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Transformations in pharmaceutical research and development, driven by innovations in multidimensional mass spectrometry-based technologies

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Abstract

In the pharmaceutical industry, there is a tremendous need for qualitative and quantitative analysis of target analytes such as peptides, proteins, drugs, metabolites, biomarkers, impurities, and degradation products in various mixtures including synthetic reactions, in vitro cultures, biological fluids, drug substances, finished products, and many others. To provide adequate specificity for analysis in these complex mixtures, multidimensional analytical techniques are required. Mass spectrometry plays a central role in many of these multidimensional approaches to mixture analysis because it provides an unparalleled combination of sensitivity and specificity that is useful for both molecular identification and quantitative applications. Recent innovations in mass spectrometry and industrial implementation of these advances have transformed many aspects of pharmaceutical research and development. Data that were previously unattainable, or were not collected due to exorbitant cost or time constraints, can now be obtained using mass spectrometry-based technologies. The impact of these innovations has been most dramatically felt in early stages of discovery, as more data are available to make critical decisions, such as selecting compounds for advancement to costly preclinical and clinical trials. New MS technologies have also accelerated the progression of drug candidates through development and toward regulatory approval. Here, five major categories of pharmaceutical applications of mass spectrometry are reviewed. They are new chemical entity characterization, biomacromolecule characterization, bioanalytical quantitation, metabolite identification, and impurity and degradation product identification. A brief historical perspective and evolution of technologies for each application area are presented. Those discussions are followed with a description of the current strategies for implementation of the tremendous capabilities of the state-of-the-art approaches, along with representative applications. In addition, emerging technologies for each application area are presented to indicate the future directions of instrumentation for mixture analysis in the pharmaceutical industry. (Int J Mass Spectrom 212 (2001) 135–196) © 2001 Elsevier Science B.V.

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1. Introduction

Early applications of mass spectrometry, such as analysis of petrochemicals in the 1950s [1], utilized the sensitivity and selectivity of the techniques available during that period. It was apparent that even a single stage of mass analysis provided a great deal of

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Dedicated to R. Graham Cooks on the occasion of his sixtieth birthday.

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information including molecular weight and, when using electron ionization (EI), structurally diagnostic fragmentation data. In addition, these data could be collected with relatively low levels of analyte, as compared to alternative analytical approaches. However, single-stage mass analysis provided only one dimension of information and was not generally useful for analysis of target components within complex mixtures. By combining mass spectrometry with an added dimension provided by on-line chromatography, more comprehensive data on mixtures could be obtained from a single experiment. However, during the 1960s and 1970s, introduction of liquid chromatography (LC) effluents into the high-vacuum environment of the mass spectrometer was considered impractical and early packed-column gas chromotography mass spectrometry (GCMS), which employed coated stationary phases, had issues that limited its utility for mixture analysis [2–4]. For example, column bleed and instability at high temperatures contributed to chemical noise, compromising detection limits. Even with the advent of capillary GC, employing covalently bound stationary phases [5,6], the applicability of GCMS was limited to analytes that could be vaporized and remained chemically stable at temperatures up to 300 °C.

In the late 1970s, Cooks and co-workers first reported the addition of a second stage of mass analysis as an alternative second analytical dimension for mixture analysis [7,8]. In this tandem mass spectrometry (MS/MS) approach, samples were directly introduced by way of a probe into a mass-analyzed ion kinetic energy spectrometer to identify and, potentially, quantify target compounds in complex matrices. By mass selecting a precursor ion and using the product ion spectrum for identification or quantitation, they reported a significant reduction in chemical noise that resulted in detection limits as low as 10 pg for certain samples. Through a series of manuscripts it became increasingly apparent that a second stage of mass analysis enabled mass spectrometry to become particularly useful for identification and quantitation of low-level target compounds in mixtures [9–16].

Since the first reports on the promise of MS/MS for mixture analysis, many advancements have occurred in three major technology categories: (A) sample introduction and ionization techniques; (B) mass analyzers; and (C) application-specific peripherals. The driver for these developments has largely been a desire to enhance the utility and extend applicability of mass spectrometry-based techniques to a wide array of mixture analysis problems. In fact, recent innovations have increasingly resulted from the direct efforts (or under the sponsorship) of instrument manufacturers, who compete to deliver the most versatile and user-friendly mass spectrometry-based tools, primarily targeted for industrial mixture analysis applications. In reflecting on the above-cited categories of progress, it seems reasonable to generalize that category-A developments largely broadened the range of compound classes and mixture types amenable to mass spectrometry-based analysis and/or provided increased sensitivity; category-B developments (e.g. mass range, spectral resolution, stages of mass analysis) most importantly increased the depth and overall informing power of data accessible through a given experiment; and category-C developments (e.g. automation hardware and software) provided the user-friendly means of accessing the power and versatility of the mass spectrometry-based hardware, which has been essential in streamlining experimental setup, minimizing analysis and data reduction time, and aiding in data interpretation and reporting.

Probe-based sample introduction, combined with EI or chemical ionization (CI), is applicable only to analytes that are stable throughout the associated thermal vaporization and relatively harsh ionization processes. The availability of softer desorption ionization techniques, such as secondary ion mass spectrometry (SIMS) [17], fast atom bombardment (FAB) [18], and laser desorption (LD) [19], beginning in the early 1980s, extended applicability to larger, thermally labile compounds. Later, the development and refinement of matrix-assisted laser desorption ionization (MALDI) [20,21] further broadened the range of applications by providing enhanced ionization efficiency, relatively low chemical background, resistance to sample matrix effects, and superior reproducibility for this static experiment.

Over time, it became apparent that (even with

MS/MS detection) most mixture analysis applications benefit from the added dimension of on-line chromatography. The chromatographic resolving power of capillary GC makes it an attractive complement to mass spectrometry. However, as utilized with EI or CI, it shares the same compound class limitations as the probe-EI/CI experiment. Creative chemical derivatization protocols have certainly extended the applicability of GC, but the more general solution has been the development of practical LCMS interfaces [22]. Moving belt [23,24] and particle beam (PB) [25] interfaces proved to be an effective means of addressing the desolvation quandary presented by the atmospheric pressure liquid to high vacuum differential in LCMS. Unfortunately, these techniques continued to include thermal vaporization processes associated with EI or CI, and were also only of limited use for low-boiling-point analytes. The advent of thermospray (TS) [26] represented the first practical use of a truly soft ionization mechanism with an LCMS configuration. Continuous-flow FAB [27], originally developed to alleviate difficulties associated with high concentrations of nonvolatile matrices used in FAB, became a second soft ionization option for low-flow LCMS experiments. Both techniques expanded the utility of LCMS to encompass an array of nonvolatile and thermally labile compounds. However, neither approach enjoyed widespread popularity, given only modest sensitivity and relatively high chemical background for most applications, as well as challenges in optimizing experimental set-up, which resulted in day-to-day and lab-to-lab reproducibility issues.

Refinement of atmospheric pressure ionization (API) techniques, including APCI [28] and electrospray ionization (ESI) [29,30], has resulted in an explosion in the use of LCMS for mixture analysis, owing to their high sensitivity, ruggedness, and, collectively, nearly universal compound class coverage. Although APCI requires some degree of analyte volatility, and can cause in-source fragmentation or degradation of labile compounds, it has proven to be more sensitive and robust than any of the aforementioned LCMS options. Of all LCMS interface types, ESI has become the most versatile and widely employed. It is also the softest of ionization techniques, typically yielding minimal in-source fragmentation, even for the most fragile of compounds, and is exquisitely sensitive for polar analytes. Further, due to its ability to produce multiply charged pseudomolecular ions, highly functionalized compounds up to 200 kDa can be routinely detected within the *m/z* range of most modern mass analyzers.

A variety of mass analyzers have evolved to deliver greatly improved performance, often in a manner that complemented advances in ion source technologies [31,32]. The commercial availability of reliable triple quadrupole mass spectrometers was arguably the most important step toward the widespread deployment of the MS/MS experiment for mixture analysis. Initially available with GC and various probe inlets, these instruments delivered unit resolution in both mass analyzers, along with relatively efficient fragment ion formation by way of collision-induced dissociation (CID). Such systems provided easy access to the three basic types of MS/MS experiments [12]: precursor (or parent) ion scan; constant neutral loss scan, useful in screening complex mixtures for compounds of known fragmentation characteristics; and product (or daughter) ion scan, invaluable for determination of unknown structures. Selected reaction monitoring (SRM), a special case of the product ion scan, has become the preferred mode for target compound quantitation.

Time-of-flight (TOF) mass analyzers are useful in qualitative applications, particularly in conjunction with analyses requiring a high *m/z* range, high speed, or possessing a low duty cycle [33]. For example, an ability to detect an entire mass spectrum for each laser pulse makes TOF the analyzer of choice for MALDI applications and spectral acquisition rates of up to thousands per second make TOF ideal for monitoring fast separations. Recent advances in TOF analyzer design, especially refinement of reflectron ion focusing technology [34] and the availability of transient digitizers with gigahertz response rates, have greatly enhanced their utility by providing mass resolution in excess of 10 000 and stability sufficient for accurate mass measurements approaching the capabilities of magnetic sector instruments. In addition, refinement of delayed ion extraction (or time-lag focusing) tech-

niques has been key to achieving this resolving power for MALDI applications, as it corrects for the kinetic energy distribution of ions caused by the laser-desorption process [35,36]. Crude MS/MS experiments can be carried out on commercially available MALDI-TOF instruments, using the postsource decay (PSD) technique [37].

The hybridization of quadrupole and TOF mass analyzers (Qq-TOF) was first achieved in 1994 [38,39]. This instrument employs a quadrupole for mass selection and a second quadrupole for collisional activation, analogous to a triple quadrupole. However, instead of a third quadrupole, an orthogonal TOF analyzer is used for the final stage of mass analysis. This instrument is capable of generating mass spectra and product ion spectra displaying superior sensitivity and resolution. As such, elemental composition of product ions can be determined, greatly aiding in spectral interpretation and structure assignment. Commercially available versions of Qq-TOF instruments can be used with MALDI, ESI, or APCI sources for ionization of a wide range of analytes.

Relatively small in both size and price, quadrupole ion trap mass analyzers (ITMS) provide high sensitivity in the full scan and product ion scan modes [40]. Certain API-interfaced ITMS systems also offer $(MS)^n$ capabilities [41,42] that may be of value in solving particularly challenging mixture characterization problems. The Fourier-transform ion cyclotron resonance mass spectrometer (FTICRMS) is another type of ion trap device. Although large, costly, and expertise intensive, this research-grade alternative is now finding applications in molecular characterization due to a combination of $(MS)^n$ capabilities, excellent mass accuracy, and achievable resolutions commonly exceeding 50 000 [43].

The advances in ionization techniques and mass analyzers have been complemented by an increase in the level of automation and instrument control, which was largely driven by the rapid evolution in computer hardware and software. In addition, advances in data mining algorithms and storage capabilities have added considerable value. This use of information technology also resulted in a decreasing degree of human intervention by allowing automated instrument optimization and sample injection, as well as unattended data acquisition for batch runs and, in many cases, automated reduction of data. These advances have also made the instrumentation more user-friendly and, as a result, the mass spectrometry-based techniques have seen much broader deployment and increased usage in recent years.

In considering the above key advances, as applicable to mixture analysis, it is worth noting that some have resulted in obsolescing older approaches. For example, MALDI has superseded SIMS and FAB for most static-sample mass spectrometry applications and API-based LCMS interfaces have replaced moving belt, thermospray, continuous-flow FAB, and, largely, particle beam. Conversely, a great many of these advances simply added complementary tools to the arsenal of the mass spectrometrist or (especially with application-specific peripherals) enhanced the value of mass spectrometry techniques already available.

It is our belief that, over the past 25 years, the remarkable advances in sample introduction and ionization techniques, mass analyzer instrumentation, and mass spectrometry-associated computer technology, have been felt most strongly in the pharmaceutical industry. Supporting this view is the observation that, until the 1980s, mass spectrometry was a highly expertise-intensive tool in pharmaceuticals, primarily employed as a complement to other qualitative analytical techniques and rarely used for quantitative analysis. Today, mass spectrometry is woven into the fabric of literally all aspects of drug discovery and development and is now of equal importance for quantitative measurements as it is for molecular characterization applications. An example of this trend for widespread usage is the increase in the number of mass spectrometers within Procter & Gamble's Health Care organizations. In 1986, a total of only 4 major mass spectrometers were utilized for health care applications and by mid-2001, that number has grown to 44.

In net, the availability and appropriate implementation of these mass spectrometry-based tools have driven the trend to learn more about properties of new chemical entities at a much earlier stage of drug

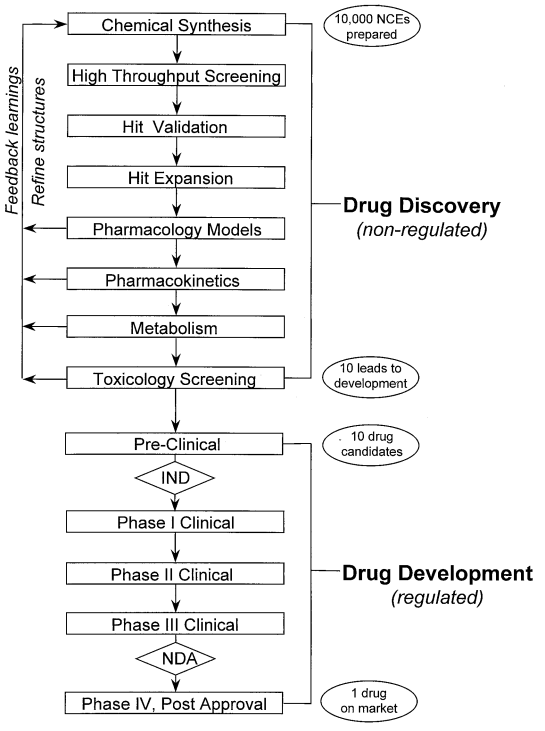

Fig. 1. General overview of the stages of drug discovery and development.

discovery, permitting more rapid and predictive assessments of their ultimate value as therapeutic agents [44]. Moreover, the power and speed of these technologies have played a major role in reducing the time required to progress a drug to market. The importance of mass spectrometry to the pharmaceutical industry has become so great and so widely recognized that it is even influencing the Wall Street community. Portfolio managers are recommending the stocks of mass spectrometry manufacturers because mass spectrometry has become "the leading analytical instrument in drug discovery" [45]. In addition, biotech companies with particularly strong mass spectrometry capabilities are perceived as having a significant advantage over competition, "What I find appealing . . . is their use of mass spectrometry. It's really powerful and qualitative, and they seem to have a lead in that area" [46].

Although the flow diagram provided in Fig. 1 is a simplified depiction of the drug discovery and development processes, it serves as a general indication of the great diversity of activity types that require mass spectrometry support. Milestones and submission documents used in the USA are provided here as an example, although these differ to some degree in other countries around the globe. Drug discovery is illustrated as having a feedback loop due to the iterative efforts to incorporate learnings from in vitro and in vivo studies into the synthesis of next generation compounds. Development is illustrated as a linear process, although it also benefits from learnings that can be reapplied from other stages, other projects or external sources. However, this process is not as highly integrated and rapid as it is for drug discovery.

More so than in any other industry, a major challenge in pharmaceuticals is to properly match the specialized needs presented in each of these areas with the most appropriate combination of MS-based tools to efficiently address the scientific needs. Implicit in this is the practical necessity to appropriately balance the capital costs, people resources, and analysis turnaround requirements for each category of applications. Thus, the successful implementation of mass spectrometry strategies to support each aspect of pharmaceutical research and development requires both an in-depth knowledge of the needs within each major business area and each stage of the drug discovery and development process, as well as familiarity with the now-extensive arsenal of associated mass spectrometry-based instrumentation. For this reason, expertise is often developed within various groups to specialize in given application types.

In this article, an overview of the impact of mass spectrometry on the pharmaceutical industry is provided by highlighting five major application categories: new chemical entity (NCE) characterization; biomacromolecule (BMM) characterization; bioanalytical quantitation; metabolite identification; and impurity and degradation product identification. Table 1 provides a general overview of the analytical challenges presented within each of these application areas. Each corresponding section contains background information on the pharmaceutical significance, followed by a brief history, and current strategies for implementation of state-of-the-art mass

Table 1
General overview of the major analytical challenges impacted by mass spectrometry in drug discovery and development

Activity	Type of data	Mixture type	Adequate or current typical* sensitivity	Current throughput capability (/MS/day)	Regulated?
NCE characterization					
Open-access	M.W., verified structure, purity	discrete NCEs + impurities	ppm	10s–100s	No
Combinatorial chemistry	M.W., verified structure	equimolar NCE mixtures	ppm	10s–100s	No
HTOS	M.W., verified structure	discrete NCEs	ppm	1000s	No
HTOS	M.W., verified structure, purity	discrete NCEs + impurities	ppm	100s	No
Purification	M.W., purity	reaction products	ppm–ppth	10s–100s	No
BMM characterization					
Protein modification	position and modification	digested proteins	fmol*	1s	No
Structural biology	M.W. and 2D, 3D structure	pure proteins	nmol	1s	No
Proteomics	protein identification	peptide mixtures	fmol*	100s	No
Bioanalytical quantitation					
Discovery	quantitative	in vitro, in vivo biomatrices	ppt–ppb*	100s	No
Development	quantitative	in vivo biomatrices	ppt–ppb*	100s	Yes
Metabolite identification					
Discovery	proposed structure	in vitro, in vivo biomatrices	ppb–ppm*	1s	No
Development	confirmed structure	in vivo biomatrices	ppb–ppm*	1s	Yes
Impurity identification					
Process impurities	confirmed structure	drug substances	ppb–ppm	1s	Yes
Degradation products	confirmed structure	drug products	ppb–ppm	1s	Yes

spectrometry capabilities now available for industrial drug discovery and development. In addition, future directions and emerging technologies are discussed. Due to length considerations, a comprehensive review is not possible for all sections. However, key advances and selected examples are provided that illustrate the tremendous impact of modern mass spectrometry within the pharmaceutical industry.

2. New chemical entity characterization

2.1. Pharmaceutical significance

The drug discovery process typically begins with the design and synthesis of NCEs by medicinal chemists, for testing in a variety of in vitro and in vivo biological models and screens. Structural characterization and purity assessments are obtained at various points throughout the synthesis and compound storage processes to assure the quality of the test compounds prepared and, therefore, the validity of conclusions drawn from subsequent biological screens. By obtaining this information on key synthetic intermediates and final products, the speed and certainty of multistep synthesis and purification processes are enhanced. Recharacterization of NCEs, either after a period of storage or postbiological testing, provides assurance of adequate stability and further supports the validity of the screening data. Thus, in drug discovery, the need for NCE characterization is driven more by practical work process and business considerations, rather than by requirements of regulatory agencies. For this reason, the speed of obtaining such data is generally more important than the completeness of the NCE characterization package, provided there is a good probability that the scientific conclusions are correct.

Over the past decade, there have been radical shifts in the strategies for producing and testing NCEs, with the aim of increasing the number of compounds being evaluated per therapeutic indication. The desire to increase NCE evaluation rates is driven by a statistical

numbers game, as implied in Fig. 1. Typically, tens of thousands of compounds must be synthesized and tested, for every drug that is eventually marketed. The theory is, therefore, that by increasing the rate of NCE synthesis and screening, a greater number of high quality drugs will be discovered and developed. The ability to deliver this higher throughput has been fueled by innovations in automation and robotics technology, which have been implemented for both compound synthesis and biological screening. Thus, the rapidly shifting strategies have come about through the need to properly balance the NCE production rate with the throughput of the biological screens. The balance at any given time will have a great effect on the analytical strategy employed for NCE characterization, as implied below.

The recent development of combinatorial chemistry capabilities provides ready access to a much broader range of molecular diversity for study in drug discovery [47,48]. Following its origins in the successful development of solid-phase peptide synthesis in the 1980s [49–51], combinatorial chemistry has rapidly evolved and is now regarded as an essential component of drug discovery strategies throughout the pharmaceutical industry. The introduction of associated automation tools for peptide synthesis and "split and mix" pooling strategies [52–54], for the simultaneous synthesis of large numbers of peptide and peptidomimetic mixtures, also spurred continuing refinement of associated synthetic strategies and enabling instrumentation. This facilitated the implementation of automated parallel high-throughput organic synthesis (HTOS) [55]. For the present discussion, the term HTOS is associated with the preparation of arrays of discrete small organic molecules, while combinatorial chemistry relates to the production of large (usually equimolar) mixtures of small organic molecules. Both approaches yield many NCEs, but only in quantities intended for in vitro screening (typically 1–20 mg total).

Through the mid-1990s, in vitro biological screens were considered low to moderate throughput, by today's standards. Thus, strategies to test compounds in mixtures (originating from natural products, postsynthesis pooling of NCEs, and/or combinatorial chemistry) grew in popularity, as a means of evaluating the greatest number of NCEs. More recently, true high-throughput screening (HTS) assays, capable of performing first-tier evaluations for tens of thousands of compounds per month, have become available for a growing number of therapeutic areas. Where available, HTS capabilities have encouraged a trend away from the preparation of combinatorial mixtures and toward the use of HTOS. This alternative offers many advantages in the interpretation of biological screening results. For example, complex deconvolution protocols are unnecessary to identify the primary active. True quantitative structure-activity relationship (QSAR) models can also be developed because the degree of binding or functional activity is measured for each discrete NCE. Finally, by screening NCEs, the potential for generating misleading biological results (due to, e.g. the presence of antagonists and agonists, or the superposition of multiple weakly active compounds, in the same mixture) is eliminated.

Whether the NCEs originate from combinatorial chemistry or HTOS, drug discovery generally proceeds according to the outline in Fig. 1. Test substances showing the most potential, based on HTS, are designated "hits." These hits are next taken through a hit validation process, which first involves characterization of the test substance to verify the presence of the intended NCE or (in the case of mixtures) deconvolution of biological and analytical data to determine the structure of the NCE responsible for the observed activity. The identified hit is resynthesized, usually on a larger scale (tens or hundreds of milligrams) by using more traditional organic chemistry techniques, purified and characterized, and then retested in the in vitro screens. If this newly prepared compound demonstrates sufficient activity to account for the original HTS results, the hit is termed validated. This validated hit is not considered a lead compound but, rather, serves as a starting point for hit expansion. In this process, conventional medicinal chemistry or HTOS is employed to prepare many related compounds, which will lead to NCEs with optimized biological activity. The most promising of these NCEs are progressed, as potential lead compounds, for evalua-

tion in more-refined in vitro and in vivo pharmacological models. Regardless of the precise early drug discovery tact, tremendous challenges are presented by the fast pace, large number of NCEs, process complexity, and interdependency of synthetic strategies, screening results, and NCE characterization data. To efficiently manage these challenges, medicinal and analytical chemists, as well as biologists, work closely together at all points in a given program, from strategy development through lead compound selection.

With the notable exception of mixture deconvolution, the general objectives in providing NCE characterization to assist compound synthesis and purification, verify stability upon storage, and validate the structures of proven-active compounds, remain unchanged. However, with the advent of HTOS, combinatorial chemistry, and HTS, the demands placed on analytical instrumentation or methods to meet these objectives are staggering. For example, combinatorial chemistry programs may require a quality control (QC) verification of reaction success, through detailed characterization of numerous 100-component NCE mixtures. From a practical standpoint, the speed versus accuracy trade-off equation dictates that evidence for only a certain percentage (e.g. 80%) of intended compounds is required, for such complex mixtures to pass the QC test. Depending on the screening approach, such as on-bead enzyme binding assays, there may also be a need to characterize NCEs present on a single solid support bead (as small as 100 μm in diameter). Characterization requirements for HTOS more closely mimic those of traditional medicinal chemistry, which now include near real-time reaction monitoring capabilities, QC for verification of identity and purity, and assistance with purification of final products, typically presented in arrays of tens or hundreds of discrete compounds.

Unlike most other categories of analytical needs described in this article, method sensitivity is not typically a major issue in NCE characterization. Also, because the chemistry is designed to yield predetermined structures, identification of synthetic products is largely a verification process for which a single qualitative analytical technique may be sufficient. Instead, the major challenges here reside in the design of instrumentation and strategies to provide adequate characterization and purity assessments for extremely large numbers of diverse compounds, requiring the analytical chemist to also address issues such as data archival, retrieval, and integration into corporate database platforms, as well as providing access to analytical instrumentation and data for nonexpert end users.

