjoyed by either Edman sequencing or amino acid analysis is the ability to locate and characterize post-translational modifications occurring in recombinant protein therapeutics. These modifications are usually introduced by the cellular machinery and include glycosylation (98–99), sulfation (100), phosphorylation, and disulfide bond formation (101). Other modifications that can be readily characterized by mass spectrometry are those that occur during purification and storage of the product (e.g., oxidation and deamidation (102), racemization (103), glycation (104)), or by chemical reactions to improve the pharmacokinetics (e.g., pegylation (105)). The identification of the cysteines involved in disulfide bond formation, and the characterization of N- and O-linked glycosylation sites are the most challenging tasks encountered in recombinant protein therapeutic characterization. These two challenging areas have led to development of many innovative strategies for their characterization.

The most common approach used for disulfide mapping entails enzymatic digestion without a preceding reduction and alkylation step (106). The peptides thus formed are then analyzed by mass spectrometry to determine which cysteines are involved in the formation of disulfide bonds. A limitation of this method is that some proteins are resistant to proteolysis if they have not undergone reduction and alkylation (107). Furthermore, some cysteines are located adjacent to one another so that enzymatic cleavage does not occur between them, and as a result, the identification of the cysteine involved in disulfide bond formation remains ambiguous. Wat-

son and colleagues have combined protein chemistry with mass spectrometry to develop a method capable of identifying disulfide bonds occurring in the most challenging positions (108).

Characterization of glycosylated proteins presents a significant analytical challenge due to the tremendous complexity presented by oligosaccharides. This complexity has fostered the development of many creative mass spectrometric-based approaches. Electrospray ionization in conjunction with either an ion trap or a triple quadrupole mass analyzer is the system of choice. The most common approach utilizing this instrument configuration requires that the skimmer-to-nozzle voltage in the ESI interface is elevated for a fraction of the instrument scan time to induce in-source fragmentation and produce diagnostic carbohdydrate ions. This approach identifies the peptides that contain oligosaccharides. The sequence of glycoforms present on the peptide are then characterized by MS/MS techniques (98). The complexity of the problems encountered in thoroughly characterizing glycoproteins is demonstrated by the three-dimensional separation necessary to provide a detailed characterization of the glycans on tissue plasminogen activator (93). After the glycans have been thoroughly characterized for one batch of protein, analytical methods are still needed to monitor subsequent lots for changes in the glycoform distribution. MALDI-TOF can be used to assess the changes in glycans that can occur during process recovery. MALDI-TOF was shown to provide quantitatively similar results to the slower, but well-established, method of high pH anion-exchange chromatography with pulsed amperometric detection (109). Figure 4 demonstrates the type of data readily available by the MALDI-TOF approach, following enzymatic release of the N-linked glycans.

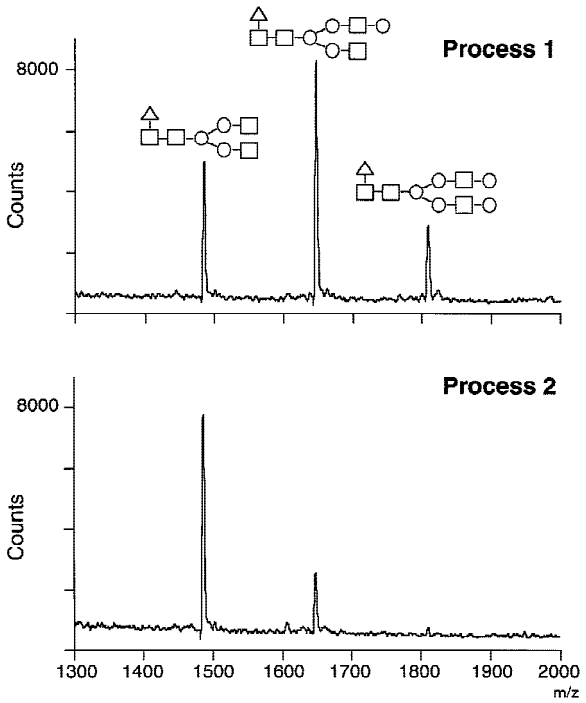


**Fig. 4.** Positive-ion MALDI-TOF mass spectra of glycans released enzymatically from a glycoprotein expressed under two cell culture conditions. The symbols represent deoxyhexose (□); Hexose (△); N-acetylhexosamine (○).

Figure 4 emphasizes how MALDI-TOF can be used to evaluate the impact of changing cell culture conditions on the carbohydrates expressed on an antibody. Quantitative mass spectrometry in such cases is invaluable to improving cell culture conditions in development before scaling up in manufacturing.