2.2. Evolution of mass spectrometry technology for NCE characterization

For several decades, mass spectrometry has been one of several complementary spectrometric tools employed to verify or assign structures of NCEs prepared by medicinal chemists. Most commonly, nominal molecular weight and, occasionally, fragmentation information were gathered, and combined with NMR and elemental analysis (CHN) data, as proof of the final-product structure. During the course of multistep synthesis, the chemist would typically assess reaction progression or characterize intermediates by using thin layer chromatography, GC, and often NMR. Mass spectrometry data was not viewed as a guide to synthesis but, rather, as simply a piece of information required to check a box on the characterization package for each NCE. The barrier to broader utilization of mass spectrometry had been the usual long delay between sample submission and availability of interpreted data (averaging more than two weeks in many settings). Technological limitations contributed to this relatively poor responsiveness. Samples were individually prepared and analyzed on complex mass spectrometry systems that required careful optimization by an expert operator, employed vacuum interlocks to introduce samples, and necessarily offered a variety of complementary (but difficult and time consuming to switch between) inlets or ionization modes (FAB, probe-CI or EI, GC, etc.) to cover the range of compound classes requiring characterization.

Within the past decade, state-of-the-art, mass spectrometry-based techniques have been adapted to enhance NCE characterization efforts in drug discovery.

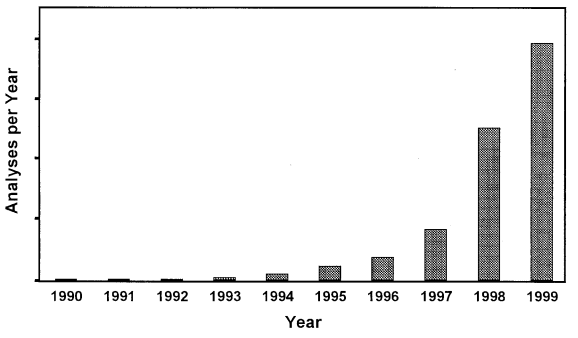

Fig. 2. Relative trend in annual NCE characterization analyses performed at P&G in support of medicinal chemistry, HTOS, and combinatorial chemistry from 1990 until 1999.

These developments have provided: nonexpert end users with direct and near real-time access to molecular weight data on individual samples; phenomenal throughput for large batches of discrete NCEs requiring molecular weight determinations or more comprehensive characterization; and a high level of versatility needed to creatively adapt to a wide variety of specialized problems presented by combinatorial mixture characterization. The impact has been so profound that mass spectrometry has become the technique of choice for first-line NCE characterization, and has even transformed many early drug discovery processes. To the medicinal chemist, mass spectrometry has become an indispensable tool used to increase the speed and certainty of their daily work. For HTOS and combinatorial chemistry, mass spectrometry-based technologies provide unparalleled throughput and make possible many types of NCE mixture deconvolution protocols that would be impractical with any other analytical technique. A measure of the increased importance of mass spectrometry in NCE characterization is provided in Fig. 2, which represents an explosion in mass spectrometry utilization within our own drug discovery organization over the past decade. In a trend typified across the pharmaceutical industry, the molecular weight determination rate of several hundred samples per year (early 1990s), had increased 100-fold by the end of the decade.

For NCE characterization, the most impactful technological advancements have been the implementation of automated sample processing and analysis, rugged APCI and ESI interfaces for flow injection analysis (FIA) and LCMS, and user-friendly software, which offered the nonexpert (medicinal chemist) direct access to mass spectrometry data. Also, for combinatorial mixture analysis, in particular, a premium was placed on creative/custom protocols for interrogating complex mixtures, employing various mass spectrometry-based tools, as described later in this section.

2.2.1. Mass spectrometry-based tools for rapid characterization of discrete NCEs

Recognizing the potential benefit that ready access to molecular weight data could have on the progression of medicinal chemistry programs, pharmaceutical mass spectrometry labs began implementing automation tools for sample preparation and instrument control, as well as data processing and reporting, as early as 1992. The first description of such a streamlined approach [56] employed a standard GC autosampler that loaded test sample onto a resistively heated filament, mounted on a robotic probe. Using a data system controlled vacuum interlock, the probe was introduced into an EI/CI ion source for analysis. This novel batch approach typically provided next-day mass spectrometry data for the chemists. Response times were markedly reduced by employing FIA techniques in conjunction with LCMS interfaces that were initially based on TS or PB [57,58] and later employing APCI [59] or ESI [60]. Atmospheric pressure ionization techniques provide maximal compound class coverage, easy-to-interpret spectra, and are relatively rugged. These efforts demonstrate the utility of open-access mass spectrometry (OAMS), which typically employs a sample log-in computer, with simple-to-use software with which a user can choose from a standard set of data acquisition methods. The entire process, from log-in through mass spectrum printout, typically requires less than 5 min, providing a responsiveness that makes OAMS of value even for in-process reaction monitoring.

A logical extension of this work was OA-high-performance liquid chromatography (OA-HPLC)

mass spectrometry for rapid qualitative profiling, also employing broadly applicable (template) methods. Unfortunately, traditional HPLC methods (i.e. long columns, low flow rates, and shallow gradients) were lacking for OA or other high-throughput applications. However, effective alternatives were developed, making use of short, wide-bore analytical HPLC columns, in combination with steep gradients and high mobile phase flow rates [61,62]. Reported results included high-speed analyses (2–5 min, inject-to-inject cycle time), with minimal loss of column performance. These early OAMS works clearly set the stage for the very high-throughput analysis systems, devised to support the NCE characterization needs of HTOS, as described in Sec. 2.3.

2.2.2. Techniques for combinatorial mixture characterization

No single protocol is adequate to address the many and highly varied needs in combinatorial mixture characterization, which stem from the diverse nature of combinatorial chemistry programs across the pharmaceutical industry. For example, mixtures may originate from solution-phase or various types of support-bound chemistries, of widely varying yield; consist of from 5 to more than 100 NCEs; contain NCEs of redundant molecular weight, including structural isomers; and undergo a range of screening protocols (including on-bead). Each characterization protocol must, therefore, be customized to meet the specific needs of the combinatorial chemistry program it supports. For this reason, techniques for combinatorial mixture characterization are often novel, almost always evolving, and difficult to generalize. Presented here is a brief discussion of a variety of techniques, devised to meet these special needs.

The breadth of analytical applications for combinatorial chemistry has been recently reviewed [63–66]. Many of the initial qualitative and quantitative analytical techniques for evaluating combinatorial chemistry products evolved from the classical colormetric tests used in peptide chemistry (e.g. ninhydrin color test for detection of free terminal amines or the bromophenol blue test for detection of free amines) [67,68]. In addition, Fourier transform infrared spectroscopy has been used for direct “on-bead” characterization of compound functional groups [69,70]. This approach provides a quick and nondestructive method for the rapid confirmation of a successful reaction coupling, which is of particular value in synthesis optimization procedures. In a similar fashion, magic angle spinning NMR has been used for on-bead characterization of reaction products [71,72].

Although true nondestructive on-bead analysis is not practical using mass spectrometry, MS-based techniques have nevertheless developed into the key tools supporting combinatorial chemistry [73–77], owing to their speed and versatility. Because combinatorial chemistry developed from solid-phase peptide synthesis, many of the earliest mass spectrometry-based applications employed MALDI-TOF [78–80]. This technique has a number of attributes that match the needs in this area, for example sensitivity and limited sample size requirements; capability to analyze solution-phase products or single beads (in-matrix cleavage); ability to rapidly characterize simple mixtures; and straightforward experimental setup and design. In one example, a novel approach for the rapid characterization and sequencing of peptide libraries, synthesized on a solid support by using a *N*-acetyl-*D,L*-alanine capping reagent, was demonstrated [80]. During each reaction coupling step, a small amount of the capping reagent was added in order to effect the partial termination of the peptide sequence. When the capping sequence is conducted over a number of iterations of the reaction, a solid support contained not only the intended peptide sequence, but also a small percentage of a series of shorter terminated peptide chains. Thus, not only could the peptide molecular weight be directly measured by MALDI-TOF mass spectrometry, but its precise sequence is indirectly determined, by simply reading the map provided by the lower-level capped peptides. This approach was successfully employed in the preparation of acetylated and nonacetylated pentapeptides, in order to identify ligands for a HIV-neutralizing antibody.

As combinatorial chemistry efforts pushed the limits of the practical size of synthetic mixtures from tens up to hundreds or more NCEs per test sample, it was recognized that MALDI-TOF mass spectrometry was ill-suited to provide adequate characterization. Nominal mass redundancies due to isobaric components in the reaction scheme quickly became problematic and the nominal mass resolution capabilities of these early systems did not allow for unequivocal characterization of the reaction mixture. In an attempt to overcome characterization issues due to mass redundancies, the potential of FTICRMS was explored for analysis of combinatorial mixtures [81,82]. The very high mass resolution and accurate mass measurement capabilities of this technique, provided a plausible solution to the issue of molecular weight redundancies in reaction mixtures and was shown to be useful for characterization of medium-sized combinatorial library mixtures. Obviously, FTICRMS could offer only a limited solution to this problem, as it cannot resolve differences in isobaric ions of identical empirical formulae (isomers).

The use of GCMS to facilitate characterization of combinatorial mixtures has also been reported [83]. However, because GCMS and EI/CI ionization modes are applicable only to a limited range of pharmaceutically relevant compounds, the majority of applications involve indirect characterization of solid-phase combinatorial library components (prepared using split-mix synthesis protocols), through the use of chemical “tags” [84]. A chemical tag, which may be readily cleaved and analyzed by GCMS, is attached to the solid-phase reaction bead to represent the functionality of the reactant added during a particular reaction sequence. In a typical, multistep combinatorial reaction sequence, discrete chemical tags are coupled to the solid-phase reaction bead simultaneously with the execution of each step in the primary reaction sequence. Because a unique tag is preassigned to represent each potential reactant, the combination of tags present on any given bead serves as a code for all reaction steps associated with a particular bead. Following on-bead biological screening, a small percentage of the tags are cleaved from isolated beads, determined to be biologically active, and characterized by GCMS. Identification of the compound tags allows rapid, indirect deconvolution and identification of active compound structures.

Reports of methods for characterization of solution-phase combinatorial mixtures are less common than those for solid-phase synthesis. This may be attributable to the advantages and ease of use of resin-supported chemistries. However, solution-phase combinatorial chemistry has a distinct advantage in the breadth of synthetic schemes that are available without the need to adapt the chemistry to a solid-support environment. Unfortunately, chemical tagging and other coding procedures are not easily designed into solution-phase protocols. Hence, identification of NCEs of interest in such mixtures must be addressed with novel sample pooling formats (indexed, scanning, etc.) and subsequent guided deconvolution of the mixture using appropriate analytical techniques [85–87]. In one example, this approach was employed in the preparation of a series of “Churchkey Cauldron” libraries, as part of a search for novel antibacterial agents [88]. A 10 000 member indexed semicarbazone library [87] was synthesized in pools of 100 equimolar NCEs in two sets of 100 vials each, 100 reagent variants per vial per set. In subsequent biological screening, two particular vials exhibited strong activity. Deconvolution of the library, based on the indexed array synthesis protocol, did not prove sufficient for identification of an NCE responsible for the observed activity. Profiling of the active pools, using HPLC-(ESI)MS, with parallel UV detection, provided evidence that the activity was not due to an expected product but, rather, resulted from a side product of the original reaction scheme. This side product was later validated to exhibit broad-spectrum antibacterial activity. This experience illustrates that, even with the strictest care in library design, use of pooling strategies and deconvolution methods alone are not always sufficient to identify active compounds in even simple mixtures. Further, these results typify the need for rigorous analytical methods to support the characterization of library synthesis and screening assay results.

2.3. Current strategies for NCE characterization

2.3.1. General approach

The recent developments in mass spectrometry-based instrumentation and associated automation provide the means to achieving the high responsiveness and throughput for NCE characterization, now demanded in accelerated drug discovery programs. Given the diverse nature of these needs, the critical challenge is to appropriately select, adapt, and deploy these tools and techniques to optimally meet the needs in each of several categories of NCE characterization. Reflecting what appear to be pharmaceutical industry norms, mass spectrometry now has a major (usually leading) role in the following: OA support for medicinal chemistry; array characterization by both FIAMS and LCMS for HTOS; high-throughput purification (HTP) for HTOS; and combinatorial mixture characterization. Although there is an acknowledged trend away from mixture approaches, and toward NCE array library production by way of HTOS, special-case combinatorial characterization needs will continue to be important for the foreseeable future. Because the examples of customized mass spectrometry-based mixture characterization protocols presented in Sec. 2.2.2. have provided an indication of the adaptability required in this area, this category will not be further discussed here.

Although the mass spectrometry-based measurement needs of the traditional medicinal chemists and the HTOS chemist are qualitatively similar, and both now incorporate mass spectrometry information to speed and guide their work processes, the manner in which these scientists typically acquire these data is (by design) quite different. Briefly, medicinal chemists (and HTOS chemists that may be optimizing synthesis processes, prior to array library production) have a need to verify the identity and (sometimes) purity of one or a few test samples at a time. These data often dictate or guide next steps in their daily work, so near-real-time access to mass spectrometry data is the key for them. In the case of HTOS production support, throughput of large batches of distinct NCEs and data quality are essential. These important distinctions factor heavily into mass spectrometry technology deployment and utilization strategies, as described in the following sections. Finally, mass spectrometry is now viewed as an important tool to either guide or support HTP processes. A brief review of these mass spectrometry-based HTP strategies is also provided.

2.3.2. Open-access mass spectrometry

Because nominal molecular weight data are the primary information required in OAMS applications, ESI and APCI interfaces are most commonly employed in conjunction with single quadrupole mass analyzers. Selection of a particular instrument is more driven by data system considerations, such as compatibility with high-speed autosamplers, ability to control preferred HPLC pumps, automated data processing capabilities, and ease-of-use for the nonexpert customer. This latter consideration is reflective of the fact that, whereas mass spectrometry data is vitally important to the chemist, it is unrealistic to expect that all chemists have a high degree of knowledge in mass spectrometry-based instrumentation. Based on an informal survey of pharmaceutical companies, each OAMS system is typically available to support between 20 and 50 chemists, of widely varying analytical skills. Thus, because rapid availability of mass spectrometry data are critical to all chemists, the user interface and associated procedures must be exceedingly simple. Included in this are simple and clear sample dilution protocols, which must be strictly enforced, given the broad negative impact of gross contamination of the instrument.

Most commonly, modern OAMS systems are configured to provide FIAMS data in under 3 min. This response time is essential for reaction monitoring and to minimize instrument time required per sample. Based on our experience, for this speed to be of practical benefit, OAMS instruments need to be distributed to locations that are no more than about a 2 min walk for any chemist. Further, to provide optimal benefit, an OAMS system is considered fully utilized once cumulative run times occupy 30% of an 8 h day. This reflects the practical OA reality that a high-speed analysis is of use only if there is no long cue of samples.

Although OAMS systems are often deployed remotely from central analytical functions, mass spectrometry experts take responsibility for selection, implementation, and maintenance of the instrumentation. This includes standardizing software packages across OA instruments, as well as development of a short list of template methods, most generally applicable to the chemistry groups nearest a given instrument. In ideal cases, broadest compound class coverage is provided through multiple instruments with complementary methods (e.g. APCI and ESI). Systems configured for OA-LC-UV-MS are also becoming more prevalent, as a complement to the very rapid OA-FIA-MS. In the OA-LC-UV-MS mode, the user can select from a short list of broadly applicable separation-detection methods. The benefit is that, in addition to qualitative data on reaction mixture components, a crude estimate of purity can be obtained through use of serial UV detection. The LCMS alternative also offers the advantage of separating reaction products from excess ionization-suppressing reagents that are typically present, particularly in reaction monitoring applications. Data are automatically processed and reported, in a manner customized to best meet local needs. This flexibility costs instrument time, typically requiring about 10 min to profile a sample (equilibration through analysis), which is why such systems are often implemented in addition to dedicated OA-FIA-MS capabilities.

By providing medicinal chemists with ready access to dependable OAMS instrumentation and broadly applicable methods, as well as very basic training in instrument use and data interpretation, the use of mass spectrometry data to guide chemical synthesis work has now become an expectation in the pharmaceutical industry. In delivering the data by way of the OA mechanism, tremendous numbers of samples are analyzed with a relatively modest effort from analytical personnel. This has had an indirect benefit of allowing analytical scientists to focus their attention on the design of high-throughput systems and methods to address NCE characterization needs associated with HTOS, as described in Sec. 2.3.3.

2.3.3. Ultrahigh-throughput FIAMS, supporting HTOS

Because of the tremendous rate of NCE preparation now attainable with HTOS technology, the throughput of characterization methods must be extremely high (ideally, up to thousands of compounds per system per day). This clearly distinguishes HTOS from OA support strategies that must focus on fast turnaround, rather than overall throughput. Thus, for high efficiency and to assure data integrity, analyses supporting HTOS are carried out by analytical experts, typically located in centralized facilities. Based on speed considerations, FIA methods are typically employed for first-tier characterization. Although high-throughput autosamplers have recently been coupled with NMR to provide more rapid qualification of HTOS products [89], NMR-based tools do not currently offer the throughput potential of mass spectrometry-based systems. In addition to the speed of the actual analysis, data reduction and interpretation requirements also factor into the throughput equation that favors mass spectrometry. Central to this point is the fact that, whereas NMR data are rich in structural information, it is much more complex to interpret and less definitive than, for example, extracting molecular weight information from an ESI-MS spectrum and verifying consistency with the intended NCE. This latter exercise is readily automated and is typically considered sufficient for initial QC of large HTOS libraries. Thus, there has been a recent focus on the refinement of automated/robotic mass spectrometry-based systems to address the throughput and data management demands of HTOS library characterization [73,90,91]. These efforts not only include first-tier ultrahigh-throughput FIAMS approaches, but also high-speed gradient LCMS-based systems for more detailed characterization and purity assessment, and strategies for using mass spectrometry to speed and improve the quality of preparative (prep) LC purification protocols for HTOS, as described later.

Products of HTOS are usually presented in arrays of (intended) discrete compounds, formatted in deep-well plates. Over the past few years, throughput for FIAMS analysis of NCEs within typical 96-well formats has increased by more 50-fold per mass

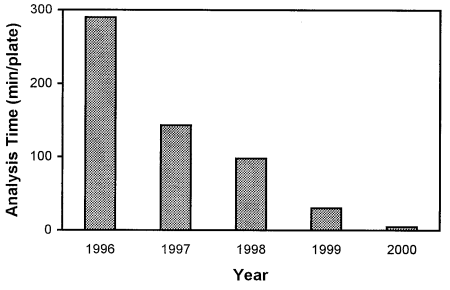

Fig. 3. Total analysis time per 96-well plate for NCE molecular weight determination, using FIAMS, over the past five years. The trend reflects the dramatic impact of the rapid development of associated analytical technology.

spectrometry system, as illustrated in Fig. 3. Initial applications employing array autosamplers (in 1996) required about 280 min to complete one plate (based on 3 min per sample run time) [60]. Since then, the analysis time per plate has been continuously reduced, to the present best of less than 5 min per 96-well plate [92]. This increased throughput has been essential for addressing the increasing NCE characterization demand (Fig. 2), of which the largest application segment has recently been FIAMS.

In progressing to this throughput level, several key technologies were developed and integrated with single quadrupole mass spectrometry systems. For example, a Gilson 215 multiprobe liquid handling system was interfaced to a single quadrupole mass spectrometer to increase FIAMS throughput [93]. In this design, the Gilson system provides for multiple autosampler injectors to be preloaded prior to FIAMS analysis, thus eliminating a significant fraction of the sample analysis cycle time (i.e. probe washing and sample loading between each run). With this design, which is now commercially available, nearly a fourfold increase in throughput was realized over conventional single probe autosampler systems, reducing cycle times to as low as 12 min per plate. Building on this work, the per-plate analysis was improved to a cycle time of under 5 min, by increasing the speed of sample transfer through the autosampler [94]. By increasing the linear velocity of the sample and mobile phase through the autosampler and painstakingly

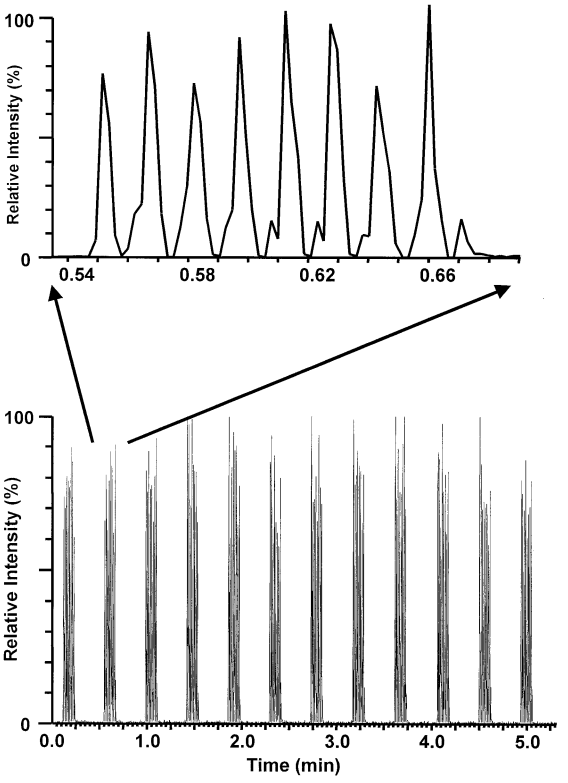

Fig. 4. Ultrahigh-throughput FIAMS analysis of a 96-well plate, containing quinine ($C_{20}H_{24}N_2O_2$, molecular weight 324.2) in each well. The analytical system consisted of a Gilson 215 multiprobe liquid handling system, modified for reduced dead volume, and a Micromass LCT ESI-TOF mass spectrometer, acquiring *m/z* 100–1000. Displayed is the extracted *m/z* 325.2 trace (quinine MH^+), for all 96 injections (bottom). These conditions provided 6.6 s analysis times for an eight-sample injection sequence, as highlighted in the expanded scale (top).

minimizing dead-volume, flow-injection sample peak widths of approximately 0.5 s [full width at half maximum (FWHM)] were achieved, along with an injection sequence time of 6.6 sec for each eight-sample autosampler loading. The per-plate throughput resulting from these modifications is illustrated in Fig. 4. Due to the narrow sample peak widths and the typical need for scan ranges up to (on the order of) *m/z* 1000, scan speeds available with conventional quadrupole mass analyzers are inadequate to yield high quality mass spectra. This work was, therefore, conducted using an ESI-TOF mass spectrometry instrument.

2.3.4. High-throughput LCMS, supporting HTOS

Although FIAMS provides a very rapid mechanism to verify the presence or absence of an intended NCE, based on molecular weight, it is not suitable for purity assessment. Philosophies regarding the extent of purity profiling required to support HTOS programs vary, from spot-checking representative test samples, to analyzing 100% of reaction products. Purity profiling is accomplished using LCMS to map major components based on molecular weight, primarily to determine the retention time of the intended NCE. One or two additional auxiliary detectors are configured in series or parallel (by way of postcolumn split) with the mass spectrometer to yield purity or NCE concentration data, which are collected and stored within the electronic data file corresponding to each sample. Evaporative light scattering (ELS) and UV detectors are now most commonly used for this application. However, chemiluminescence nitrogen detectors represent an attractive alternative, offering the capability of absolute quantification of the NCE, provided the structure contains one or more nitrogen atoms [74].

Because chromatographic separation is required, the throughput challenge in HTOS purity assessment is particularly formidable, fueling the need for very high-speed LCMS. Recent strategies fall into one of two categories: high-throughput serial (single column) LCMS [95,96]; or parallel column LC, combined with a multisprayer mass spectrometry [97]. An example of the practical utility of rapid serial LCMS for HTOS product purity assessment has been reported [95]. Short LC columns, high flow rates, and steep chromatographic gradients were employed to reduce per-sample profiling times to approximately 1 min (injection-to-injection). Under ideal conditions, analysis times of as little as 42 s were demonstrated.

Parallel chromatography systems allow simultaneous analysis of multiple samples on identical LC columns, thus, reducing the time required to analyze a batch of samples by a factor roughly equal to the number of columns. For purity assessment, each post-column fluid path is interfaced (in series or parallel, if split) to an auxiliary detector. Flows from all columns are subsequently passed on to one mass spectrometer. Obstacles precluding the practical utilization of parallel LC systems with a single mass spectrometer were successfully overcome by incorporating an ESI ion source configured with mechanical segmentation for sequential sampling of multiple LC flow paths [97]. Automated software control of the flow segmentation process allows direct and unambiguous correlation of the sample identity, qualitative LCMS data, and purity profile from the auxiliary detector, for each of up to eight LC columns. Total analysis times of as little as one hour have been demonstrated for the LCMS purity assessment of a 96-well microtiter plate, using this parallel characterization approach. Based on the potential throughput benefits and the commercial availability of multicolumn and multisprayer ionization sources, use of this new technology is growing rapidly for NCE characterization applications.

2.3.5. Mass spectrometry-based high-throughput purification

Having described methods for assessing the purity of discrete NCEs, the next issue is appropriate utilization of these data. Certainly information is provided regarding the quality of a particular reaction product, or an array library of discrete NCEs, which may be used to optimize synthesis conditions or serve as a QC test that must be passed prior to biological testing. NCEs verified to be present, but failing a QC purity criteria, are typically subjected to purification. Further, there is an increasing appreciation for the value of high-purity NCEs in drug discovery, given experiences demonstrating the potential for minor or trace-level impurities producing errant bioassay results. This issue is particularly acute in the hit expansion stage, where reliable QSAR data is a must. For this reason, many pharmaceutical companies have adopted the strategy of purifying 100% of NCEs. This raises throughput challenges in purification that precisely parallel those encountered in NCE characterization. In turn, the most successful and now commonly employed responses to this challenge parallel the LCMS solution for high-throughput purity assessment (Sec. 2.3.4.). That is, high-speed separation principles, originally developed for analytical-scale

NCE characterization, were extrapolated to prep-LC for compound purification [98–100]. With these approaches, adequate separations are achieved for purification of up to 100 mg of compound in well under 10 min. For perspective, typical prep-LC conditions include: 20 × 100 mm C18 (10 μm) column; flow rate of 30 mL/min; and a gradient from 10% to 100% organic in 5 min. Volatile modifiers, such as ammonium acetate or trifluoroacetic acid (TFA), may also be included in the mobile phase.

Although these rapid prep-LC methods provide the potential for achieving the purification throughput requirements faced with HTOS libraries, final product quality and recovery are enhanced using real-time data-driven fraction collection, the so-called detect-before-collect strategy [98]. This general approach employs the output signal of an on-line detector to initiate and terminate the fraction collection process. This offers major efficiency and product quality advantages over traditional collect-before-detect fractionation protocols, in that: fewer fractions are generated because collection only occurs upon elution of detectable peaks; compounds are more cleanly isolated from closely eluting peaks; and a compound of interest is usually confined to a single fraction. One of three general approaches is followed in implementing rapid prep-LC with detect-before-collect methodology, all of which are enabled by mass spectrometry-based detection, in some way. As briefly highlighted below, each approach has its strengths and the strategy adopted is driven by many factors, including throughput and quality requirements, and differences in philosophy related to overall production efficiency versus risk of losing compounds.

For the highest possible throughput, the use of parallel prep-LC columns, with UV detection to trigger fraction collection is the method of choice [99,100]. In a now commercially available system (Parallex, Dyax Corp., Charlottesville, VA), four parallel systems (each aligned for purification of one HTOS library plate) process and track samples. Depending on UV threshold settings and the complexity of each of the original reaction products, multiple fractions are typically collected for each sample. Fractions are then characterized by high-throughput FIAMS to determine which fractions contain the desired NCEs. Most commonly, these fractions are then taken to dryness and then re-analyzed by LC-MS-UV (or ELS detection), which certifies the identity and purity of the NCE in its final form. Although the entire process is tracked and controlled by vendor-supplied software, it is modular (or station-to-station) in nature, which offers workflow flexibility (e.g. slower steps, such as fraction dry-down can be easily bolstered by adding equipment). In addition to the high throughput provided by this parallel chromatography approach, by collecting all UV peaks there is less risk of discarding the intended NCE. A tradeoff, relative to alternatives described later, is that a greater onus is placed on the separation procedure, because it is difficult to achieve optimal isolation from partially coeluting components.

A second approach to implementation of detect-before-collect purification employs a single prep-LC column, with both UV and (by way of a postcolumn split) mass spectrometry detection. As previously described, the UV serves as the trigger for collecting separated components. However, the mass spectrometer also provides a mapping of molecular weights of collected compounds, as well as a preview of peak purity. In this case, the postisolation mass spectrometry analysis of each fraction is unnecessary.

In terms of final product quality, prep-LCMS is the ultimate detect-before-collect NCE purification tool [101,102]. Now available commercially, prep-LCMS systems are capable of collecting multiple fractions per sample, through output signals actuated by the intensity at each of several preselected *m/z* values. For HTOS support, a single compound is typically targeted per sample. Prep-LCMS offers advantages over prep-LC-UV systems, as only compounds of interest are collected and mass spectrometry data are immediately available for each collected fraction. Further, because of the superior selectivity of the mass spectrometry detector, the triggering process is blind to partially coeluting components of different molecular weights. This results in the highest possible target compound purity for a given prep-LC separation.

Prep-LCMS-based purification systems are being increasingly utilized throughout drug discovery pro-

grams to support not only HTOS, but also medicinal chemistry, natural products purification, and the isolation of low-level metabolites. There has been an ongoing effort, with considerable success, by the chromatography and mass spectrometry vendors to refine and improve prep-LCMS-based purification technology. Nevertheless, experience gained in the next few years will determine whether the advantages gained with these systems will outweigh the security of prep-LC-UV-based systems.

2.4. Emerging mass spectrometry technologies for NCE characterization

It is apparent that the area of NCE characterization has been transformed in a very short time, in part responding to the high-throughput demands of HTOS and combinatorial chemistry, but largely driven by innovations in mass spectrometry and automation technology. The phenomenal speed with which these transformations in NCE characterization strategies have and are occurring makes it difficult to clearly judge between what are outdated, currently established, or emerging practices and technologies. Nevertheless, considerable effort continues to be expended on improving chromatography, sample introduction, and instrumentation for providing even higher throughput support for HTOS. Examples of alternative chromatographic strategies include supercritical fluid [103,104], normal phase [105], and high-temperature [106] chromatography. There have also been investigations of alternative mass spectrometry systems, such as desorption ionization on silicon (DIOS), in combination with LD-TOF mass spectrometry [107].

Supercritical fluid chromatography (SFC) has traditionally been considered to fill a “conceptual niche” in the family of chromatographic techniques for the analysis of nonpolar analytes and, therefore, has not been employed extensively as a separation technique for pharmaceutically relevant compounds. However, there has been a recent resurgence of activity in SFCMS development within the pharmaceutical industry, as an alternative to reversed-phase LCMS. Utilizing the “enhanced fluidity” [108] conditions of SFC it is quite straightforward to operate at flow rates that are three to five times faster than LC. Thus, methods using SFC-based chromatography could dramatically increase analysis throughput. The potential utility of gradient packed-column SFC for screening combinatorial libraries has been reported [109]. Another potential application of SFC is derived from the fact that the majority of SFC separations employ carbon dioxide/methanol mixtures. The high volatility of this mobile phase composition may offer a tremendous benefit in sample purification [110] since solvent removal is the most time consuming step in the purification process.

Another, potentially exciting, new technique for high-throughput NCE characterization is DIOS for LD-TOF mass spectrometry. DIOS-MS was introduced as a method for direct laser vaporization and ionization of peptides from the surface of a silicon chip [107]. A novel aspect of the technique is that desorption and ionization is affected without the use of an organic matrix, as required in MALDI, making DIOS amenable to small molecule analysis. Applications of DIOS-MS have since been extended to the analysis of any number of small molecules from the silicon surface, including demonstration of the feasibility of collecting chromatographic effluents on the surface of the chip, followed by DIOS-MS analysis [111]. DIOS-MS may potentially overcome the shortcomings of MALDIMS and could offer advantages versus current instrumentation.

Combinatorial chemistry and HTOS will continue to grow in use across the pharmaceutical industry where it is anticipated, if not expected, that bench chemists will continue to incorporate many of the techniques described here into their everyday research efforts. Open-access and high-throughput analytical technologies will continue to rapidly advance in both performance and user friendliness. With an ongoing desire to further increase the efficiency of drug discovery, there has been a recent concerted effort to enhance the lines of communication between the vendor, analytical chemist and medicinal chemist, resulting in new technologies designed to better answer the synthesis questions of the medicinal chemist, whereas addressing instrument performance and func-

tionality concerns of the analytical personnel. It is likely that these highly collaborative initiatives will lead to a dramatically different paradigm for open-access analytical instrumentation over the next few years.

3. Biomacromolecule characterization

3.1. Pharmaceutical significance

The modern drug discovery process requires the identification of targets (proteins or enzymes, in particular) to exploit for a given therapeutic intervention. When tracking the history of medicine and pharmaceuticals, the primary mode of intervention is by way of low molecular weight chemical entities (drugs) that interact with and modulate the function of proteins. In today's pharmaceutical setting, this paradigm still holds true. In addition, a growing number of therapies now rely on biological compounds (protein therapeutics) as the active ingredient (e.g. insulin for diabetes, human growth hormone for growth hormone deficiency disorders, and tissue-plasminogen activator for acute myocardial infarction), thus proteins play a central role as both targets for therapeutic intervention and as the therapeutic agent. As such, analytical characterization of proteins is a necessary function in both drug discovery and drug development organizations.

Historically, the most common approach to drug discovery starts with identification of a protein corresponding to an enzyme active that, upon inhibition, is likely to have some therapeutic advantage. Defining the protein corresponding to the physiological activity of interest can be a considerable challenge since there may be insufficient quantities of that protein to characterize by traditional chemical sequencing approaches. Once a target protein is found and purified, additional characterization of the primary sequence and post-translational modifications of the protein usually follows. Here, once again, a challenge is to have available sufficient protein for analytical characterization (structure and properties). Next, if the protein is validated as a potential target for therapeutic intervention through biological assays, it may then be cloned, overexpressed, and purified to yield sufficient quantities for more rigorous structural characterization (e.g. three-dimensional) by NMR and/or x-ray crystallography (each requiring milligram quantities of pure protein). Depending on the type of protein being investigated, some or all of these analytical and physical characterizations may be required to facilitate the discovery of potent and specific inhibitors for the enzyme activity of the target protein.

More recently, however, a new mode of target discovery and validation, based on genomic screening and protein profiling (proteomics), has become a second paradigm in drug discovery [112,113]. Both genomics and proteomics approaches rely on detailed comparative expression profiles of control verses altered (e.g. diseased, drug-treated) cells or tissue. Genomics approaches typically refer to messenger RNA profiling, while proteomics is a term used to describe comparative protein profiling. In either case, information gained by these comparative profiles is used to generate hypotheses regarding which protein targets are the key modulators of the disease process and thus are potential targets for therapeutic intervention. As with the traditional drug discovery approach described above, once a potential protein target is established, the structural characterization begins. For proteomics studies, the analytical challenge includes how to rapidly identify the differentially expressed proteins from the comparative profiles (often more than 100 distinct low-level proteins per study).

For protein therapeutics, all of the protein structural characterization needs described above also apply but, in-addition, the development of a protein therapeutic requires extensive characterization of stability, degradation, metabolite identification and quantification, lot-to-lot variability, etc. Each of these investigations must be done in a regulated environment, with detailed documentation for filing with regulatory agencies, including analogous considerations to those required for small-molecule drugs, as described in Secs. 4–6.

3.2. Evolution of mass spectrometry technology for protein/peptide characterization

Over the past decade, several key advances in mass spectrometry-based instrumentation and software have lent themselves particularly well to addressing unique challenges now faced in biomacromolecule characterization. As a result, applications in protein/peptide mass spectrometry have become an integral part of drug discovery and development, in many cases delivering the sensitivity, specificity, and throughput needed to make new drug discovery paradigms feasible. Although the intent of this section is to provide only a sampling of the most prominent advances in protein mass spectrometry, as relevant to pharmaceutical applications, the reader is also directed to two recent compendia that provide additional information on a variety of mass spectrometric techniques for analysis of biological systems [114,115].

By about 1990, the majority of mass spectrometry-based protein and peptide characterization was being done by FAB, liquid SIMS, and laser desorption techniques, coupled primarily to magnetic sector or linear TOF mass analyzers [116]. Unfortunately, the sensitivity, resolution and/or mass accuracy of these techniques were insufficient to address many of the protein structure characterization issues faced within the pharmaceutical industry. This situation began to change with the development and/or maturation of several mass spectrometry technologies, the most impactful of which include: ESI (including microspray and nanospray versions); advanced MALDI-TOF instruments; and user-friendly computer control and data analysis interfaces that have broadened the mass spectrometry user-base to include nontraditional mass spectrometrists, such as biochemists. First, the ability to directly mass analyze biomacromolecules by ESI has perhaps had the greatest impact on the protein characterization needs in the pharmaceutical industry. Because this technique produces multiply charged ions, as represented in Fig. 5(a), proteins could be readily detected within the *m/z* range of most quadrupole instruments. In addition, the ability to calculate the intact protein mass from the combined averages of

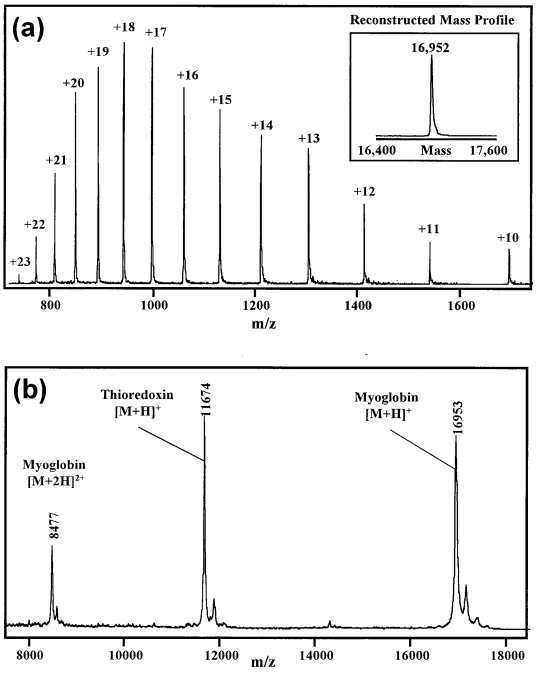

Fig. 5. ESI-MS and MALDI-TOF mass spec spectra of proteins. (a) ESIMS of myoglobin showing the charge-state envelope, as produced by flow infusion on a single quadrupole instrument. The inset represents the reconstructed mass profile of the protein, calculated as an average of all the peaks in the charge-state distribution. (b) MALDI-TOF mass spectrum of a mixture containing 0.1 pmol each of *E. coli* thioredoxin and horse myoglobin spotted in sinapinic acid matrix.

the entire charge-state distribution [Fig. 5(a), inset] produced a typical mass accuracy of about 0.01% [117,118]. For the first time, a wide variety of proteins could be analyzed with mass accuracies sufficient to detect minor mutation or modification to the proteins.

Because detection of ions by the mass spectrometer is concentration dependent, miniaturization of the ESI inlet to microspray [119] and nanospray [120] has helped push detection limits down into the low femtomole to attomole range. Further, because ESI interfaces allow direct coupling of a variety of separation instruments to mass spectrometers, the combination of low flow rates and steep gradient elution conditions can result in higher analyte concentration entering the mass spectrometer, providing improved signal amplitide. In addition, the ability to separate and analyze mixtures on-line has set the stage for such

detailed characterization procedures as mapping sites of protein modification, identification of proteins for proteomics applications, and de novo protein sequencing. For the latter, both triple quadrupole and ion trap instruments often fall short, when accurate and complete sequencing information is required from a novel protein. For de novo sequencing applications, the ESI-Qq-TOF instruments offer the best solution [121,122]. The combination of sensitivity, resolution and mass accuracy provides all of the components needed to produce accurate sequence information for a given peptide.

Second, advances in MALDI-TOF instruments have improved the resolution, mass accuracy, and detection limits required for protein and peptide analysis. It is now routine to analyze proteins of >100 kDa at sub-picomole levels and mass resolutions up to 15 000 FWHM have shown a clear benefit in peptide and small protein characterization. In spite of its value in rapid molecular weight determinations, a drawback of MALDI-TOF mass spectrometry, even with PSD, has been an inability to routinely produce robust fragmentation spectra for peptide sequence elucidation. However, a new MALDI-TOF-TOF MS/MS instrument, showing promise for sequencing peptides by CID, has recently been reported [123]. Finally, a major advantage of the MALDI-TOF instrument is an ability to simultaneously analyze protein or peptide mixtures without prior separation. This is possible because the prominent ions produced by MALDI are predominantly singly charged [Fig. 5(b)], unlike the broad charge-state distribution in ESI spectra [Fig. 5(a)]. Thus, the choice of using ESI versus MALDI will be dependent on the application needs and the sample complexity.

Finally, the use of mass spectrometry for protein characterization would not be having the widespread impact, now being experienced in the pharmaceutical industry, without the development of user-friendly instruments with simple to use computer interfaces for data collection and processing. Although many of the advances in mass spectrometry and instrument design certainly originated in academic laboratories, credit must also be given to the instrument vendors who have optimized the user-friendly interfaces to facilitate all aspects of running an instrument, collecting data and analyzing results. For example, the integration of data-dependent scanning features on an ion-trap instrument has facilitated a number of automated acquisition schemes including the "triple-play," where during an on-line LC separation the ion trap can be automatically configured to collect full scan data, higher resolution data to determine charge-state of the ion, and MS/MS fragmentation spectra throughout the entire chromatographic separation. Thus, from a single on-line LC analysis, fragmentation data from each eluted peptide can be readily collected. These user-friendly applications have been particularly critical in the area of protein mass spectrometry where many of the end-users of the instruments are often trained first as biologists or biochemists, not as traditional analytical chemists or mass spectrometrists. Clearly the availability of these advanced mass spectrometry techniques to the biological scientist, coupled with the availability of user-friendly instrumentation has been a key driving force in the growth of protein mass spectrometry over the past decade.

3.3. Current strategies for protein/peptide characterization

3.3.1. Rapid characterization of chemical and post-translational modifications of proteins

When studying proteins in biological systems or as targets of therapeutic intervention, the two most abundant post-translational modifications that require characterization are glycosylation and phosphorylation. Typically, in the pharmaceutical industry, glycosylation is viewed as a nuisance for most structural studies because it complicates application of spectrometric techniques (i.e. mass spectrometry, NMR, x-ray crystallography) used to study protein structure. However, in the arena of protein therapeutics, the heterogeneity of glycosylation requires detailed characterization and validation prior to development of biological drugs. Mass spectrometry has played a role in both the characterization of the carbohydrate portion of the molecule [124] and in the identification of the sites of glycosylation on the protein backbone

[125]. In the latter case, numerous methods have been established to selectively identify glycopeptides by diagnostic, glycan-specific, product ions produced by MS/MS experiments. For example, ESI and CID of tryptic digests from an asparagine-linked glycoprotein produce glycan-specific fragment ions at *m/z* 204 (*N*-acetyl glucosamine) and 366 (hexose–*N*-acetyl glucosamine) [126]. With these diagnostic ions, and the other peptide product ions produced by CID, the sequence and the site of glycosylation can be easily identified. For O-linked glycosylation (serine or threonine), pinpointing the exact amino acid that is glycosylated is a bit more challenging, as there is not a strict concensus amino acid sequence for glycosylation like the Asn-X-Ser/Thr concensus sequence for *N*-linked proteins. However, the combination of diagnostic product ion scanning with chemical hydrolysis methods permits the pinpointing of O-linked glycosylation sites by mass spectrometry [127,128].

In the case of phosphorylation, additional challenges have been encountered in that the stoichiometry of phosphorylation at any given site may be very low. Thus, a sensitive and selective means for identification of phosphopeptides from a mixture of peptides resulting from a protease-digested protein is essential. Analogous to the approach used for glycosylation, CID also produces diagnostic fragment ions for phosphorylation [129]. By using a triple quadrupole instrument in the precursor-ion scanning mode, phosphopeptides can be selectively detected in complex mixtures of nonphosphorylated peptides into the femtomole range. In addition to the state-of-the-art triple quadrupole-based method [130], related approaches of note include selective phosphopeptide enrichment by metal chelation chromatography prior to mass spectrometric analysis [131], methods specific for ITMS [132] and the use of inductively coupled plasma mass spectrometry (ICPMS) for selective detection of phosphorus [133].

Numerous reports describe the utility of mass spectrometry to pinpoint attachment sites of various other forms of protein modification, including disulfide bond mapping [134], nitration of tyrosines [135], covalent attachment of inhibitors [136], or even enzymatic mechanisms that result in mass shifts in the target proteins [137]. An example of mapping a covalent modification of a protein is illustrated in Fig. 6. A 17 913 Da enzyme was reacted with a potent active-site inhibitor and then analyzed, intact, by ESI-MS. The resulting spectrum, presented as a reconstructed mass profile [Fig. 6(a)], shows a 48 Da mass shift, indicating a covalent modification. Both the modified and control enzymes were then digested with trypsin and the active site peptide of each was subjected to ESI-MS/MS (product ion mode) analysis, on an ITMS instrument. The resulting spectra [Fig.

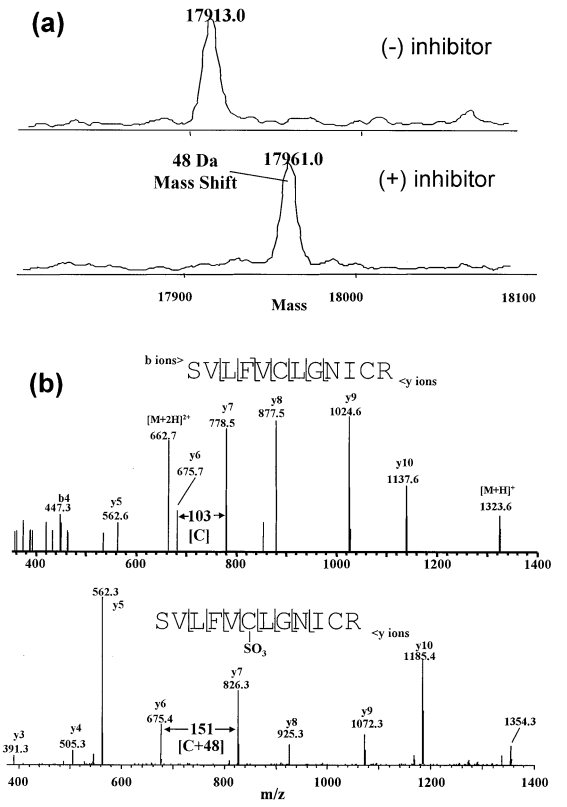

Fig. 6. Detection and site mapping of an enyzme inactivated by oxidation. (a) An enzyme reacted without (−) and with (+) a potent active-site inhibitor, assayed by on-line desalting and infusion into an ESI mass spectrometer (single quadrupole). The resulting reconstructed mass profiles show a 48 Da mass shift for the enzyme plus inhibitor. (b) Product ion spectra (obtained on an ITMS) of the protonated active-site tryptic peptides from control (−) and inhibited (+) enzymes. The fragment ion shifts confirm that the active site cysteine (residue mass 103) has been oxidized to cysteic acid (residue mass of 151).

6(b)] allowed the covalent modification to be mapped as an oxidative inactivation of the active-site cysteine to cysteic acid. Finally, whereas the above methods describe only a few examples on the study of post-translational and chemical modification of proteins, it is clear that mass spectrometry has become the method of choice to sort out any type of modification that results in a mass shift in the protein.

3.3.2. Mass spectrometry in structural biology

Structure-based drug design refers to the development of drug molecules based on a detailed knowledge of the spatial configuration of the target enzyme active site, in the presence or absence of inhibitor molecules. This approach has become an integral part of drug discovery and lead optimization processes within nearly every pharmaceutical organization. Over the past several years, mass spectrometry has played an increasing role in sorting out various aspects of protein conformation, protein–protein interaction and protein–ligand interaction. Currently, mass spectrometry is unable to produce the protein structural (spatial) resolution of other physical techniques (NMR, x-ray crystallography). However, the advantages of speed, utility with limited sample availability, and the ability to study proteins over 30 kDa has brought mass spectrometry to the table for addressing certain key issues of protein structure. One of the techniques most often used in these studies is hydrogen/deuterium (H/D) exchange. The use of H/D exchange to study protein conformation dates back some 20 years [138] where it was primarily used in NMR studies [139]. For mass spectrometry, the premise behind the H/D exchange approach is that one can readily exchange solvent-accessible hydrogen such that a mass shift, consistent with the number of deuterium atoms exchanged, can be measured. Thus, one can study protein folding by digestion of the protein and identification of peptides that contain deuterium versus those that were protected from exchange due to protein structural constraints that prevented penetration of deuterium. This technique was recently applied to study unfolding and refolding of large proteins such as rabbit muscle aldolase [140], and used as a means to address the mechanism and kinetics of the chaperonin GroEL-assisted folding of malate dehydrogenase [141]. Similarly, the role of Ca^{2+} to induce conformational changes in calmodulin [142] and troponin C [143] has been determined using H/D exchange and mass spectrometry.