*Manufacturing, In-Process Monitoring, and Quality Control*

The development of user-friendly mass spectrometers with easy-to-use data analysis software has resulted in the penetration of this technology into both manufacturing and quality control settings. Monitoring the impact of process parameters on product titer and heterogeneity is key to optimization of the manufacturing process for a biotechnology-derived product. Moreover, after an optimal process has been established, it becomes critical to demonstrate consistency in product titer and quality in all manufacturing runs. The most prevalent approach to demonstrating product quality and titer involves labor-intensive sample preparation (e.g., isolation of the product from cell culture media) and time-consuming development of HPLC- or CE- based methods to resolve and quantify the variants. Mass spectrometric techniques could accelerate such tasks because of the sensitivity and selectivity that often precludes the need for extensive sample preparation.

During the development phase, the identity and stability of biotechnology-derived products are routinely confirmed by mass spectrometry. These same techniques are just beginning to be used in the manufacturing process, and only a few examples exist. In one example, product identity for six different antibodies produced in a common manufacturing facility were unambiguously determined by ESI-MS, based on the mass/charge of both their light and heavy chains following reduction (110). In yet another example, MALDI-TOF was enlisted to monitor the stability of an antibody in response to altering animal cell culture processes. The results provided by MALDI-TOF were qualitatively similar to data obtained by SDS-CE, but with added molecular weight information that allowed identification of degradation components (e.g., antibody lacking a light chain) (111). MALDI-TOF is well suited for such analyses because of its tolerance for salts and other impurities. In the QC setting, MALDI-TOF of tryptic digests has been used as an identity test and for lot-to-lot consistency testing of recombinant protein therapeutics (112). Though mass spectrometry does not give quantitative data on the content of impurities in a product, comparability of a production lot with a reference lot can be evaluated by standardizing sample preparation methods and mass spectrometer settings. Recently, the use of mass spectrometry for quality control of drug products has gained the attention of regulatory agencies (113).

ESI-quadrupole mass spectrometer operated in the selective-ion monitoring mode has been used for faster in-process assessment of known product-related variants, without requiring prior sample purification or enzymatic digestion (114). The distribution of recombinant antibody glycoforms expressed during early cell culture production processes were assessed, where titers were too low for purification and elaborate sample treatments. A RP-HPLC method maximized recovery, ionization efficiency, and resolution of the light- and heavy-chain variants from the host cell proteins. Direct MS

analysis then provided a considerably faster, and less sample- or time-consuming, approach for glycoform monitoring than CE- or MALDI-based methods which required enzymatic digestion (114).

Real time monitoring of the product by the ESI-quadrupole MS on-line with a chromatographic purification process could potentially be used for efficient pooling strategies during large-scale purification. The application would depend on the reliable ionization of the product from complex mixtures with varying levels of impurities. This might be overcome by inclusion of internal standards, much the same way as in small molecule quantification. Future implementation of more accurate and sensitive techniques such as ESI-TOF should permit the speed necessary for in-process monitoring of product quality.

## SUMMARY

Mass spectrometry has significantly altered how the pharmaceutical and the biotechnology industries discover new therapeutics, and develop them into safe and marketable

**Table 1.** Summary of Both the Routine and the Novel Mass Spectrometric-Based Methods Used in the Pharmaceutical and the Biotechnology Industries. The Requirements Listed Here Describe the Desirable Properties for the Particular Application. The MS-Based Methods Listed Meet Some, but Not Necessarily All, of Those Requirements