Additional uses of H/D exchange have included methods to determine protein–protein and protein–ligand interaction. For example, a method employing H/D exchange, followed by pepsin digestion and MALDI-TOF mass spectrometry analysis, was used to define binding sites for a protein kinase inhibitor and adenosine triphosphate (ATP) to the catalytic subunit of murine 3′,5′-cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) [144]. This work also addressed the unknown interaction of human α-thrombin, complexed with an 83 amino acid active fragment of human thrombomodulin. These studies clearly demonstrate the utility of mass spectrometry in providing information on binding domains for both protein–protein interaction and protein–small molecule interaction.

Another mass spectrometry approach for studying protein–drug interaction is to identify noncovalent complexes. Since most drugs on the market today act by a noncovalent interaction with their target proteins, a rapid and sensitive method to assess binding affinities of drugs with targets would be of great benefit. Early feasibility studies for accessing noncovalent interaction, by way of ESI-MS, demonstrated mass shifts in various proteins consistent with the binding ratios of cofactors or regulators such as heme [145], or Ca^{2+} in calmodulin [146]. More recently, this approach was used to screen compounds based on binding affinities, and thus assist in selecting the most desirable candidates for more detailed NMR-based affinity studies [147]. Although there is still considerable debate concerning whether drug binding in the gas phase, as detected by mass spectrometry, is consistent with physiological binding in solution phase, this methodology has been effective for screening test compounds in several assay systems [147].

Finally, the use of mass spectrometry as an aid to structural biology studies has recently taken on another function, namely screening heavy metal compounds for phasing studies in x-ray crystallography.

Determination of x-ray diffraction (phasing) angles is required for interpretation of complex protein x-ray crystallography data, where no homologous protein structure is known. This is often accomplished by multiple isomorphous replacement, whereby multiple heavy-atom compounds (e.g. $HgCl_2$, $KAuCl_4$, and K_2PtCl_4) are randomly soaked into the crystals and subsequently analyzed by x-ray diffraction. The challenge for the crystallographer is that this method is purely empirical. Thus, screening greater than 100 heavy metal compounds requires time and consumes precious purified protein. A few recent reports indicate that mass spectrometry can be used as a fast and effective screening tool to determine which heavy metal compounds satisfactorily interact with a given protein and thus allow crystallographers to concentrate on the best compounds for their soaking and phasing studies [148,149].

3.3.3. Mass spectrometry in proteomics

Proteomics has become a buzz-word in the pharmaceutical industry, with the promise of being able to understand disease processes, toxicity, and mechanism-of-action of compounds, based on comparative protein profiles between normal and altered cells or tissue. The prototypical proteomics experiment involves isolation of total protein from normal versus altered biological samples, protein separation on two-dimensional (2D) polyacylamide gels, and then determination of which proteins are up- or down-regulated by comparing spot intensities from respective gels. The premise is that by understanding how proteins are regulated in normal versus altered specimens, one can gain valuable information regarding which biochemical pathways might be involved in the process. The number of proteins that are up- or down-regulated varies with each experiment. On average, however, 50–100 proteins show different levels of expression in a given experiment. Unfortunately, spots on a gel are of limited utility without knowing which enzymes they represent. In addition, the quantity of protein in each spot (femtomole range) is often below the detection capability of conventional chemical sequencing methods. As reviewed more rigorously elsewhere [150], the role of mass spectrometry in this process is to rapidly identify proteins of interest from the 2D gel spots through a combination of various techniques including peptide mass fingerprints and peptide sequence tags coupled with database searching or by de novo peptide sequencing.

First-tier identification: peptide mass fingerprinting. Peptide mass fingerprinting is the ability to correlate chemical or protease-digested proteins to known proteins in a database. This technique was first described in 1993 [151–153] and has since been established as one of the primary protein identification techniques in proteomics. As the name implies, a collection of individual protease-induced peptide masses for a given protein are often unique (like a fingerprint) to that protein. With the development of high resolution MALDI-TOF mass spectrometers, capable of routinely producing peptide mass accuracies of better than 50 ppm, a search of as few as 3–4 peptides can produce a unique protein identification. Second and third generation web-based search algorithms (ProteinProspector, Mascot, PROWL, MOWSE, PeptideSearch, MassSearch), now incorporate various post-translational and chemical modification possibilities into the search to factor in phosphorylation, nitration, and oxidation, to name a few. Finally, many of these search protocols can also be linked to the output of a mass spectrometer for automated real-time database searches, as the peptide mass fingerprint data are collected. Thus the speed, sensitivity, and automation of the peptide mass fingerprint approach has made MALDI-TOF mass spectrometry the logical tool of choice for first-tier protein identification in most proteomics programs.

Second-tier identification: peptide sequence tagging. In the event that a protein is not unambiguously identified by the MALDI-TOF peptide mass fingerprint approach, LC-(ESI)MS/MS product ion methods are used as a second tier in the protein identification process. One of the first reported MS/MS-based approaches for identifying unknown proteins was the peptide sequence tag method [154]. This strategy employs minimal manual interpretation of product ion spectra, coupled with search constraints based on the

molecular weight of the peptide and the chemical or enzymatic cleavage method used to produce the peptides. This work demonstrated the ability to unambiguously identify peptides and proteins with as few as two or three amino acids derived from a product ion spectrum. Improved algorithms were subsequently developed [155–157] for identifying proteins based solely on product ion spectra, with limited or no manual data interpretation required.

Initially, triple quadrupole instruments were most commonly used in protein identification protocols employing LC-(ESI)MS/MS analysis of peptides. However, today the instrument of choice for the high-throughput requirements of second-tier protein identification is the ITMS. Advantages of ion traps over the triple quadrupole instruments for this approach to protein identification include lower cost, the ability to more rapidly perform data-dependent product ion experiments during a LC separation, and the ability to program one set of collision conditions that will uniformly fragment most peptides. This latter advantage is a function of the collision process in an ion trap in which a general set of fragmentation parameters can be determined that produces robust (complete set) product ions from nearly all peptides. Conversely, even with the ability to scan collision energies in a triple quadrupole, the overall fragmentation spectra produced for a wide variety of peptides are not as robust as those seen for an ion trap. The only substantive disadvantage of the ITMS (relative to the triple quadrupole), for this application, is its inability to scan for low *m/z* immonium ions that can be diagnostic for specific amino acids. However, for protein identification by database searching (proteomics), the robust product ion spectra more than compensate for the loss of immonium ions, when using the ITMS as the second-tier method.

Third-tier identification: de novo peptide sequencing. The primary limitation for peptide mass fingerprint and peptide sequence tag-based approaches, is the requirement that the protein or corresponding DNA sequence reside in a searchable database. This limitation can have significant consequences when profiling rat, mouse, human or proteins from other organisms with incomplete genomic information. Thus, until the entire genome of the organism under investigation is available in a database, it will remain necessary to provide de novo sequence information from protein spots that could not be identified by first- or second-tier methods.

With creative sample preparation techniques, MALDI-TOF mass spectrometry has proven useful in generating de novo peptide sequencing data. For example, a ladder sequencing method has been reported [158], which utilizes Edman degradation-type chemistry that includes a small percentage of a noncleavable derivatizing reagent. Thus, a set of polypeptide fragments that vary by single amino acid units is produced. A similar concept to produce C-terminal ladder sequence information was achieved by controlled digests with carboxypeptidase Y [159]. In either case, the resulting set of polypeptides can then be sequenced, based on the mass differences readily detected by MALDI-TOF-MS. However, each of these ladder sequencing methods requires purified peptides of sufficient quantities (femtomole to picomole) to produce the ladder sequences prior to mass spectrometry analysis. Other derivatization protocols have recently proven to be less time consuming, require much less sample (low femtomole) and are applicable to peptide mixtures. For example, one new method produces a fixed negative charge at the amino terminus of peptides, by sulfonation [160]. This forces the addition of a mobile proton along the amide bond backbone, for detection in the positive ion mode. The net effect is an enhancement of charge site-initiated fragmentation of the backbone amine bonds and a selective enhancement of C-terminally derived fragments (*y* ions). A subsequent report demonstrates the utility of this method for de novo sequencing by MALDI-PSD-TOF mass spectrometry and ESI-MS/MS of mixtures of peptides extracted from 2D gels, following a trypsin digestion [161].

Although MALDI-TOF mass spectrometry techniques can be used for de novo peptide sequencing, with the types of sample manipulations described above, ESI-MS/MS of protein digests generally represents a faster, less problematic, and more comprehensive alternative for third-tier protein characteriza-

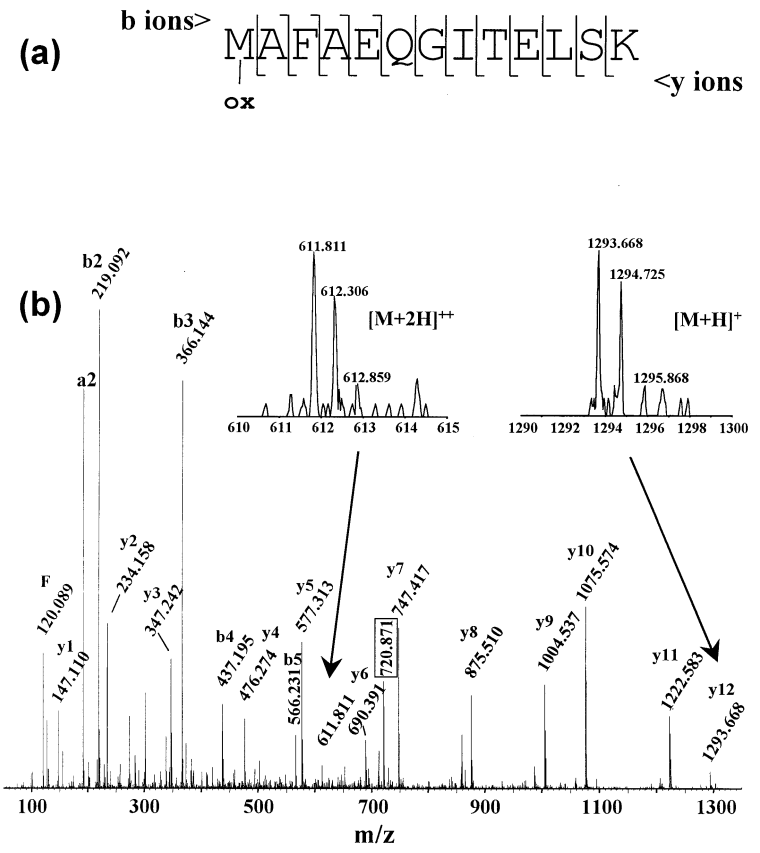

Fig. 7. Product ion spectrum from nanospray-Qq-TOF analysis for de novo peptide sequencing. A peptide mixture was recovered from an in gel trypsin digestion of a silver-stained spot (<1 pmol), desalted on a C18 Zip-Tip, and loaded into a nanospray tip. (a) Derived sequence depicting the *b* and *y* ions. (b) Representative data resulting from CID of the doubly protonated peptide ion (*m/z* 720.9), which yielded a complete set of *y*-type ions. Note the ability to detect the charge state for even the low abundance fragment ions by the isotopic distribution when the mass range is expanded (insets). These data were sufficient to define the entire peptide sequence (except L/I isobars), including the oxidized methionine.

tion in proteomics. In fact, with its recent commercial availability, Qq-TOF-based instrumentation has rapidly become the preferred option for this application. In proteomics, clear advantages of this new hybrid instrument stem from added sensitivity, resolution, and mass accuracy. For low abundance product ions, it is often difficult to distinguish real ions from background noise on ITMS or triple quadrupole systems. With the Qq-TOF, product ion peaks yielding only a few counts can be assigned as real ions based on their clearly detectable natural isotope distribution patterns (Note: such de novo sequencing experiments are typically conducted with open resolution on the quadrupole mass analyzer, resulting in transmission of polyisotopic precursor ions, thus producing polyisotopic product ions). As an example of the quality of sequencing data generated in this way, tryptic peptides were extracted from a spot on a 2D gel, desalted on a C18 Zip-Tip column and loaded directly into a nanospray tip for introduction into an ESI-Qq-TOF instrument. Each of several resulting peptide ions, produced from this single sample, was subjected to MS/MS product ion analysis and the sequence determined by mass differences in the resulting product ion spectrum. One of these spectra (products of *m/z* 720.9, an $[M + 2H]^{2+}$ ion) is shown

in Fig. 7. From this subpicomole quantity of peptide, the complete set of *y*-type ions produced was sufficient to determine the entire sequence (except leucine/isoleucine (L/I) isobars), including the oxidized methionine.

These few examples demonstrate that the concept of sequencing proteins, solely by mass spectrometry-based methodologies, has become a reality in just a few short years.

Protein mixture characterization without prior 2D gel isolation. Although the overwhelming majority of proteomics projects within the pharmaceutical industry today rely on one or more of the above mass spectrometry approaches to identify proteins from 2D gels, the mass spectrometry community has been working diligently to develop methods to circumvent the cumbersome process of separating proteins on 2D gels. Progress in this direction has been reported using a variety of alternative techniques for the identification of multiple proteins in complex mixtures. For example, a multiprotein complex from purified yeast sliceosomal U1 snRNP has been characterized by digestion of the entire complex, followed by identification of all component proteins by LC-MS/MS [162]. Similarly, trypsin digestion and a multidimensional on-line chromatographic system have been used to identify over 100 protein components from the yeast ribosomal complex, based on MS/MS sequencing and database searches from a single run [163]. Multidimensional chromatography provided the separation necessary to allow serial collection of MS/MS product ion spectra on the immense number of peptides produced by trypsin digestion of the whole protein complex. Another alternative for identifying multiple proteins in digestion mixtures involves a method described as “peak-parking,” using a variable flow LC-ESI interface coupled to a mass spectrometer [164]. This system causes the LC flow to be stopped (parked), as a peptide is detected, to allow sufficient time to generate product ion spectra on multiple peptides in a given peak, before the flow is resumed. Although this method was originally reported for the identification of multiple proteins from a single 2D gel spot, it is likely to also be amenable to characterization of multiprotein complexes without prior 2D gel separation.

Perhaps the most promising approach to protein profiling without 2D gel separation methods is the isotope coded affinity tag strategy [165]. In this methodology, two reagents are made, both containing a thiol-reactive group and a biotin tag. The difference is that one reagent contains a linker labeled with eight deuterium atoms (heavy linker), while the control reagent is unlabeled (light linker). After solubilization and reduction of proteins from a comparative protein profile experiment, the control proteins are reacted with the light linker and the experimental proteins reacted with the heavy linker. A 1:1 mixture of the two samples is digested with trypsin, followed by isolation of the tagged peptides on an avidin column. LCMS of tagged peptides provides the relative quantities of each protein (based on the H/D intensity ratio of the tagged peptide pairs), while product ion spectra provide the sequence information needed for protein identification. Although the results presented are from narrowly controlled experiments, they demonstrate an error for known concentration differences of less than 10%, with a detectable difference in concentration of the pairs of peptides in the range from 1:1 to 1:200. Of course, the limitation of this method at present is that it is only valid for cysteine-containing proteins, but this type of approach is only in its infancy.

In summary, the emergence of proteomics as a key drug discovery and characterization technology in the pharmaceutical industry is a direct result of the convergence of genomic sequence information and the advances in protein mass spectrometry. In less than ten years, identification of proteins by mass spectrometry has gone from state-of-the-art technology to a somewhat routine analysis. Now mass spectrometry is poised to be a leader in the next great challenge in proteomics, the elimination of 2D gels for protein profiling.

3.4. Emerging mass spectrometry technologies for protein/peptide characterization

Several emerging technologies in protein mass spectrometry promise to expand its utility in drug

discovery. Some of the more interesting new areas include imaging of tissue by MALDI-TOF, bacterial identification, microfabrication, and cross-linking studies to provide distance constraints for protein molecular modeling. A brief description of these emerging technologies follows.

Recent reports demonstrate the potential for direct analysis of proteins and other analytes from tissues, employing MALDI-TOF mass spectrometry [166–168]. The goal of this approach was to profile the protein content or the presence of a drug in a specific tissue. Samples were prepared in one of three ways: microdissection of specific tissues, followed by solubilization in MALDI matrix and analysis of solubilized proteins/analytes; blotting of wet tissue onto polyethylene membranes, followed by analysis of proteins/analytes that were passively diffused to the blot; or preparation of thin sections of tissue in which MALDI matrix was added directly onto the features of interest, followed by direct analysis of the proteins/analytes from the section. These methods were able to show differential expression of proteins in various sections of mouse colon [168], rat pancreas, rat pituitary, and human buccal mucosa [166]. The few implications of such methods for drug discovery would include the possibility of profiling diseased versus nondiseased tissues for protein markers or as a direct method to determine whether a drug compound gets to the required in vivo target.

Whether the interest is to serotype a pathogen from a clinical sample or to test for contamination of food products, the need for fast and efficient methods of bacterial identification continues to be a challenge in the health care industry. Several recent studies have indicated that MALDI-TOF mass spectrometry analysis of intact bacterial cells or bacterial cell extracts can yield data on diagnostic proteins, characteristic of the specific microbe of origin [169–171]. Therefore, this approach shows promise as a fingerprinting technique for rapid identification of bacteria.

Another area of protein mass spectrometry that has drawn a great deal of interest has been microfabrication. This area has developed out of the need for lower flow rates of infusion into an ESI source to increase sensitivity, coupled with requirements for high throughput protein identification in proteomics projects. One of the first reports in this area described a multichannel etched inlet device that could be attached to the spray source of a mass spectrometer for automated nanospray applications [172]. Several other microchip-type inlets have also been described [173–175], including a multichannel silicon etched device containing a miniature sample reservoir and a 5×10 μm channel spray tip for each sample [176].

Finally, a method using bifunctional cross-linking reagents, protease digestion and mass spectrometry, to provide distance constraints for a native protein tertiary structure has been described [177]. In these preliminary studies, basic fibroblast growth factor 2 (FGF-2) was used as a model system. The tertiary structure was probed with bis(sulfosuccinimindyl) suberate, a reagent specific for Lys–Lys cross-linking. Eighteen unique intramolecular cross links were identified. The intramolecular distance constraints, coupled with a computational molecular model were all consistent with the tertiary structure on FGF-2, identifying it as a member of the β-trefoil fold family. Although there are still considerable technical challenges to overcome before this type of technique will be widely applicable, it has the potential to significantly impact structural biology and molecular modeling.

4. Bioanalytical quantitation

4.1. Pharmaceutical significance

From a fundamental standpoint, quantitative measurement of drugs, metabolites, and biomarkers in biological matrices as a function of time or experimental conditions facilitates the understanding of changes to both a drug and its environment. Measurement of drug and metabolite concentrations provides absorption, distribution, metabolism, and elimination (ADME) information that define the effect of the environment on a drug. Measurement of biomarker levels, in addition to other pharmacodynamic effects such as efficacy or adverse reaction, indicate the effect of a drug on its biological environment. From a

pharmaceutical perspective, bioanalytical data generated throughout discovery are used to evaluate and/or predict important biological effects of a drug such as efficacy, toxicity and drug/drug interactions. Bioanalytical data also play a major role in drug development efforts such as selecting a route of administration, optimizing the formulation, identifying the dose level, and refining the dosing regimen.

Prior to the early 1990s, bioanalytical quantitation was largely utilized for selected applications in drug development. However, as data became available showing that 40% of drug candidates were failing in development due to poor pharmacokinetic (PK) properties, there was a concerted effort to obtain these data earlier in discovery to aid in the decision to advance drug candidates to preclinical trials [178]. Over the past decade, the applications of bioanalytical quantitation have expanded as the advances in instrumentation have made it more practical to obtain high throughput measurements at low part-per-billion or even part-per-trillion concentrations of target compounds in the presence of salts, proteins and other endogenous materials. In addition, the value of having more bioanalytical data earlier in drug discovery has been repeatedly realized by increasing the probability that selected drugs will produce favorable results in costly and time-consuming clinical trials.

As a result, generation of quantitative data to determine the ADME characteristics of a drug occurs very early in discovery using a variety of in vitro and in vivo experiments [179] (Fig. 1). For example, to predict or model drug absorption into the bloodstream, in vitro measurements of drug concentrations are often performed using Caco-2 cells or various membranes [180]. Other examples of in vitro studies include metabolic stability in hepatocyte or microsomal incubates, protein binding, and early identification of metabolites (see Sec. 5.). Metabolites that are of significance (i.e. particularly abundant, potentially active, or toxic) are often quantified throughout the development program to answer many of the same questions as described in the following for parent compounds.

Pharmacokinetic data are also gathered early in the discovery process using small animals, such as rats. Drug levels are determined as a function of time in matrices such as plasma, urine, and tissues to define systemic absorption and elimination. For given indications, other biomatrices may also be probed such as saliva, synovial fluid, gingival crevicular fluid, feces, bone, cartilage, and various target organs to determine site-specific drug delivery and elimination. The quantitative data generated in these studies are rapidly collected for a large number of compounds; typically in the range of several dozen for a given program. The nature of these studies requires rapid development of high-throughput assays where complete optimization of conditions and thorough validation are not necessary [181].

As test compounds progress into preclinical studies, further animal data are obtained; however, the number of candidates decreases to a few compounds per program, whereas the amount of data collected for each drug greatly increases. In addition, validation and quality control requirements for the bioanalytical methods increase in rigor [182]. As progression continues into phases I and II clinical studies, human data are obtained that define safety and then efficacy. Concomitantly, bioanalytical data are generated to correlate these pharmacodynamic effects with levels of parent compound and target metabolites and to optimize drug delivery. These measurements are usually performed in plasma, to determine systemic exposure, and possibly in other selected matrices for specific indications. In phase III clinical studies, bioanalytical assays are used to process thousands of samples from a diverse human population to establish the suitability of the drug for a variety of genotypic and phenotypic conditions. After FDA approval of a drug, phase IV investigations increasingly involve PK studies to further optimize safety, efficacy, route of administration, formulation, dose level and dosing regimen so that second generation products can be more effective and/or increase the level of patient and physician acceptance. These data may also ultimately support a switch from prescription to over-the-counter distribution.

In addition to measuring drugs and metabolites, a very common bioanalytical measurement need is the in vivo and occasionally in vitro quantitation of

biomarkers. Changes in the endogenous levels of these compounds may be predictive of and/or correlate with a biological effect caused by an applied stimulus such as drug administration. These data can provide insight into mechanism-of-action and determine the effect(s) that a given dose has on various biochemical pathways. For example, reducing the rate of metabolism of arachidonic acid to form eicosanoids, such as leukotriene B_4 and prostaglandin E_2, would be indicative of anti-inflammatory properties [183]. Similarly, catecholamine levels are indicative of sympathetic nervous system activity [184].

4.2. Evolution of mass spectrometry technology for bioanalytical quantitation

Bioanalytical applications in drug development have both benefited by the advances in mass spectrometry and driven the development of mass spectrometry-based techniques that provide lower detection limits, improved specificity and higher throughput for the determination of target compounds in complex biological mixtures. These advances have provided flexibility for the pharmaceutical researcher to utilize the optimal combination of analytical instrumentation that produces the desired level of speed, sensitivity and ruggedness to efficiently answer business questions with a predefined level of assurance in the quality of data that are generated. Key instrumental configurations that have been used over the past 20–30 years are highlighted in the following.

From the early 1970s through the early 1990s, mass spectrometry-based bioanalytical work was largely performed using GC separations with electron or chemical ionization [185–187]. An example is the determination of 5-methoxyflavone in rat and dog plasma using EI and GC-MS/MS for PK applications [188]. With this approach, a 1 ng/ml lower limit of quantitation (LLOQ) was achieved with a throughput of seven samples per hour. Another example is the determination of the anti-inflammatory drug, tebufelone, in human plasma [189]. This semivolatile ketone is amenable to direct analysis by GC-MS/MS; however, a nonvolatile acidic metabolite and an unstable hydroxy metabolite were not able to be analyzed under the same conditions. To extend the applicability of this method, flash derivatization was performed in the injector port to increase the volatility of the metabolites so that quantitation of the parent drug and both metabolites could be performed in a single assay [190].

Derivatization has also been commonly used to increase sensitivity for GC applications. In particular, electron attachment negative ion GC-MS/MS is extremely sensitive, with on-column detection limits in the high attogram to low femtogram range for compounds with high electron affinities. A pentafluoropropionic anhydride derivative was shown to lower detection limits for norepinephrine (NE) determination in rat and dog plasma. This electronegative reagent improved both the chromatography and the electron capture properties, which resulted in a method capable of the determination of NE in small volumes of plasma containing as little as 1 pg per sample [191]. Other examples of the extreme sensitivity of negative ion GC-MS/MS include the low pg/mL detection limits achieved by derivatization of thromboxanes, prostaglandins and other arachidonic acid metabolites in plasma [192] and the 7 pg/mL detection limit reported for fluprostenol in a 0.1 mL plasma sample volume [193].

Derivatization successfully extends the applicability and sensitivity of GC-MS/MS; however, it lengthens the time for method development and sample analysis. It also adds complexity to methods, raises the level of expertise needed for analysis and increases the possibility for batch failure. For this reason, during the 1970s and 1980s many bioanalytical methods for nonvolatile compounds were developed using LC-based systems with ultraviolet or fluorescence detection. In many cases, these techniques provided adequate data, but the poor specificity of the detectors generally necessitated extensive sample preparation and lengthy separation. This resulted in significant time required for method development and the instrumental throughput was very low, at a few samples per hour. In addition, detection limits were often much higher than desired, at mid-ng/mL to μg/mL levels, depending on the chromophore of a given molecule [194]. During that time

period, enzyme-linked immunosorbent assays or radio immunoassays were alternative approaches that were most practical when used for a large number of samples as would be encountered for biomarkers or drugs in clinical trials. The development of antibodies is a time consuming process and even after completion, these assays often suffer from cross reactivity with chemical analogs, such as metabolites, and generate data of poorer accuracy and precision than chromatographic-based techniques. Sensitivity, however, is usually quite good with pg/mL levels attainable [195].

It became obvious that the marriage of LC with mass spectrometry would provide superior attributes for bioanalytical quantitation, compared to many of these technologies. Bioanalytical assays were reported with early LC interfaces including continuous flow FAB [196], particle beam [197], thermospray [198], and moving belt [199]. These interfaces had varying degrees of success, but the introduction and development of ESI and APCI interfaces provided excellent sensitivity, a broad range of compound applicability, superior ruggedness and lower chemical noise that resulted in an explosion of LC-MS/MS bioanalytical applications. Flow injection using these interfaces has also been performed, but for bioanalytical applications, the additional specificity and assay ruggedness gained with a LC separation is usually preferred [200].

ESI and APCI have been successfully used in conjunction with a number of mass analyzers for bioanalytical quantitation such as ion trap [201], time-of-flight [202] and sector mass spectrometers [203]. However, the quadrupole, and particularly the triple quadrupole, has become the instrument of choice for pharmaceutical bioanalytical analyses. Using the SRM mode, this instrument provides the best combination of sensitivity and specificity for quantitation of target compounds in biological fluids. Also critical for quantitative applications, it has accuracy and precision advantages over ion traps, a wider linear dynamic range than TOF detectors and is cheaper, more rugged, and easier to use than sector instruments.

Single quadrupoles have been used for bioanalytical applications [204]; however, the selectivity of triple quadrupole instruments for complex mixture analysis is far superior. The dramatic improvement in signal-to-noise ratio, when using MS/MS, is illustrated in Fig. 8, for the determination of dextromethorphan (DEX) in a liquid/liquid extract of human plasma. In Fig. 8(a), SIM of the protonated molecule produced a high background with the analyte peak barely detectable, whereas the SRM chromatogram in Fig. 8(b) shows a clean background and a signal-to-noise enhancement of more than 50-fold. This selectivity advantage results in the lowest quantitation limits, when needed, or allows flexibility with method development when extreme sensitivity is not required. For example, small sample volumes can be used; extractions can be designed for speed rather than for cleanliness; and/or problems such as ionization suppression and poor chromatography due to matrix effects can often be obviated solely by sample dilution. This level of selectivity provides insurance when

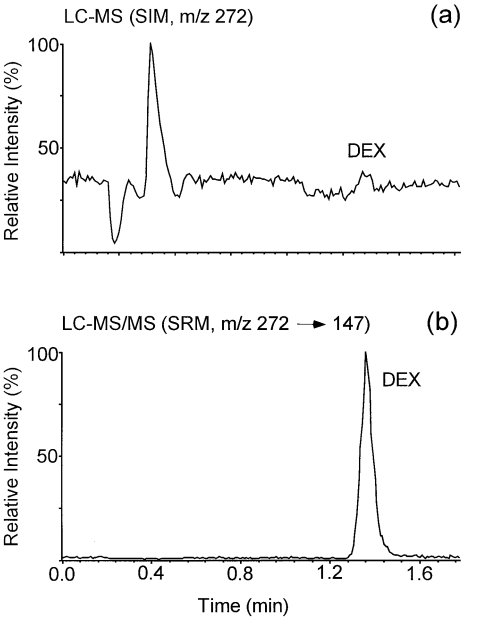

Fig. 8. Analysis of a 1 mL human plasma sample spiked with 100 pg of DEX and prepared by liquid/liquid extraction. These chromatograms were produced by (a) selected ion monitoring of the protonated molecule by using a PE Sciex API III$^+$ and (b) selected reaction monitoring of the transition from *m/z* 272 to 147 using the same sample, LC and mass spectrometer.

transferring methods from one animal species to another, or from matrix to matrix, by minimizing the potential of encountering new interferences. It also facilitates higher sample throughput because there is a reduced need for lengthy separations to resolve analyte peaks from responses due to endogenous materials.

4.3. Current strategies for bioanalytical quantitation

4.3.1. General approach

The use of a stable-isotope-labeled internal standard along with 96-well sample preparation and LC triple quadrupole mass spectrometry, with ESI or APCI, is the current gold standard for most bioanalytical quantitative methods in the pharmaceutical industry. The use of stable isotopes provides important advantages for mass spectrometry-based assays versus other analytical approaches. First, stable-isotope labeled variants are widely used as internal standards because they closely track the analyte through sample cleanup, extraction, storage, separation, ionization and dissociation in the mass spectrometer. They provide the best accuracy, precision and assay ruggedness and are often desired when critical data will be generated for a given compound. The major issues with stable-isotope-labeled compounds are the cost and time involved in the synthesis. For these reasons, chemical analogs are often used for mass spectrometry assays in exploratory or short-term investigations. Second, stable-isotope-labeled analogs can be co-administered with the unlabeled compound, by way of different routes, to measure PK parameters within a single subject to reduce variability, as described further in Sec. 4.3.3.

There are many approaches for preparation of biological samples that are assayed by LC-MS/MS. For samples that are solids such as tissue or bone, an initial grinding or shearing step is necessary to provide extractable solutions. For the resulting solutions and for liquid biomatrices, cleanup is commonly performed using the 96-well plate format by protein precipitation [205], solid phase extraction (SPE) [206], liquid/liquid extraction (LLE) [207], dilute-and-shoot (DAS) [208], or filtration [209]. The choice for sample preparation depends on the matrix and the LLOQ required for a given application. Often, SPE and particularly LLE produce cleaner samples, more rugged methods and lower detection limits than the other options. However, with these approaches, a 96-well plate takes 0.5–2 h to prepare. When exploratory work is performed, or higher quantitation limits are acceptable, a simpler and more universal cleanup such as protein precipitation or DAS may be appropriate. These approaches result in faster preparation times at 10–30 min per 96-well plate. In addition, on-line sample cleanups with systems such as on-line SPE [210], turbulent-flow LC [211] or other column switching apparatus [212] have been shown to be viable alternatives for some applications. The advantages of these systems include minimal need for sample manipulation prior to analysis and the ability to directly inject a significant amount of plasma or other matrix, which is especially useful when analyte stability is an issue. Problems with these approaches include the dedication of LC-MS/MS time to wait for on-line preparation of each sample and the complexity of method development and experimental setup, relative to the generally preferred modular batch approaches for preparation and analysis. Direct injection of biological matrices also presents biosafety as a greater concern, within the instrument laboratory.

Autosamplers for analysis of biological extracts are now designed to inject directly from 96-well plates using single needle or recently, multiple needle configurations. This newer alternative has been developed because run times for some methods have decreased below the cycle times for single needle autosamplers. Typical column lengths have decreased from more than 200 mm to the range of 30–50 mm and column diameters have also decreased from more than 4 mm to typically 2 or 1 mm and, in some cases, below 1 mm to increase sensitivity and throughput. The general ruggedness of column diameters at or below 1 mm, however, has yet to be established. In addition, flow rates have been increased to several hundred microliters per minute and in some cases more than 1 mL/min for narrow-bore columns to obtain higher throughputs. This is now possible due to recent advances in the desolvation capabilities of ESI

interfaces. For quantitative applications, the basic concept of using MS/MS with the triple quadrupole remains, but instruments continue to evolve toward more universal ionization, greater sensitivity, smaller footprints and lower costs. Software has become easier to use, with a trend toward regulatory compliant packages for electronic acquisition and storage of data.

With skilled method developers, the use of SPE or LLE preparation in combination with LC-MS/MS, can produce very high quality assays with low part per trillion sensitivity, analysis times of a few minutes per sample, accuracy and precision in the range of 3%–10% and linearity over more than three orders of magnitude. For rugged methods, batch sizes often consist of several plates or several hundred samples. Each of these bioanalytical method attributes can be optimized for a given application and a given stage in the pharmaceutical development process at the discretion of the method developer; for example, speed can be gained at the expense of sensitivity as would be important in an early animal PK study.

4.3.2. Survey of applications

A diverse array of molecules can be ionized and detected when using APCI or ESI with MS/MS. To extend the applicability of this general approach, various types of chromatography have been employed to provide separation prior to mass analysis. By far the largest area of pharmaceutical application utilizes isocratic or gradient reversed-phase separation for quantification of small organic molecules possessing at least one readily ionizable functional group. A diverse array of functionalities have been successfully quantitated including hydroxamic acids [213], synthetic prostaglandins [214], estrogen sulfates [215], benzodiazepines [216], tamoxifen analogs [217], quinolonyl beta-lactams [218], and aminohydantoins [219], to name only a few.

By employing alternative separation approaches, the applicability of LC-MS/MS can be extended to a broader range of compound classes. For example, the quantitation of the endogenous tripeptide glutathione and its oxidized dimer has been accomplished from a cell culture matrix within a single analysis, even with respective concentrations differing by up to 10 000-fold, using ion exchange chromatography with an amino column and a formic acid gradient [220]. However, peptide quantitation is often performed using ion-pairing chromatography with a reagent such as TFA [221,222] or heptafluorobutyric acid (HFBA) [223] to improve the separation. Ion-pairing chromatography has also been reported to improve the retention of particularly polar organic compounds, such as catecholamines [224] or aminoimadazoline compounds [225] with the use of HFBA as the ion-pairing reagent.

Since chiral compounds are not readily distinguishable with mass spectrometry, a LC separation must be used for chiral specificity. Many chiral LC separations are performed using normal phase solvents. With proper safety precautions, these separations can be successfully interfaced to mass spectrometers; however, improved sensitivity is reported when adding a reversed-phase solvent as a makeup flow to improve the ionization efficiency [226]. Simpler experimental setups and very good detection limits are reported with the use of reversed-phase conditions and chiral stationary phases. Examples include quantitation of ketoprofen [227] and salbutamol [228]. As with GC-based analysis, chemical derivatization can be used to improve the performance of LC-MS/MS assays. For example, derivatization of 5-aminosalicylic acid with propionic anhydride has been shown to increase chromatographic retention and improve sensitivity [229], whereas formation of dimethyl aminoethyl esters increases sensitivity for analysis of fatty acids in plasma [230].

Even with the preference of LC-MS/MS for many bioanalytical assays, there are, of course, alternative approaches that are used for given situations. For example, GC-MS/MS is still particularly useful for volatile analytes that do not separate or ionize by LC-MS/MS. In cases where extreme sensitivity is needed, negative ion GC-MS/MS continues to play an important role [231].

4.3.3. Multiple component assays

LC-MS/MS is also well suited for the analysis of multiple analytes in a single assay. Often, a generic

gradient can be used to elute target compounds with some degree of structural diversity. For example, this approach has been implemented to evaluate activity of seven major cytochrome P450 (CYP) enzymes in vitro, by monitoring diagnostic metabolites of seven specific substrates in a single assay. By applying this method, CYP inhibition or induction can rapidly be determined for new drug candidates and these data can be used for evaluation of potential drug/drug interactions [232]. Similarly, the stability of parent drugs to metabolic processes can also be performed in vitro, using hepatocytes or microsomal fractions. Incubations are often performed with individual compounds, but the specificity and sensitivity of LC-MS/MS allows the individual incubations to be combined, prior to analysis, to reduce the number of analytical samples [233].

To assess in vivo PK parameters in discovery using small animals, an *N*-in-one, or cassette, dosing strategy has been employed. Drugs are often dosed as a cocktail by administering several chemically similar drugs to a given animal and collecting samples at various times postdose. All analytes are measured in each sample with one LC-MS/MS method, which not only reduces the analytical effort, but it also reduces the animal usage [218,225,234–237]. With this approach, usually one of the administered compounds is well characterized and used as a control to insure that the PK data obtained from single compound administration are consistent with data obtained when administered as part of a cocktail.

Another example of multiple compound analysis involves intravenous administration of a stable-isotope-labeled compound and simultaneous administration of the unlabeled version, by way of another route, to determine absolute bioavailability. This approach greatly decreases biological variability and hence reduces animal usage and sample load. Using a [^{13}C,^{18}O]-labeled analog, absolute bioavailability of orally administered tebufelone was reported using GC-MS/MS [238]. Both oral and opthalmic bioavailability of timolol were measured by using the two different labeled versions of the parent drug and employing LC-MS/MS [239].

An example of an experiment that used both stable-isotope co-administration and *N*-in-one dosing is detailed in Fig. 9 [240]. Two test compounds, PGE8753474 and PGE2601204, were both administered perorally (p.o.) to a monkey. In addition, a trideuterated version of PGE8753474 and a tetradeuterated version of PGE2601204 were administered intravenously (i.v.) to the same animal. Plasma samples were prepared by protein precipitation and the analysis was performed with a rapid gradient, using ion-pairing LC-MS/MS with HFBA. A chemical analog was the internal standard for all analytes. Chromatographic data are shown in Fig. 9 for a plasma sample collected 10 h post-dose. From a single timecourse of plasma samples, PK parameters such as Tmax, Cmax, AUC, and half-life were obtained for two parent compounds from both i.v. and p.o. routes of administration. In addition, absolute bioavailabilities for both compounds were calculated and PK data for the key metabolites arising from both forms (i.v. and p.o.) of each parent compound were provided by this rich data set. Renal clearance data were also generated by analyzing urine from the same animal, using DAS preparation and ion-pairing LC-MS/MS. The use of stable-isotope co-administration and *N*-in-one dosing in a single experiment greatly reduces the analytical sample load. In addition, by acquiring all of these data simultaneously, a significant cost reduction is realized and animal use is minimized.

4.4. Emerging mass spectrometry technologies for bioanalytical quantitation

Instrumentation for bioanalytical applications is becoming smaller, cheaper, easier to use and more automated while maintaining a focus on lower limits of quantitation. Given the large sample load in the pharmaceutical industry, another major area of advancement has been increasing sample throughput. Recently, parallel sample introduction using two non-indexed sprayers and one mass spectrometer was reported [241] and effluent from four parallel separations was interfaced to a single mass spectrometer with a four-position ESI interface [242]. Column switching was demonstrated for gradient separations so that equilibration occurs on one column while

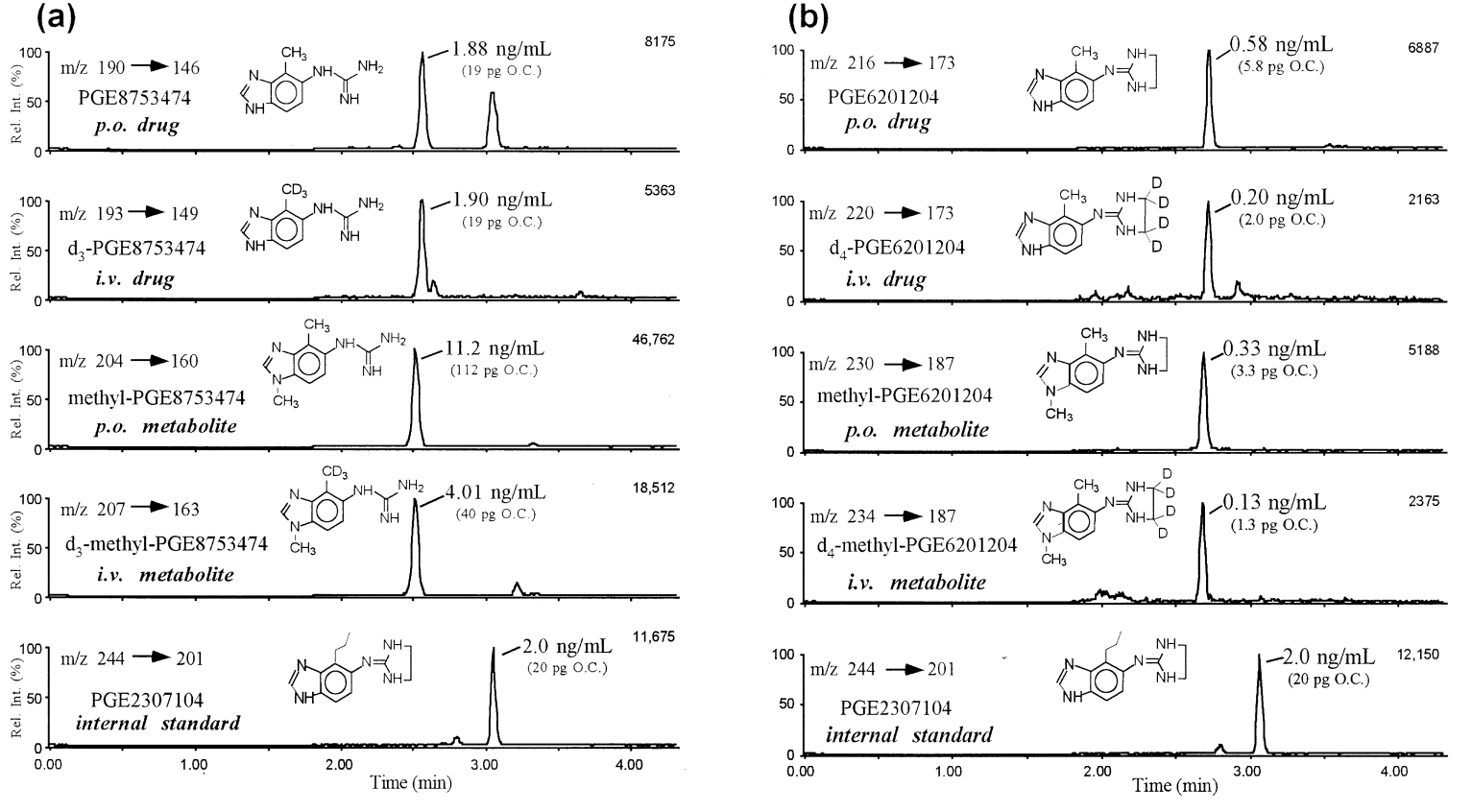

Fig. 9. SRM chromatograms showing (a) the analysis of unlabeled and labeled versions of compound PGE8753474 and key metabolites along with a chemical internal standard. (b) Analysis of the same sample reinjected showing data from a second drug candidate, PGE6201204. A second injection was performed due to instrumental restrictions on the PE Sciex API III$^+$ that limited the number of transitions to eight; newer instrumentation allows the analysis of all nine channels in a single experiment.

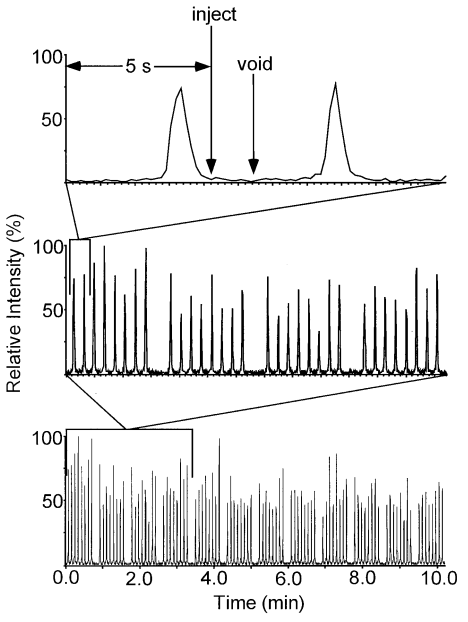

Fig. 10. Ninety-six sequential injections of d_3-DEX in MeOH (40 pg on-column) demonstrating the use of pcSFC-MS/MS to reduce the time for quantitative analysis of an entire 96-well plate to ~10 min.

separation of analytes occurs on the other column to increase throughput by twofold using a single sprayer [243]. In a more complex configuration, up to four columns have been used with a single sprayer interface by staggering the elution times so that the effluent from each column is directed to the mass spectrometer only during the retention time of interest [244]. The staggered elution approach utilizes a simpler mass spectrometer interface and allows faster scan speeds and no chance for cross talk when compared with co-elution of analytes that typically occurs with the multiple sprayer methods.

A serial approach to increasing throughput for bioanalytical applications was recently reported using packed column supercritical fluid chromatography (pcSFC). The advantages of pcSFC, compared with the more commonly used LC separations, are that flow rates can been increased to over 5 mL/min without back pressure limitations and, even with these high flow rates, the entire effluent can be directed to the mass spectrometer interface because of the higher volatility of the CO_2-containing mobile phase. Applications include the chiral determination of an acidic analgesic, ketoprofen [245], and nonchiral determination of a basic antitussive, dextromethorphan [246]. With the latter application, quantitation of DEX from an entire 96-well plate in 10 min, with part per trillion sensitivity, has been demonstrated (Fig. 10). To keep pace with the advances in the analysis portion of bioanalytical measurements, increases in sample preparation throughput are also being reported. For example, instrumentation for sample preparation has been designed to increase the number of wells per sample plate by fourfold, to 384 [247].

To minimize instrument size and to increase throughput, electrophoretic separations on a chip have been demonstrated for determination of carnitines in human urine [248]. Although amazingly small and potentially of lower cost, better sensitivity and ruggedness will be required to encourage broader acceptance. For quantitation of compounds without authentic standards, ICPMS may prove useful for analytes containing atoms that are not commonly found in biofluids such as bromine and certain metal ions [249]. The theory is that these compounds can be quantified without standards because the detector response is specific for an atom and not a compound. Accelerator mass spectrometry (AMS) has been used for PK studies, due to the attomole sensitivity for measuring selected radioisotopes. Because of this extreme sensitivity, relatively small samples are required and compounds with low bioavailability or high toxicity, where dose levels must be kept low (<1 μg/kg), can be studied using AMS [250]. Each of the approaches discussed here needs further refinement prior to routine use in an industrial setting; however, these examples provide evidence for continued advancement of the capabilities of mass spectrometry to perform bioanalytical quantitation for pharmaceutical applications.

5. Identification of metabolites

5.1. Pharmaceutical significance

The human body, and those of mammals in general, has a number of mechanisms for eliminating

xenobiotics (foreign chemical substances) [251]. One of these is to metabolize or biotransform the chemical structure of the xenobiotic. Typically, metabolism tends to be an alteration that results in a more polar chemical structure. This increases a compound's hydrophilicity and facilitates renal clearance (excretion of the substance into the urine through the kidneys) [252]. For a number of reasons this phenomenon is of interest to research, development and ultimately in the utilization of pharmaceuticals.

Metabolism of a compound may instigate, alter, prolong or halt a desired physiological effect. Additionally, biotransformation can sometimes produce a compound that has undesired toxicity, when the original drug or parent compound did not. The pharmacokinetic properties of a metabolite may be other than the expected change in elimination. Further, an efficacious metabolite must be patented to avoid potential loss of precious assets. Clearly, there are many reasons to understand the nature of a drug's metabolism in humans and in species used for safety testing prior to human exposure. The result is considerable effort to define the chemical structure of significant metabolites, particularly those present in plasma.

The types of matrices examined and the extent to which metabolites are characterized varies. During the drug discovery process, the metabolism of candidates may be explored by in vitro incubation in hepatocytes or microsomal fractions (human and toxicology species). This information can be utilized in the subsequent synthesis of improved drug candidates. The levels of metabolites formed in vitro can be relatively high and the sample matrix clean, compared to other biofluids. Further, since metabolites formed in vitro, by design, reflect in vivo biotransformations, these initial experiments can provide important data that will facilitate more challenging identifications in plasma, urine, and other matrices. Form-specific in vitro experiments are also routinely conducted to determine which CYP enzymes metabolize a drug candidate. These results are used to predict potential issues with drug–drug interactions. For early identification of metabolites formed in vitro, the exact structure of the biotransformation products may not be required. For example, it may be fairly straightforward to determine that a candidate has been hydroxylated, a phase I biotransformation, but the exact location of the oxidation is unknown. Glucuronidation (phase II metabolism) of a molecule may be readily ascertained, but the position of attachment might not be obvious, due to the availability of multiple "handles" (e.g. hydroxyl functionalities). Initially, the type and general extent or rate of metabolite formation are often adequate to provide the information required to progress a project.