| Application | Requirements | MS-Based Methods |
|---|---|---|
| **Combinatorial Library Generation** | | |
| Verify identity and determine purity | Rapid; Automated; Mass accuracy < 10 ppm; Universal detection | –Flow injection analysis MS (FIA-MS)<br>–Parallel multiple column with on-line MS<br>–Ultra-fast HPLC-ESI/TOF<br>–Supercritical fluid chromatography MS (SFC-MS)<br>–Parallel evaporative light-scattering and MS detection |
| Purify compound | Rapid; Automated; Reliable | –Dual column m/z-triggered fraction collection |
| **High-Throughput Screening** | | |
| Screen combinatorial libraries for activity | Simultaneous analysis of mixtures; Predictive of receptor/drug interactions; Minimal use of target; Structurally informative (MS/MS capabilities) | –Affinity capillary electrophoresis MS (ACE-MS)<br>–Pulsed ultra-filtration MS (PUF-MS)<br>–Affinity MALDI-TOF<br>–Library affinity selectoin MS (LAS-MS) |
| **ADME** | | |
| Determine PK parameters in animal models | Rapid; Quantitative; Low detection limits; High selectivity | –On-line SPE or 96-well disc extraction plus column switching for sample concentration plus HPLC/MS/MS for quantification.<br>–Cassette dosing or plasma sample pooling<br>–CE-MS for direct urine or plasma analysis |
| In vitro screens for permeability and metabolism | Rapid; Quantitative; High selectivity | –On-line SPE or 96-well disc extraction plus column switching for sample concentration plus HPLC/MS |
| Metabolite identification | High sensitivity; Capable of $MS^n$ ($n \geq 2$); Mass accuracy < 5ppm | –ESI-triple quadrupole, ESI/Q-TOF, ESI/ion trap<br>–Intelligent software (INTAMS) |
| Mass balance | High sensitivity; High selectivty; Universal detection | –Chemical reaction interface MS (CRIMS)<br>–Accelerator mass spectrometry (AMS)<br>–Continuous flow-isotope ratio MS (CF-IRMS) |
| **Biomolecules** | | |
| Proteomics | Rapid; Automatable; Femtomole sensitivity; Mass accuracy <10 ppm; MS/MS capabilities | –MALDI-TOF of intact or enzyme digestion mixture<br>–Capillary LC with nano-spary interfaced to MS/MS for sequencing (triple quadrupole, ion-trap, or Q-TOF detector)<br>–CZE or SPE-CZE coupled to ESI/MS/MS |
| Variant and degradation product characterization | Tolerate salts and buffers; High resolution; Mass accuracy <200 ppm; MS/MS capabilities | –MALDI of enzymatic digestion mixture<br>–On-line HPLC ESI-MS/MS for sequencing |
| QC ID testing; Lot-to-lot consistency | High selectivity; Simple and reproducible | –MALDI-TOF of enzymatic digestion mixture |
| In-process monitoring | Rapid; High selectivity; Semi-quantitative; Tolerate salts and buffers | –On-line HPLC-ESI/MS with quadrupole or TOF detection |

Abbreviations: CZE, capillary zone electrophoresis; ESI, electrospray ionization; ID, identity; MALDI, matrix-assisted laser desorption and ionization; SPE, solid-phase extraction; QC, quality control; TOF, time-of-flight mass analyzer; Q-TOF, tandem quadrupole and time-of-flight mass analyzer.

drugs. Table 1 summarizes the MS-based methods described in this review and how they address the industry's needs.

In the realm of small-molecule drug discovery, innovations in MS source design, improved mass accuracy, and automation have accelerated the purification and characterization of compounds derived from combinatorial libraries to thousands per month. The effect of mass spectrometry on high-throughput bioactivity screening remains to be seen. The new genre of mass spectrometers has increased the throughput in pharmacokinetics and metabolism studies of NCEs. The high sensitivity of AMS is enabling the determination of the *in vivo* metabolic fate of drugs with minimal exposure to radioactivity. Another potential means to conduct mass balance studies more safely is offered by the use of stable isotopes in conjunction with either CRIMS or CF-IRMS.

In the biotechnology arena, the road from the genome to the proteome has been paved by nano-technologies, data mining, and bioinformatics in conjunction with high sensitivity mass spectrometric techniques such as MALDI-TOF and ESI-Q-TOF. The accuracy, sensitivity, ruggedness, and ease-of-use of the current mass spectrometers has led to the penetration of MS-based technologies into most development sectors of the biotechnology industry. As instrument cost and size come down and reliability increases, mass spectrometers will eventually become an invaluable tool for routine monitoring of the manufacturing process, and in the quality control of drug products.

On the horizon are the affinity-based methods which, coupled to mass spectrometry, promise rapid and selective identification of both synthetic and biological molecules. In the latter case, protein array chips have been developed by Ciphergen that contain covalently linked receptor or antibody target molecules to selectively capture compounds from crude biological mixtures and to quantify their changes in response to pathological or biochemical conditions using MALDI-TOF (115). The next generation of technologies that will simplify the investigation of the complex fate of novel pharmaceutical entities *in vitro* and *in vivo* are chip-based approaches coupled with mass spectrometry. Many companies including Caliper and Microfluidics have now designed microfabricated modules that use electro-osmotic flow to conduct assays and perform chemical reactions on a chip (116). These microfabricated devices have also been sensitive enough to support proteome research by generating gradient separations at nL/min prior to ESI-MS detection (88). Microfabrication promises to impact the bioanalytical field much as microchips have revolutionized the electronics and computer industry.

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