Once a candidate has been progressed to preclinical and then clinical stages of development, characterization of metabolites becomes predominately in vivo based and more rigorous. This begins with identification of significant metabolites in species utilized for safety testing. At this point, the work is typically facilitated by the presence of a radiolabeled (e.g. ^{3}H or ^{14}C) analog of the drug and characterization generally takes place in urine, bile, and plasma. Care is taken in locating the radioactive atom at a position within the drug molecule that is unlikely to be biotransformed (i.e. eliminated). Identification of metabolites in toxicology study animals and subsequent matching of those metabolites to those formed in humans ensures that safety studies anticipate human exposure to the drug and its metabolites. In rare cases, where unique metabolites are formed in man, they must be chemically synthesized and tested in separate preclinical toxicology evaluations.

5.2. Evolution of mass spectrometry technology for metabolite identification

Until the mid-1980s, the only practical approach to the study of metabolism was to administer a radioisotope-labeled analog of the test compound and collect biological specimens postdose. The radiotracer was necessary to measure the total compound equivalents recovered in each sample and served as a tool to selectively determine relative levels of drug and individual metabolites, employing HPLC or TLC, with on-line radioactivity detection (RAD), or using liquid scintillation counting of isolated fractions [253,254]. Fractions containing significant radioactivity were further purified and preconcentrated to permit

elucidation of the metabolite structures, using various instrumental techniques. The effort required to prepare labeled compounds, combined with laborious sample processing and analysis procedures, made it impractical to study the metabolism of a drug candidate until it had progressed well into the preclinical or even clinical stages of development.

Historically, mass spectrometry has been a key tool in helping to assign structures for isolated metabolites. However, the rapid evolution in mass spectrometry-based technologies has more recently resulted in a dramatic enhancement in the amount and quality of structural information available, as well as the ease and speed of obtaining such data on low-level metabolites in complex biological matrices. This directly impacted the drug discovery and development process in two important ways. First, metabolite structural data is now obtained at a much earlier stage, contributing to a more rigorous drug discovery process, which in-turn increases the potential for subsequent success in the extremely costly development program. Second, structural assignments are now made more rapidly and with greater certainty at all stages, speeding the overall drug development process. As reflected in the following discussion, the availability of MS/MS, ESI interfacing for LCMS, and most recently ESI-TOF mass spectrometry, have had the greatest positive impact on metabolite identification strategies.

The initial commercial availability of MS/MS instruments, particularly triple quadrupoles, provided an opportunity to survey even crudely prepared biological samples for metabolites, without the need for radioisotopes [255,256]. The selectivity afforded by this new technology was essential in this type of work because metabolites are present at extremely low levels, within a variety of fantastically complex biological samples. However, the challenge in metabolite identification is not only to find the proverbial needle in the haystack, but also to assign a precise molecular structure to each subtly distinct “needle.” In an early example [257], raw urine was loaded directly onto a solids probe and thermally vaporized into an EI ion source. Ions produced were interrogated using MS/MS precursor ion scanning to survey for molecular ions of metabolites expected to yield a product ion analogous to that of the pyridazine-based parent drug. Once likely metabolite molecular ions were found, the experiment was repeated in the product ion scanning mode to obtain compound-specific structural information on each metabolite. This approach worked well for the metabolites of this structurally rugged compound. However, a softer ionization approach, such as FAB, was essential for more general metabolite characterization, particularly when considering common metabolic conjugates. For example, FAB/MS/MS, employing constant neutral loss scanning, was shown to be useful as a general screen for glutathione-conjugated drug metabolites excreted in bile [258,259]. Similarly, FAB/MS/MS, using precursor ion experiments, has been employed to reveal a glucuronide conjugate of a monohydroxy metabolite of tebufelone, present in crudely prepared (SPE desalting only) urine [260].

The direct interfacing of LC to mass spectrometry detection offered additional advantages for metabolite characterization. Not only could HPLC-UV or HPLC-RAD profiles then be directly correlated with mass spectrometry structural data, but the mass spectrometry data could be background subtracted to yield higher quality spectra for individual metabolites. This also provided a means of assessing the chemical purity of the chromatographic peaks and the physical separation of metabolites permitted characterization of isomers that would otherwise be indistinguishable through mass spectrometry or MS/MS detection alone. A moving belt interface, combined with methane chemical ionization, was shown useful for the LCMS profiling of nonlabile metabolites of budesonide [261]. Subsequently, as the first relatively soft ionization technique compatible with conventional HPLC flow rates, thermospray facilitated much broader use of LC-MS/MS for metabolite profiling, including the characterization of labile conjugates [262–264]. However, the advent of API interfaces, particularly ESI, has essentially obsolesced alternative ionization techniques for LCMS, as applied to metabolite characterization. This is reflected in the observation that 96% of LCMS-based metabolite identification reports, published 1998–2000, employed API [265]. Of these, ESI is favored over APCI

by greater than a 3:1 margin. In net, the ability to chromatograph almost any drug and metabolite without derivatization, extensive sample clean-up, or consideration of thermal lability has made identification tasks easier, faster, and in many cases simply feasible.

Owing to their ruggedness, ease of use, and (in the case of MS/MS) access to the most versatile set of scanning functions, quadrupole mass analyzers have evolved to become the most commonly used instruments for metabolite identification, as represented in their use in 85% of LCMS-oriented reports, 1998–2000 [265]. However, ion trap mass spectrometers have also been utilized to identify metabolites [266,267]. In addition to their relatively low cost, these mass analyzers are more sensitive in the full-scan mode than conventional scanning instruments [268]. Although precursor ion and constant neutral loss scanning are not practical with ion traps, their unique ability to perform $(MS)^n$ product ion experiments has proven useful for solving certain metabolite structure elucidation problems [269]. Although less sensitive than triple quadrupole instruments for SRM applications, the ion trap offers potential value in its ability to simultaneously generate quantitative and qualitative data, as demonstrated in a recent report of simultaneous metabolic stability measurement (parent drug loss) and metabolite identification, from in vitro systems [270].

In spite of their obvious complementary advantages, ion traps have not been widely utilized for identifying metabolites. Arguably, in their commercial forms, they have not been mated to the LCMS interfaces and computer software most suited for this work. For example, it has been our experience that the heated capillary ESI interface, used in most ion trap instruments, presents a somewhat harsher ionization-desolvation environment (relative to cone-based interface designs), making it more prone to degrading labile metabolites, such as conjugates. Ion traps are also susceptible to problems associated with an over-abundance of ions, resulting from the analysis of biological samples with little or no prior clean-up, as is common in metabolite identification work. A high abundance of co-eluting matrix ions can make it difficult to exploit the fundamental full-scan sensitivity advantage of the ion trap, as required for characterization of trace-level metabolites. Further, it is impractical to carry out all but the most basic $(MS)^n$ experiments on a chromatographic time scale. Typically, detailed $(MS)^n$ genealogical mapping of an unknown compound first requires isolation (fraction collection), followed by slow infusion of the fraction, while collecting $(MS)^n$ data.

More recently, and therefore not yet broadly reflected in the literature, commercially available ESI-TOF-MS and ESI-Qq-TOF-MS instruments are beginning to demonstrate their superiority over other mass spectrometry-based systems in most metabolite identification applications. State-of-the-art TOF mass analyzers offer two key features that translate into major benefits in this sort of work. First, unlike scanning instruments that discard most of the formed ions while acquiring a spectrum, TOF analyzers are inherently more sensitive in obtaining full mass spectra. Although ions are not as efficiently transferred from the source into the TOF mass analyzer as with a quadrupole instrument, it retains a net sensitivity advantage of more than an order of magnitude [271], for *m/z* ranges typically employed in metabolite identification. Second, ready access to mass resolutions of approximately 10 000 for small molecules ($<$1000 Da) provides empirical formula data on low-level metabolites and elemental compositions for product ions (in the case of Qq-TOF instruments). Relative to ESI-quadrupole-based systems, the advantages of ESI-TOF for the identification of metabolites outweigh the disadvantages associated with this technology. These are added cost (about 50% greater); data storage/manipulation (files are much larger); and a slightly higher skill level required to optimally operate the instruments and analyze the data. In addition, as with ion traps, a shortcoming of Qq-TOF technology is that screening for metabolites using precursor ion or constant neutral loss strategies is currently impractical.

5.3. Current strategies for metabolite identification

5.3.1. General approach

Tremendous technological advances provide the mass spectrometrist with a variety of powerful new

tools that, when properly implemented, offer unparalleled speed and certainty in metabolite structure elucidation. As a result, mass spectrometry has become the first and, in many cases, the only technique needed for identification of metabolites. The current challenge resides in developing strategies for optimal deployment or utilization of these tools. As the objectives driving the need to study metabolite structures differ between drug discovery and development, so too will the strategies for providing mass spectrometry support. In discovery, for example, there is a greater emphasis on speed versus thoroughness. Often the objective is to elucidate at least partial structures, to determine the portion of a molecule most susceptible to metabolic attack, in a time frame that can benefit the next round of compound synthesis strategies. In these cases, mass spectrometry will be the first and only analytical tool employed and, therefore, must be well integrated into the overall planning of in vitro or in vivo studies to assure seamless compatibility with sample generation and preparation procedures.

Although early integration is also important in development metabolism projects, mass spectrometry is not the first analytical procedure employed when supporting more definitive ADME studies and there is also a greater emphasis on completeness in providing metabolite structural assignments. Dosing of radiolabeled drug remains a requirement at this stage, to provide quantitative measures of the drug and its biotransformation products. All study samples are typically profiled by LC-RAD, with the resulting data used to determine the significance of each observed metabolite. Only metabolites deemed significant are then identified, at least initially, through subsequent LCMS analyses of representative samples. It is essential that chromatographic conditions employed for the LCMS work be identical to those used in the LC-RAD profiling, to assure a clear correlation between data sets and that the correct metabolites are identified. Ideally, this is accomplished by using the same model of LC and, if possible, literally the same column for both sets of work. In addition, when reanalyzing the representative samples, use of a RAD in parallel with mass spectrometry detection serves to validate this correlation. The RAD signal can be captured by the mass spectrometry data system, allowing the two chromatograms to be overlaid. Because the RAD trace displays only the drug and metabolites, attention is drawn to significant metabolites, allowing their mass spectra to be readily extracted at the exact retention time, from what is typically a complicated total ion chromatogram. A metabolite is deemed definitively identified, only after synthesis of an authentic reference compound and a match of its analytical characterization data with that of the incurred metabolite is demonstrated. Thus, the need for high certainty in the structural assignments stems from the significant synthetic effort required to prepare metabolite reference compounds, as well as the costly delays due to rework, should a proposed structure be incorrect.

With the versatile array of mass spectrometry tools now available, there are many different strategies that can accomplish the basic tasks in metabolite identification. However, it is clear that ESI-TOF mass spectrometry technology (highlighted in Sec. 5.3.2.), is fast becoming the centerpiece of rigorous metabolite identification strategies in both drug discovery and development. Further, where detailed structural information is required, the Qq-TOF is the instrument of choice. In practice, due to the high cost of Qq-TOF systems, metabolite identification work is ideally stratified, judiciously aligning a portion to less costly instrumentation. For example, our philosophy in drug discovery is to employ LC-TOF mass spectrometry for investigatory metabolite profiling, with analyses transferred to an LC-Qq-TOF system only when more detailed structural data is required. Alternatively, for development metabolism projects, a LC-Qq-TOF system is employed to support the first definitive ADME study of a drug candidate, facilitating the most rapid and certain assignment of metabolite structures. For subsequent work, such as an ADME study in a second species, an LC-TOF-MS analysis suffices, given that the focus would likely be on identity verification, rather than de novo structure elucidation.

Although ESI-TOF-based systems are preferred for most applications, there will continue to be a need for complementary tools, such as ITMS $(MS)^n$ and

triple quadrupole instruments, to play certain roles. For example, in the case of unlabeled test compounds dosed at low levels, metabolites might best be located within a chromatographic separation using precursor ion or constant neutral loss scanning on a triple quadrupole [272,273]. Also, SRM scanning capabilities of triple quadrupoles provide superior sensitivity for detecting suspected metabolites in plasma. Because metabolite levels are much higher in bile or urine, in vivo identification begins there. After defining significant metabolites in these matrices, attention turns to verification of the presence of these (or related) metabolites in plasma. However, plasma metabolite levels may be too low to acquire clear, full-range mass spectrometry or MS/MS spectra. In these instances, a SRM experiment is carried out, monitoring one or more product ions that are characteristic of the metabolite of interest. A SRM chromatographic peak, at the retention time of the corresponding metabolite observed in the excreted matrices, is normally sufficient verification of the targeted metabolite.

In spite of the power of LCMS-based approaches, GCMS will continue to offer complementary value in low-level metabolite identification, particularly when needing to precisely define positions of functionalization. GCMS provides high resolution separations, conventionally interpretable EI spectra, and access to a wide array of creative chemical derivatization procedures [274], all of which can facilitate the structure elucidation process. An earlier example illustrating these three virtues is found in elucidation of the precise structure for a glucuronide-conjugated hydroxy metabolite of tebufelone [260]. Urine collected from subjects dosed with a 1:1 mixture of tebufelone and [^{13}C]-tebufelone (carbonyl position) was previously shown to contain this metabolite, although the site of substitution was unknown. A SPE fraction containing this metabolite was taken to dryness and then subjected to a three-step chemical derivatization process, as follows: methylation with diazomethane; acetylation with acetic anhydride; and trimethylsilation with bis(trimethylsilyl)trifluoroacetamide (BSTFA). The intent was to create, in turn, a methyl ester of the glucuronic acid moiety, ethyl esters of free aliphatic hydroxyl groups, and a trimethylsilyl (TMS) ether at the phenolic position (Note: the order of derivatization was based on prior verification that the phenolic group of the parent compound was readily trimethylsilated, but not amenable to methylation or acetylation). The sample was then analyzed by GC-(EI)MS, with the resulting total ion chromatogram and EI spectrum (Fig. 11) providing a wealth of structural information. The molecular ion isotope cluster indicated a molecular weight of 704, consistent with formation of the expected methyl and three ethyl esters on the glucuronide moiety, plus a single TMS ether. This verified that the phenol position of the metabolite was not conjugated. The presence of two closely eluting, but distinct, isomers yielding identical EI spectra suggested that metabolism had resulted in formation of diasteriomers, indicating that initial hydroxylation had occurred at one of the three methylene carbons. Further interpretation of the EI spectrum, including a classic gamma-hydrogen rearrangement, ultimately led to the proposed structure.

5.3.2. ESI-TOF in metabolite identification

The resolution advantage of a TOF instrument is demonstrated in Fig. 12, which displays the MH^+ region of two ESI mass spectra of a molecular weight 358 test compound. The spectrum in Fig. 12(a) was obtained with a quadrupole, whereas the spectrum in Fig. 12(b) was collected using a TOF mass spectrometer. For most metabolite identification experiments, the mass accuracy afforded by TOF-MS allows measurement of a *m/z* value to the third decimal place, without sacrificing sensitivity. Using postcolumn addition of an appropriate reference lock-mass compound, this provides mass accuracies of better than 5 ppm. This information, along with the ability to limit elemental stoichiometry based upon the substrate (test compound) structure, typically allows elucidation of an unambiguous empirical formula for an unknown metabolite. So instead of beginning structure elucidation with only a molecular mass, as one would with nominal mass data, the first information obtained also includes the empirical formula. Although a number of biotransformations can be easily deduced, based upon nominal molecular mass of the metabolite alone (e.g.

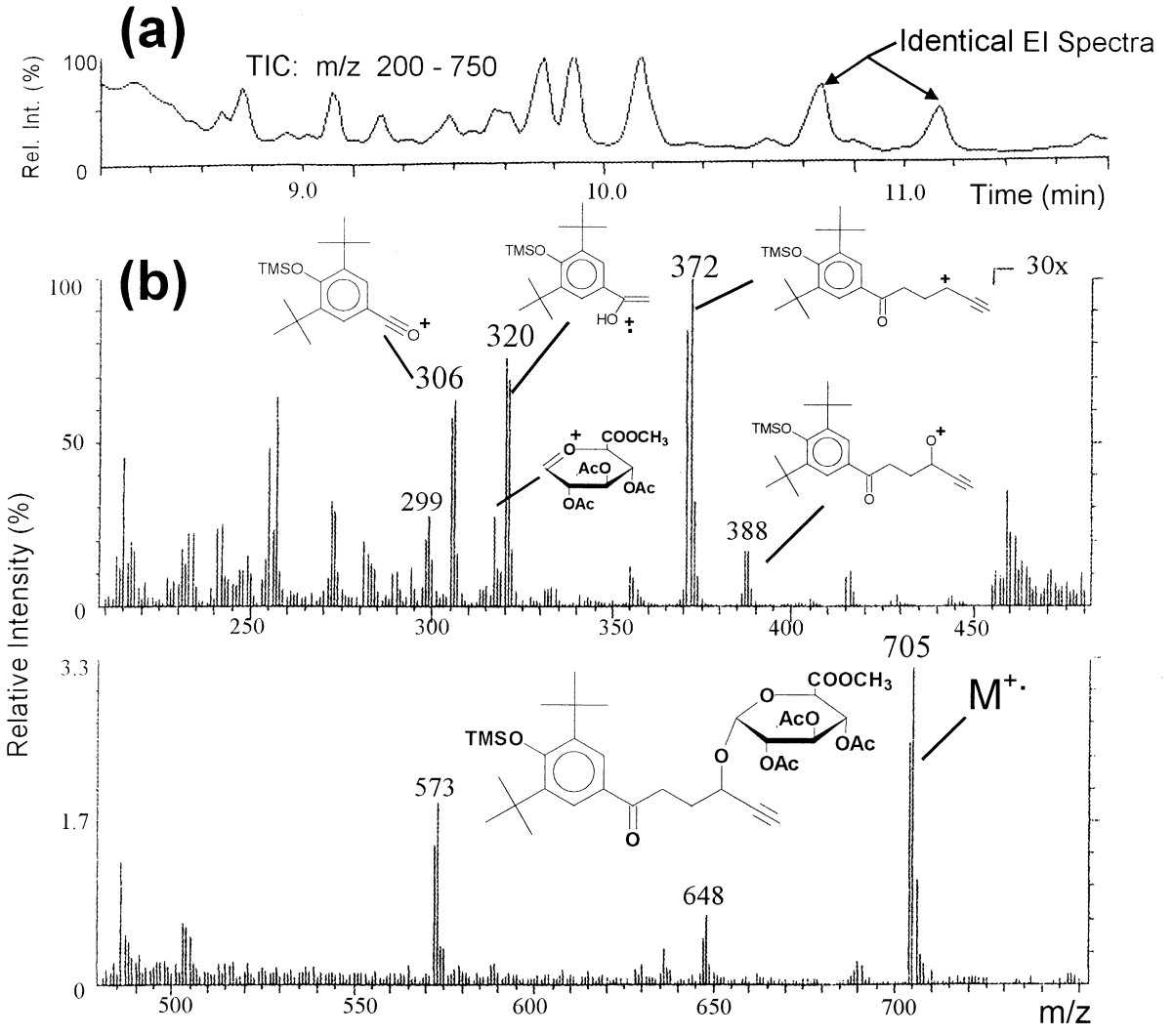

Fig. 11. (a) Expanded TIC chromatogram resulting from GC-(EI)MS analysis of a crudely isolated human urinary glucuronide-conjugated metabolite of tebufelone, following a three-step derivatization process (methylation, acetylation, and trimethylsilation). (b) One of two identical EI spectra, corresponding to a pair of fully derivatized diasteriomeric metabolites, displays structurally diagnostic fragments. Ions retaining the carbonyl carbon appear as isotope doublets, as a result of dosing 1:1 unlabeled: ^{13}C-labeled tebufelone.

glucuronidation, hydroxylation, etc.), there are ample cases where nominally isobaric possibilities occur, but can be readily discerned with knowledge of the empirical formula. Moreover, the elemental composition of metabolite product ions can readily be obtained when operating a Qq-TOF instrument in the MS/MS mode.

A perhaps under-appreciated advantage of TOFMS, relative to nominal resolution mass spectrometers, is related to analysis of data by viewing mass chromatograms. When searching for unknown metabolites in a complicated matrix, it is common to extract mass chromatrograms that would correspond to the *m/z* of potential biotransformation products (e.g. mass of hydroxy metabolite = mass of substrate + 16). With high resolution TOF data, it can be

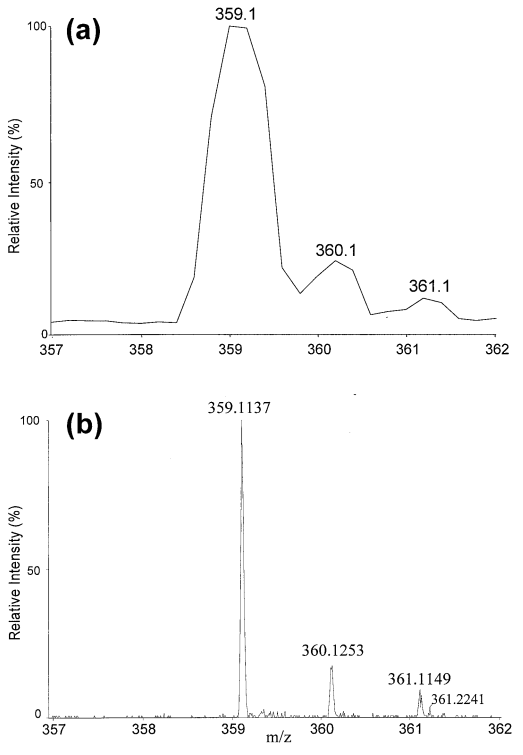

Fig. 12. Protonated molecular ion regions of ESI mass spectra resulting from analysis of a molecular weight 358 test compound, using (a) quadrupole and (b) TOF mass analyzers.

useful to extract a much narrower *m/z* window, as illustrated in Fig. 13. Dog urine, collected 8–12 h after p.o. dosing of a drug candidate, was analyzed directly by gradient LC/(ESI-TOF)MS. The resulting total-ion chromatogram (TIC) (*m/z* 100–800) is expectedly complex. A metabolite of empirical formula $C_{15}H_{19}NO_8S$ had been previously found in other species, but was not initially evident in the dog, based on LC-RAD profiles. Display of a *m/z* 374.09 (calculated mass of the metabolite, plus one proton) chromatogram, using a ±0.5 *m/z* window, mimics results expected from a nominal resolution quadrupole instrument. The result [Fig. 13(b)] was a forest of false positives that, following examination, largely turned out to be nominally isobaric substances present in the urine matrix. However, display of *m/z* 374.09, using only a ±0.05 *m/z* window greatly simplified the profile, now including peaks corresponding to the metabolite in question and two related compounds [Fig. 13(a)]. (Note: the unlabeled peaks in the ±0.05 *m/z* display result from the natural heavy isotope form of more abundant metabolites of nominal molecular weight 1 Da lower than the metabolite of interest. Their natural ^{13}C–MH^+ ions have a calculated exact mass of 374.1102.) The targeted metabolite was estimated to be present at about 1 ppb in urine and was clearly detected with approximately 100 pg analyzed (on column). Finding low-level metabolites in this way can be important from a standpoint of verifying whether or not a metabolic pathway is species dependent. For example, in this case, observation of this metabolite in the dog provided evidence that the pathway giving rise to it was accessible in all species studied, albeit less preferred in the dog.

5.3.3. Isotopic labeling to facilitate metabolite identification

An isotopically labeled analog of a test compound can be particularly useful in identification of metabolites using mass spectrometry. By employing radiotracers (a low-level radioisotope-labeled version of a test compound, blended with unlabeled compound), in metabolism studies, metabolites produced can be selectively detected in complex matrices by using LC-RAD. Incorporating a RAD in parallel with mass spectrometry detection, as described earlier, permits a direct correlation between an eluting metabolite and structural information collected at that retention time.

In cases where radioisotope-labeled analogs are not available, use of the stable-isotope cluster mass spectrometry technique [260,275,276] can provide an alternative means to help locate metabolites in the presence of complex biological matrix components. This approach typically involves the study of a 1:1 mixture of stable-isotope-labeled and unlabeled test compounds. Metabolites that retain the labeled portion of the molecule will display a conspicuous isotope doublet within their mass spectra. The utility of this concept is demonstrated in a study of PGE8753474 [240] metabolism in human liver slices, in which an equimolar mixture of this test compound and a trideutero analog were incubated for 24 h. Representative results from LC-(ESI)MS analysis of the supernatant media are provided in Fig. 14. The

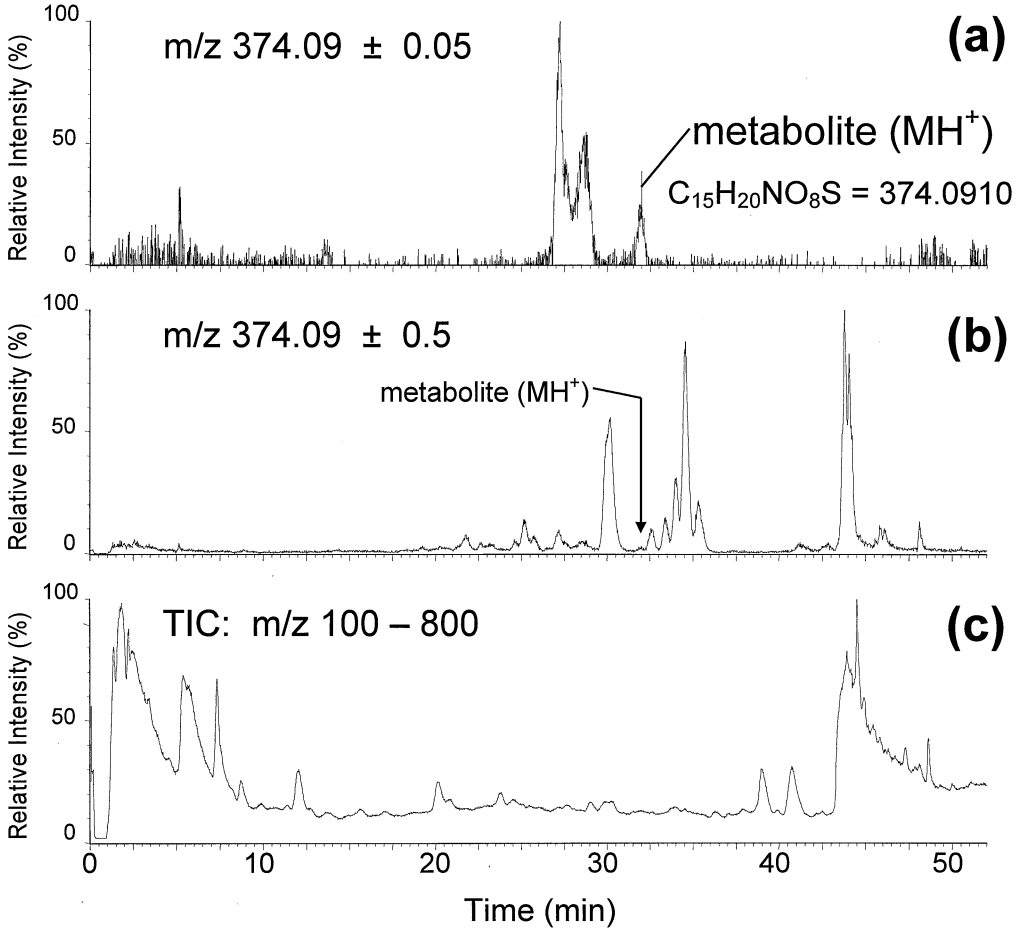

Fig. 13. (c) TIC mass chromatogram resulting from gradient LC/(ESI-TOF)MS analysis of a dog urine sample, collected 8–12 h after p.o. dosing of a drug candidate. Employing (b) ±0.5 and (a) ±0.05 *m/z* display windows demonstrates the resolution advantage of the TOFMS, in screening for a low-level metabolite.

TIC chromatogram provides an indication of the complexity of the biological matrix. Mass chromatograms at *m/z* 190 and 193, corresponding to the MH^+ of unlabeled and labeled PGE8753474, respectively, show peaks of equal intensity at the retention time of the parent compound. A background-subtracted spectrum from this portion of the chromatogram [Fig. 14(b)] displays the corresponding isotope doublet. A second isotope doublet was observed in this spectrum, suggesting the presence of a closely eluting metabolite, with a molecular ion 14 mass units greater than that of PGE8753474. Mass chromatograms at *m/z* 204 and 207 showed coincident peaks of equal intensity within this retention window, verifying the existence of a molecular weight 203 metabolite. Certainly, without use of the stable-isotope cluster technique, this metabolite may have been overlooked.

Not only do stable-isotope-labeled analogs provide a means of locating metabolites in biological matrices, but they can also be used to generate structurally diagnostic data. Continuing with the PGE8753474 example, because all three deuterium labels are conserved in the metabolite one can automatically conclude that metabolism did not occur at the labeled methyl position. Further, the ability to contrast product ion spectra of the unlabeled and labeled parent

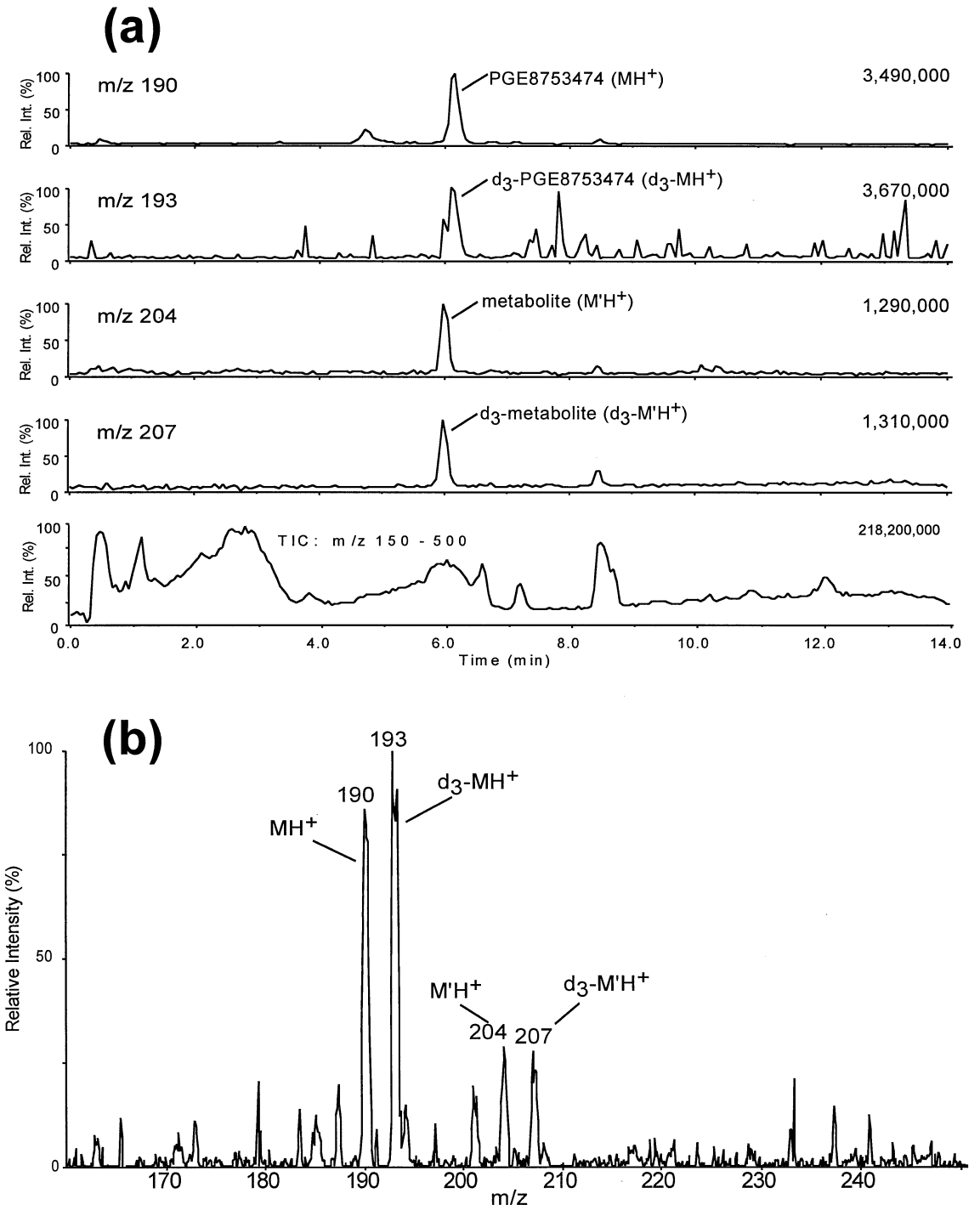

Fig. 14. (a) TIC and mass chromatograms resulting from gradient LC/(ESI-quadrupole)MS analysis of supernatant media from a human liver slice, following a 24 h incubation with 20 μM of 1:1 unlabeled: d_3-labeled PGE8753474. (b) The background-subtracted ESI mass spectrum, corresponding to the retention time of the parent compound (M), also shows evidence for a closely eluting metabolite (M′), based on the second isotope doublet (*m/z* 204/207).

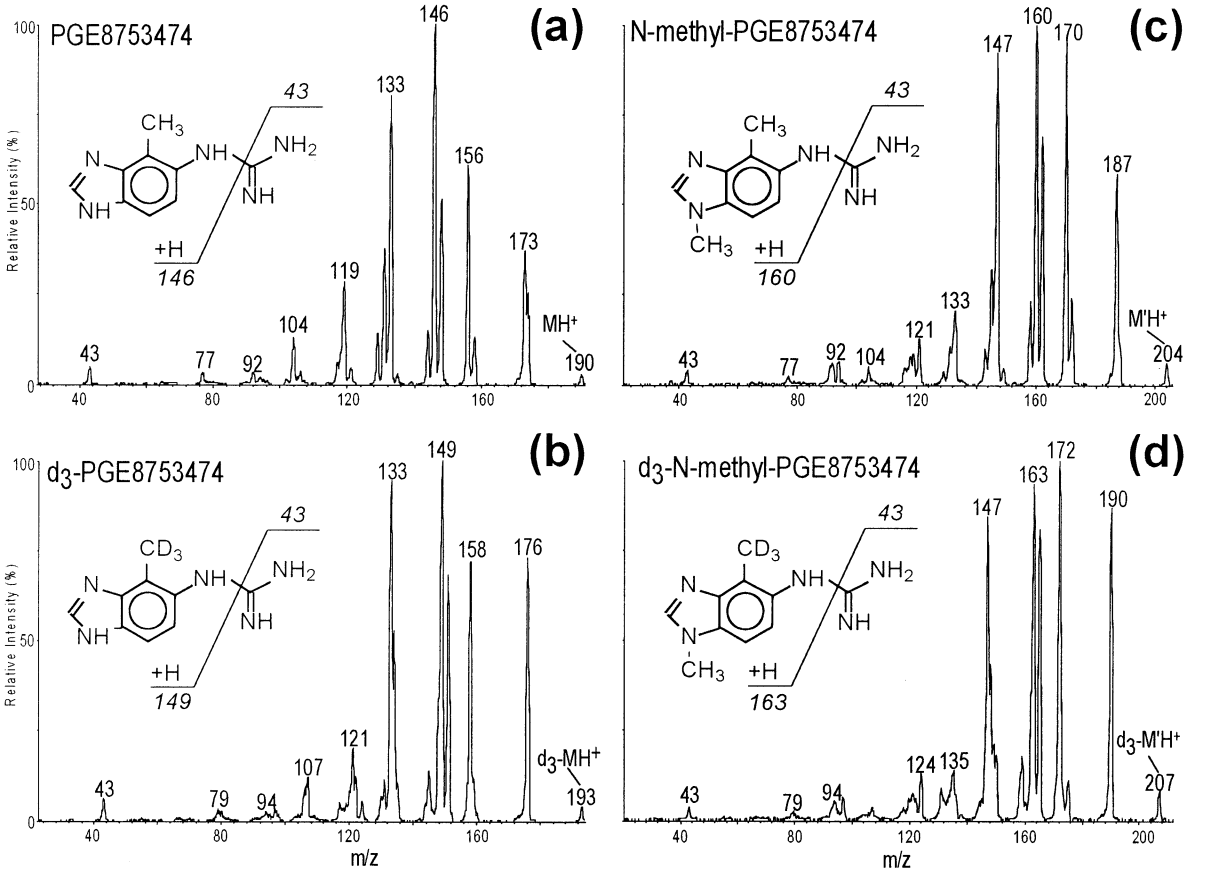

Fig. 15. ESI-MS/MS product ion spectra, obtained on a triple quadrupole instrument, for (a) PGE8753474, (b) d_3-PGE8753474 as well as (c) an *N*-methyl metabolite of PGE8753474 and (d) the corresponding *N*-methyl, d_3-analog, demonstrating the utility of comparing product ion spectra for elucidating metabolite structures.

compound [Fig. 15(a) and (b)], and then compare these with analogous data from the unlabeled and labeled forms of the metabolite [Fig. 15 (c) and (d)], aids in interpretation of fragmentation mechanisms and, therefore, in assigning structures. These data, indicated, among other things, that the amino guanidine portion of the molecule was unaltered and the resulting structural modification did not enhance fragmentation, relative to the parent compound. This led to assignment of the *N*-methyl-PGE8753474 structure shown.

5.3.4. Complementary role of NMR in metabolite identification

In many cases, mass spectrometry can provide adequate and even unambiguous identification of metabolites. However, there are often cases where structural assignments remain tentative, especially when structural (positional), conformational, or optical isomers must be rigorously identified. In those situations NMR is the key technique for ascertaining the nature or location of functional groups. Unfortunately, owing to the comparatively poor sensitivity (relative to mass spectrometry), isolation and preconcentration of metabolites from biological matrices are generally required to provide sufficient material, in a clean matrix, to permit acquisition of quality NMR data. However, recent improvements in magnet field strength, probe technology, and the successful interfacing of HPLC with NMR offer the capability of obtaining direct (i.e. without sample cleanup) struc-

tural information for metabolites in biological matrices [277–279]. To date, such applications have been limited to identification of major metabolites, generated at relatively high levels, either through in vitro incubations or by characterizing excreted biological fluids following administration of relatively high doses of previously characterized compounds. Even though the trends in NMR sensitivity improvements are promising, further advancements will be needed to render the on-line HPLC-NMR technique more universally applicable to metabolite identification problems.

5.4. Emerging mass spectrometry technologies for metabolite identification

It is not easy to predict what new instrumental development will cause the next significant improvement in the ability of mass spectrometry to further impact the identification of metabolites. More confidence can be assigned to the prediction that a number of significant improvements in various types of software will improve one's ability to accomplish this task, in terms of ease and speed. The major vendors of mass spectrometry equipment are all rapidly improving their software, designed specifically to mine data for metabolite information. Spectra that were once manually extracted can be automatically obtained from expected mass chromatograms or triggered off analog signals (e.g. RAD output). Data can be obtained by automated difference comparisons between LCMS profiles of samples and controls. Further, databases designed to compile existing knowledge or even to predict metabolism of substrates are available [280,281] and can be expected to improve. Successful integration of mass spectral data with these databases could facilitate metabolite identification efforts. Where heavy-isotope labeling is employed (or for test compounds that include an element with a significant natural heavy isotope abundance, such as Cl or Br), isotope pattern recognition techniques may become more prevalent. Such an approach may involve data-dependent scanning, where mass spectrometry acquisition is immediately switched to MS/MS (product ion scan) acquisition upon recognition of isotope patterns characteristic of the parent compound and metabolites [282]. Other work has shown how correlation analysis of MS/MS spectra can be used to distinguish drug metabolites from endogenous matrix and even match biotransformation products to substrates in mixtures of test compounds [283]. In sum, it seems certain that the future of metabolite identification by means of mass spectrometry will be as interesting as the past.

6. Identification of impurities and degradation products

6.1. Pharmaceutical significance

Impurities in pharmaceuticals arise from a variety of sources. For example, drug substances may contain synthetic reactants, intermediate products, by-products, residual solvents and/or process impurities [284]. In drug products, impurities may be formed by degradation as a result of instability to temperature, pH, humidity, light or reaction with containers. Often, degradation products are initially identified in accelerated stability studies or by forced degradation under controlled conditions. Common chemical pathways for degradation include oxidation, hydrolysis, dehydration, deamidation, dimerization, rearrangement and formation of adducts with excipients or package components [285].

Determination of the structures of these impurities and degradation products must be performed for a variety of reasons. Knowing the structures of reaction impurities can help to optimize the synthesis and scale-up of compound production and help to minimize the levels of impurities that are produced. In addition, identification of the impurities can lead to more secure patent protection, as certain impurities may be indicative of a particular synthetic route. Understanding the mechanisms of degradation can help chemists synthesize new compounds with improved stability and also help formulators use existing compounds to create products that are more stable. In addition, these impurities and degradation products may possess toxicologic or pharmacologic properties

that must be determined from both a quality and safety standpoint [286].

Because of the importance in understanding the role that impurities and degradation products play in pharmaceuticals, regulatory agencies throughout the world consider the assessment of the purity of new drug substances and drug products a critical step in the development process. The International Conference on Harmonization has drafted specific guidelines for qualification, identification, and reporting thresholds for impurities in both drug substances and drug products depending upon the expected maximum daily dosage. For drug substances, the identification and qualification thresholds for an impurity are the lower of 1 mg intake per day or 0.1%, for a maximum dose of $\leq$2 g per day. For this dose range, the reporting threshold of a designated impurity is set at 0.05%. If the maximum daily dose exceeds two grams per day, the identification and qualification thresholds are 0.05%, whereas the reporting threshold is 0.03% [287].

For new drug products, identification, qualification and reporting thresholds are also provided [288]. For reporting purposes, the threshold is 0.1% for a dose of $\leq$1 g and 0.05% for $>$1 g. For identification and qualification, thresholds are provided for four different ranges of possible maximum daily doses that allow the lower of a given percent of the maximum dose or a total daily intake (TDI) of the impurity to be present. For identification, a maximum daily dose of $<$ 1 mg allows 1% or 5 μg TDI of an impurity. Through the intermediate ranges, the percentage of an impurity that is allowable is gradually reduced to the highest specified maximum daily dose of $>$2 g, which allows 0.1% of a degradation product to be present. For qualification, the threshold of the lowest specified range is less stringent at 1% or 50 μg TDI for doses of $<$10 mg and the threshold of the upper range is the same as it is for identification at 0.1% of the degradation product allowed for a maximum daily dose of greater than 2 g.

Clearly, the thorough identification and characterization of impurities and degradation products presents analytical challenges. There are relatively low levels of unknown compounds in the presence of the parent compound and, in the case of drug products, analysis is even more difficult due to the presence of excipients, package materials and possibly other actives. These comprehensive efforts to identify impurities and degradation products begin with early safety and clinical lots and build on information generated from the rapid characterization and cursory stability assessments carried out in upstream discovery, as described in Sec. 2. These efforts are most intense when compounds are initially investigated or when changing synthetic routes, suppliers or formulations.

6.2. Evolution of mass spectrometry technology for identification of impurities and degradation products

In the late 1970s and early 1980s, identification of impurities and degradation products was often performed by isolation and collection of material followed by direct analysis. The role of mass spectrometry was often to provide a molecular weight. Much of the structural elucidation work was performed by spectroscopic means. An example is the determination of impurities in sulfasalazine [289]. Impurities were isolated by thin layer chromatography and liquid extraction. After which, they were characterized by off-line mass spectrometry, IR, UV, and NMR. The off-line approach can provide good structural information and it is even used in certain cases today. However, the preferred approach is the use of a chromatographic separation prior to on-line structure elucidation, as it is a faster and easier method of analysis. In the late 1970s, the only real on-line option for mass spectrometry was GCMS. Reported examples of on-line impurity identification, based on GCMS, include the determination of volatile impurities of clofibrate [290] and norethindrone [291]. Despite the fact that many pharmaceutical compounds were not amenable to GC, due to thermal instability or insufficient volatility, even in the early 1980s the preferred separation approach was still GC and GCMS was highly preferred to the available LCMS techniques [292].

Evolution of LCMS interfaces through the 1980s greatly facilitated on-line analysis as many small

organic molecules developed by pharmaceutical companies are semivolatile and more amenable to LC than GC. Examples include the use of thermospray to identify impurities in a series of potential anticancer drugs [293] and in Nolvadex [294]. However, as is the case with many other pharmaceutical applications, the advent of ESI and APCI provided a superior option for interfacing LC with mass spectrometry for impurity identification and has changed the way this work is performed. These sources can be used with adequately high flow rates and can effectively ionize a wide range of pharmaceutical compounds, which has made LCMS the preferred first-line approach for obtaining structural information for many impurity and degradation unknowns. Because of this, HPLC methods are now often developed with volatile buffers that can be directly used with LCMS. With newer ESI sources, even nonvolatile buffers can be used. However, mass spectrometry sensitivity is greatly reduced when using many of the traditional LC buffers, such as phosphate or citrate.

Quadrupole mass analyzers were utilized in the largest number of reported impurity applications over the past few decades; however, recent developments in ion traps and TOF analyzers provide significant advantages for this type of work. When performing identification at low levels, full scan sensitivity is critical. This is an advantage that both ion traps and time-of-flight instruments possess versus quadrupoles. The $(MS)^n$ capabilities of ion traps can also be helpful for solving difficult structure elucidation problems. The high resolution and exact mass measurement capabilities of TOF instruments are particularly valuable for providing empirical formulae data on unknown impurities or degradants. Other high resolution mass spectrometers have also been used for impurity identification. For example, a magnetic sector instrument was used for identification of impurities and degradation products of a novel oligosaccharide antibiotic [295], whereas a FTICR mass spectrometer was used for impurity determination in fluoxetine hydrochloride [296]. However, recent advances in TOF technology combined with relative ease-of-use for on-line applications and low cost make these analyzers more attractive than other high resolution instruments for elucidation of impurity and degradation product structures. In addition, the ability of Qq-TOF instruments to obtain elemental composition information on product ions can aid in the understanding of fragmentation pathways and help to better ascertain the structural relationship of product ions recorded from an unidentified drug-related compound.

On-line tandem mass spectrometry is advantageous and is often utilized for impurity identification versus a single stage of mass analysis; however, MS/MS is not as critical for these applications as when analysis is performed in more complex matrices (e.g. biological fluids). Often, a good separation is adequate to provide an eluant that is clean enough so that the mass spectrum is not cluttered with ions generated from matrix components. Pseudo-MS/MS spectra can also be obtained by inducing dissociation in the interface region with a single stage instrument and pseudo MS/MS/MS can be performed with tandem instruments by the same means. The resolution advantage of TOF detectors also provides a great deal of specificity which even further reduces the need for MS/MS. The overall combination of attributes arguably makes TOF the most readily accessible and widely useful analyzer for identification of impurities and degradation products.

Many of the analytical instruments and associated attributes required for identification of impurities and degradation products are similar to those discussed in Sec. 5. for the identification of metabolites. However, in general, the structures of metabolites are more predictable than those of impurities and degradation products, whereas metabolite identification often requires better sensitivity and specificity for analysis of in vitro incubations or in vivo biological fluids. Also, the instrumentation and overall strategies for impurity and degradant identification are necessarily more varied than most other pharmaceutical applications. The needs of a particular program, as well as the chemical and physical properties of the analytes, dictate the required analytical approaches.

6.3. Current strategies for identification of impurities and degradation products

6.3.1. General approach

Developing a strategy for the identification of drug impurities and degradants is essential in the course of drug development. It is a collaborative effort involving many functions including chemists, formulators and analytical experts in a variety of techniques such as HPLC, MS, NMR and others that may be required to elucidate specific structures [297]. Often, much of the impurity identification work occurs with the initial establishment of an impurity profile in early safety and clinical lots or when changes are made to a supplier, synthetic route or formulation. Most commonly, drug related impurities or degradation products are discovered using LC profiling with a diode array or UV detector [298]. Sometimes GC, TLC, or CE are also used, depending on the preferred methods for separation of the compound class. Impurities that are inorganic in nature or not related to the drug may require analysis by other analytical techniques, not discussed here.

Because of the sensitivity and the relative ease of using on-line LCMS, it is often the first technique applied to obtain structural information on impurities. In the event that a reference standard exists, a retention time match, along with a MS/MS "fingerprint" consisting of the molecular weight and three product ions that match with data obtained from a standard, constitutes compound identification [299]. Alternatively, a retention time match of the unknown entity with an authentic standard in three chromatographic systems can also suffice for structural confirmation [300].

If a standard compound is not available, a structure is proposed after comparison of mass spectrometry data such as molecular weight, empirical formula and product ions of the unknown, with data obtained from closely related compounds. An example is the structure elucidation of drug-related substances of acebutolol, where specific impurities were initially discovered by HPLC-UV and structures of the unknown compounds were proposed based on LC-MS/MS data [301].

The combination of LC separation, along with diode array detection and TOF mass analysis, provides a very powerful solution to many identification needs. The LC separation is amenable to many types of molecules and by obtaining UV data and high resolution mass spectra, many impurity problems can be solved. Although data provided by MS or MS/MS are often adequate to suggest a structure, sometimes more information is needed, especially in the case of indistinguishable isomers. NMR is usually the technique of choice for providing more refined characterization data. Typically, LC peaks are collected off-line, but increasingly on-line LC-NMR data are being obtained and occasionally LC-NMR-MS data are all collected within one experiment [302].

With a structure proposed for the unknown, quantities of the compound are then obtained either by preparative chromatography or by organic synthesis. The isolated unknown then undergoes complete spectroscopic characterization, and is used as a reference standard for accurate identification and quantitation of the target impurity in drug substance and/or drug product. Subsequent lots of material continue to be monitored, usually by HPLC-UV, to quantitate impurity levels and assure that no significant new impurities are found.

Latent peak purity assessment, for potential co-eluting minor impurities with the drug peak in HPLC systems, can also be performed using mass spectrometry [303]. However, peak purity of the major component(s) has more commonly been determined by ratiograms, in conjunction with LC and PDA detection methods [304]. Although mass spectrometry has the selectivity to distinguish nonisobaric impurities from the mass of the drug substance, LCMS and LC-MS/MS experiments may not always detect co-eluting components at the 0.1% level, due to compound dependent ionization efficiencies and the likely ionization suppression caused by an excess of the major component. Best results are obtained through comparison with a reference standard sample that is considered pure [305].

6.3.2. Examples of impurity and degradation product identification

The types of analytical instrumentation used for impurity and degradation product identification vary widely. Many chromatographic approaches, as well as instruments, are required to meet all potential needs, due to the diversity of both pharmaceuticals and to the structures of the impurities or degradation products that are observed. The following provides a survey of applications, which begins by describing the various chromatographic approaches required to solve these problems. This is followed by examples where different types of mass spectrometric data and spectroscopic information were obtained to elucidate structures of impurities.

Reversed-phase LC is very commonly used for impurity identification. An example is the identification of a famotidine degradation product, when interfaced to a triple quadrupole detector [306]. LC-MS/MS was also used for the identification of protein impurities in *r*-hirudin sequence variant 1 [307]. Often, the major component in a mixture elutes near the impurities and can cause difficulty when attempting to identify a minor component. A solution was devised by using a UV-actuated valve to divert the flow away from the mass spectrometer when major components are eluting. This approach has been shown to increase sensitivity up to fivefold for identification of cimetidine impurities [308]. To improve the separation of losartan and associated degradation products, TFA was used as an ion-pairing reagent. This separation assisted in the identification of acid-catalyzed substitution as the primary pathway for degradation [309]. Ion exchange chromatography has also been reported for this purpose. An example is the on-line identification of an ionic bisphosphonate impurity in alendronate with LC-MS/MS [310]. A modified APCI source was used to generate molecular weight information on drug raw materials with a SFC separation [311]. In addition, capillary electrophoresis is increasingly reported with on-line mass spectrometry configurations to elucidate structures of impuri-identify dimeric derivatives of an antiartherosclerotic drug, along with trace levels of the starting material in the drug substance [312].

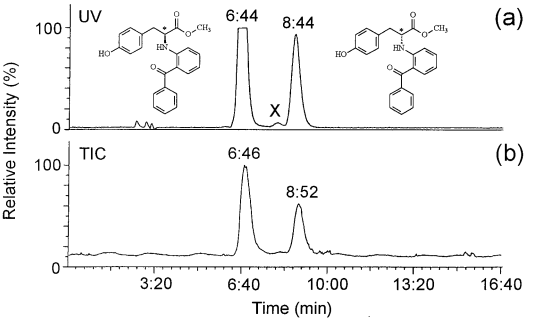

Fig. 16. Normal phase separation of (2S)- and (2R)-2-[(2-benzoylphenyl)amino]-3-(4-hydroxyphenyl)-propionic acid methyl ester from unknown impurity X. Both the (a) HPLC-UV chromatogram and (b) TIC chromatogram are displayed.

As enantiomers can have quite different pharmacological effects, the monitoring and identification of chiral impurities by normal-phase LCMS is becoming increasingly prevalent in pharmaceutical development [313]. The APCI source has been found to be a good option for many normal-phase chiral HPLC analyses [314]. However, because the vaporizer of the APCI source can reach temperatures from 300 to 600 °C, there is a potential fire hazard when producing aerosols of organic-based, normal-phase solutions. Care must be taken to ensure that the corona discharge region is sufficiently void of oxygen to prevent fire or explosion hazards when using such mobile phases.

An example of a normal-phase separation with APCI triple quadrupole MS is shown in Fig. 16 [314]. The UV and TIC chromatograms were produced by injection of a mixture of the (2S) and (2R) enantiomers of 2-[(2-benzoylphenyl)amino]-3-(4-hydroxyphenyl)-propionic acid methyl ester. Between these two peaks is an unidentified component, labeled X. The product ion spectrum of the (2R) parent compound is shown in Fig. 17(a), and that of unknown X is shown in Fig. 17(b). In the single stage mass spectrum (not shown), the 3:1 ratio of isotopes at *m/z* 424 and 426 indicates the presence of a chlorine atom in the unknown. In addition, the mass shift of the impurity is 48 Da from that of the parent. The major fragment ion at *m/z* 316 is generated by loss of acetic acid from the structure shown in Fig. 17(a). This same ion is also present in the product ion spectrum of the

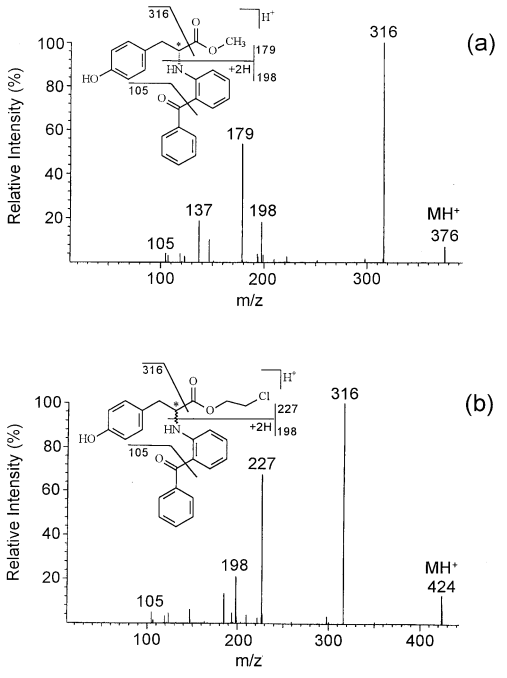

Fig. 17. MS/MS product ion spectra of (a) the (R)-isomer of 2-[(2-benzoylphenyl)amino]-3-(4-hydroxyphenyl)-propionic acid methyl ester and (b) an unidentified component. Based on the MS and MS/MS data, the proposed structure for the impurity is provided in the lower spectrum.

unknown, which indicates the site of modification. From these data, the structure in Fig. 17(b) was proposed for the unknown.

Most of the on-line impurity identification examples provided here and in the literature were generated with triple quadrupole instruments; although, the $(MS)^n$ capability of ion traps can be helpful for structure elucidation, as demonstrated in the characterization of dextromethorphan [315] and erythromycin [316] impurities. Also of practical utility is the empirical formula of an unknown. An example of using an integrated LC-UV-TOF-MS approach is provided in Fig. 18. The unknown impurity, at 15.0 min, is shown in the UV trace in Fig. 18(a). The UV spectral profiles for the parent and the impurity readily indicated a change in the chromophore (data not shown). The full scan TOF spectrum used to obtain the exact mass is shown in Fig. 18(d) after centroiding the data and calibrating with the lock mass. Possible empirical formulae were generated with the exact mass data and user-provided restrictions for the number of double bond equivalents (DBEs) and for the number of each atom likely to be present (Fig. 19). When considering these data along with other information described in Fig. 19 such as the nitrogen rule, isotopic ratio and the chemistry involved in the synthesis, $C_{20}H_{21}N_{10}$ was proposed as the empirical formula of the impurity. The exact mass, product ion information available with Qq-TOF instrumentation augments single stage TOF data. This is especially important when working with complex samples that may produce co-eluting peaks. This advantage was demonstrated in product ion elemental composition assignments to within 5 ppm for structure elucidation of cimetidine drug substance impurities [317].

Often in the process of impurity identification, several types of mass spectrometry in addition to other analytical techniques are required to determine structures of impurities that may have diverse functionalities, even through they are associated with a single compound. As an example, the degradation of ibuprofen results in several products that are primarily formed by oxidation at the benzyl positions [318]. Both an aldehyde and a ketone are produced that retain the acidic functionality, as shown in Fig. 20. Therefore, these two compounds were able to be analyzed under the same conditions that were used for the parent compound, reversed-phase LC with negative ion ESI-MS/MS detection. However, oxidation at the 2-propyl position results in loss of the acidic functionality. One of the degradants, 4-isobutylacetophenone, was observable by LCMS only when using positive ion APCI. Oxidation at both benzylic positions produces a compound in relatively low abundance that was not observed in either positive or negative ion mode when using APCI or ESI. However, the corresponding unknown LC-UV peak was collected and analyzed by GC-(EI)MS, which resulted in the proposal of the 4-acetylisobutyrophenone structure. Each of these four degradation products, initially identified by mass spectrometry, were subsequently confirmed using on-line LC-NMR.

Older examples of impurity identification using

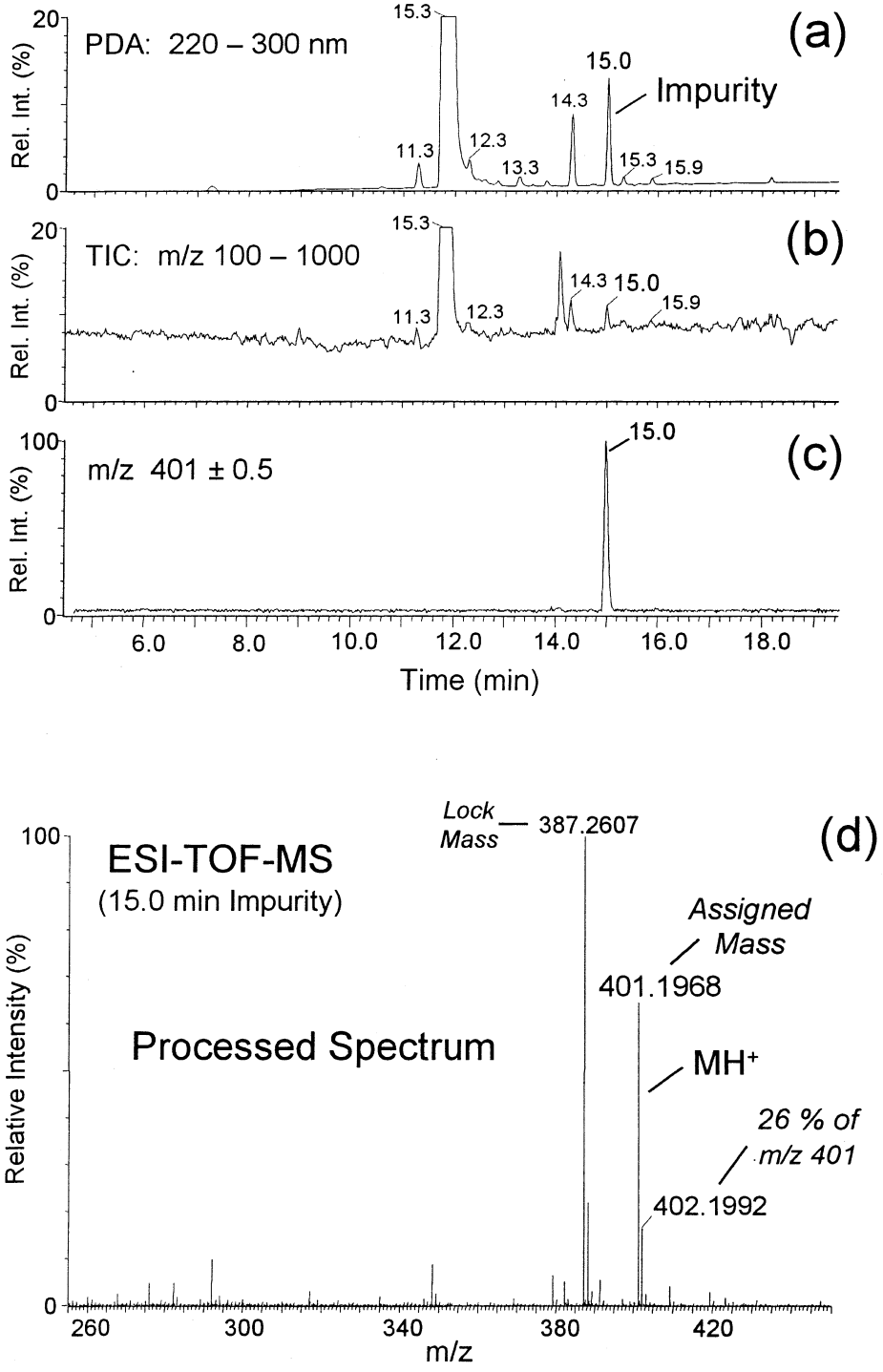

Fig. 18. Data showing the integrated approach of LC-UV-TOF-MS for empirical formula determination of impurities. The UV trace (a) is more useful than the TIC (b) for identification of the retention time of the impurity because of a better signal-to-noise ratio. The mass chromatogram of *m/z* 401 (c) also provides a much better signal-to-noise ratio than the TIC. The centroided, high resolution TOF mass spectrum is shown in (d) after calibration with the lock mass.

Calculated Mass	Δ ppm	DBE	Formula
401.1996	-6.5	1.5	~~$C_{22}H_{28}N_6O_9$~~
401.1996	-6.5	7.0	~~$C_{25}H_{23}N_4O_4$~~
401.1991	-5.7	14.0	~~$C_{26}H_{27}N_2O_3$~~
401.1983	-3.7	2.0	~~$C_{10}H_{27}N_5O_8$~~
401.1978	**-2.5**	**14.5**	**$C_{24}H_{25}N_4O_2$**
401.1969	-0.2	2.5	~~$C_8H_{25}N_{12}O_2$~~
401.1964	**1.0**	**9.5**	**$C_{23}H_{29}O_6$**
401.1964	1.0	15.0	~~$C_{22}H_{23}N_7O_1$~~
401.1956	3.0	3.0	~~$C_8H_{23}N_{10}O_6$~~
401.1951	4.2	10.0	~~$C_{21}H_{27}N_5O_5$~~
401.1950	**4.5**	**15.5**	**$C_{20}H_{21}N_{10}$**
401.1937	**7.7**	**10.5**	**$C_{19}H_{25}N_6O_4$**

All except 4 are eliminated ⊗ due to:

whole # DBE; failed Nitrogen Rule; and/or low carbon #, based on m/z 402:401 ratio

Remaining Formulae	Calculated m/z 402:401	Assessment
$C_{24}H_{25}N_4O_2$	28.4 %	Δ ppm < 5; high m/z 402:401
$C_{23}H_{29}O_6$	25.7 %	Δ ppm < 5; matched m/z 402:401; least consistent with chemistry
$C_{20}H_{21}N_{10}$ (selected)	**26.0 %**	**Δ ppm < 5; matched m/z 402:401; consistent with chemistry**
$C_{19}H_{25}N_6O_4$	23.7 %	Δ ppm > 5; low m/z 402:401

Fig. 19. Steps involved in the determination of the empirical formula of the impurity shown in Fig. 18. The exact mass measurement along with estimated ranges for the number of DBEs and the number of atoms of each element generate a list of possible formulae. Each of these formulae, except for the correct one, was eliminated by considering (1) that the number of DBEs cannot be a whole number if the molecule is protonated, (2) the nitrogen rule, (3) the number of carbon atoms based on the isotopic ratio of *m/z* 402:401 and (4) the chemistry involved in the synthesis.

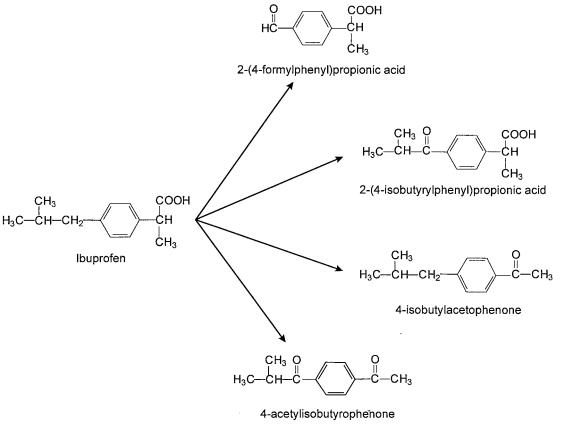

Fig. 20. Structures of the major degradation products of ibuprofen identified in a tablet formulation. Because of the functional group variation, many analytical techniques were used to identify all four structures including negative ion ESI-MS/MS, positive ion APCI-MS/MS, GC-(EI)MS, and on-line LC-NMR.

NMR were performed by collection of sample peaks followed by off-line analysis, as with the reported identification of 3-oxosteriod impurities [319]. However, more recent examples, including the prior ibuprofen example, demonstrate the feasibility of performing on-line LC-NMR, without the need for preparative isolation to facilitate structure elucidation. Examples where LCMS and LC-NMR were integrated for structural identification include the identification of six different degradation products of a protease inhibitor in a dosing solution [320]. The use of LC-NMR was important in this particular study to differentiate structural isomers. Another example is provided by determination of the structure of a bulk drug impurity of a glycinamide ribonucleotide transformylase inhibitor [321]. On-line LC-NMR more clearly lends itself to impurity and degradation product identification than, for example, on-line metabolite identification given higher relative levels of unknowns and much simpler matrices.

6.3.3. Natural products and adulterant characterization

Impurity and degradation product identification is a major application area for mass spectrometry in pharmaceutical development. Other applications of mass spectrometry-based molecular identification that are crucial to the industry, but not as frequently required, are the characterization of natural products and the identification of unknowns in product adulteration cases. Strategies and regulatory requirements for natural product characterization are somewhat different from those described here for impurities and degradation products of synthetically prepared pharmaceuticals, although many of the same analytical approaches are used. These have recently been reviewed [322].

Although it is important to identify substances that are used to adulterate pharmaceutical products, there are very few reported examples of using mass spectrometry for this purpose. Many of the same analytical

approaches described here for impurities and degradation products can be applied. Although, for these investigations, compounds that are responsible for adulteration are often known and may be identified using library searching, which is especially useful for volatile compounds that may be analyzed by GC-(EI)MS. In addition, standard compounds can often be directly purchased. An example of a nonvolatile adulteration study is the use of capillary electrophoresis in conjunction with ESI-MS to identify coptisine, berberine and palmatine that were illegally introduced into the Chinese medicine wuyoufun-13 [323].

6.4. Emerging mass spectrometry technologies for identification of impurities and degradation products

The arsenal of techniques and approaches for molecular identification has been rapidly growing. In the future, applications involving LC separations may increasingly use narrow-bore columns (~1 mm i.d.) in contrast to the traditional 4.6 mm i.d. columns for sensitivity enhancement. The use of pcSFC may also increase. Both of these chromatographic trends would be driven by the capability to produce higher resolution separations and by the ecologic–economic trend to save money and reduce waste. For mass spectrometry detection, it is likely that ion traps and especially high resolution TOF instruments will be employed more heavily for many applications, including those that utilize LC and/or GC separations. In addition, a more integrated approach, using data-dependent scanning, may be employed to minimize the time needed for complete mass spectrometry characterization by reducing the number of chromatographic runs. The ability to automatically obtain MS and MS/MS spectra in a given run, depending on the observed data, is currently available but is not yet broadly used in this application area.

The use of multiple hyphenated techniques in conjunction with mass spectrometry, such as HPLC-UV-NMR-MS and HPLC-UV-NMR-FTIR-MS will be more thoroughly evaluated and the practicality will dictate if such integrated systems will be heavily used [324]. Going forward, it is likely that on-line UV in conjunction with mass spectrometry will be more commonly used in the course of routine impurity and degradation product profiling. This will be driven by several factors, including cheaper and easier-to-use mass spectrometers. In addition to providing a complementary mode of detection, the mass spectrometer would be particularly useful where greater sensitivity is required for quantitation. Possible applications include situations where impurities or degradation products do not contain an adequate chromophore for quantitation by UV.

Although not yet in widespread use throughout product development, the techniques and instruments described above provide promising options for the future of impurity and degradation product identification. However, in the product development environment, where many activities are subject to review by regulatory agencies, it often takes longer to implement new technologies. Although speed of analysis is an important consideration, a higher premium is placed on thoroughness, accuracy and detailed documentation. This hierarchy contrasts that of the discovery environment where, for most applications, speed and high throughput are major driving forces for the implementation of many analytical approaches. In the development world, some of the hurdles to on-lining new technologies include initial qualification, operational qualification and performance qualification of instrumentation. In addition, procedural and instrumental standard operating procedures (SOPs) must be written and approved, and data collection and archival must be compliant with the electronic records and electronic signatures requirements. In the USA, these requirements are described in 21 Code of Federal Regulations part 11.

7. Conclusions

Over the past few decades, a very rapid pace of innovation in mass spectrometry-based technologies has resulted in an array of multidimensional

analytical tools that provide practical solutions for many types of pharmaceutical mixture analysis problems. The major advances that have impacted this industry over the past 25 years include the development or improvement of interfaces that allow the use of mass spectrometry with separation techniques such as GC, LC, or CE. Older ionization techniques have been improved and several new ionization techniques, such as ESI and APCI, have emerged to interface LC with mass spectrometry. In addition, development of MALDI to allow direct, soft ionization of biomolecules has proven extremely useful. There have been advancements in readily accessible instruments that can perform multiple stages of mass analysis for structural elucidation, including the triple quadrupole, ion trap and Qq-TOF. The availability of high resolution instruments for determination of empirical formulae and fragment ion composition is most practically provided with modern TOF instruments. Using a separation, along with two dimensions of mass analysis, has proven to be an optimal combination of sensitivity and specificity for quantitative applications and is most usefully implemented with the triple quadrupole. Innovations in computer hardware and software to control and operate instruments, as well as collect, mine, reduce and store data have greatly improved ease-of-use and automation for widespread instrument deployment and efficient sample throughput. This collection of innovations and improvements has provided a substantial arsenal of practical multidimensional analytical tools for pharmaceutical applications including NCE characterization, biomacromolecule characterization, bioanalytical quantitation, metabolite identification and molecular identification of impurities and degradation products.

Industrial implementation of these advances has also proceeded rapidly due to the monumental needs for mixture analysis. Those working within each application area on a daily basis are required to develop and/or align techniques and instrumentation with specific pharmaceutical mixture analysis problems in order to optimally distill useful knowledge from samples. This must be done efficiently with consideration of time, cost and personnel required. Often, existing approaches are available; however, there are many opportunities to develop novel or improved solutions. New instruments or technologies are often provided by instrument vendors, academic laboratories or in collaboration with pharmaceutical researchers. In many cases, technologies developed or targeted for one application area have also been successfully reapplied to other areas. For example, mass spectrometry-based approaches using 96-well plates were initially developed for support of HTOS, but have since been adapted for bioanalytical quantitation. This approach has had a major impact on quantitative bioanalytical throughput and efficiency. Also, ESI was originally developed for use with biomolecules; however, it is now perhaps even more widely used for analysis of small organic molecules in applications ranging from NCE characterization to bioanalytical quantitation to identification of metabolites, impurities, and degradation products.

As a result of these advances and the practical industrial implementation, the utilization of mass spectrometry has increased for all phases of drug development and particularly for drug discovery. From an analytical perspective, these advances have most importantly improved sensitivity, specificity, ease-of-use and sample throughput, as well as increase the amount of structural information. From a pharmaceutical industry standpoint, these developments have changed the game. As briefly as a decade ago, a relatively small amount of analytical information was obtained in any of the stages of drug discovery, described in Fig. 1, largely due to sensitivity and throughput limitations. Now, hundreds of thousands of synthetic compounds can be practically characterized and proteomics is a feasible approach for intelligent drug design. In addition, PK and metabolism data are obtained in discovery to provide a much deeper level of understanding of the in vivo properties of therapeutic agents. That information leads to the design of better drug molecules and more successful selection of compounds for advancement to development.

From preclinical through phase IV, all activities involving mass spectrometry such as metabolism, PK

and identification of impurities and degradation products have been greatly accelerated due to these technological advances. In addition, new approaches to old problems are now available. For example, it is now practical to obtain PK data at the site-of-action, in a rapid and iterative manner. Such capabilities are increasingly applied to optimize formulations for improved product performance, prior to proceeding to costly and time-consuming clinical trials [208].

Given the continuing rapid pace of emerging instrumentation, new mass spectrometry technologies will likely continue to transform pharmaceutical research and development. Key analytical attributes such as sensitivity, specificity and sample throughput are likely to continue to improve for all application areas and to generate new applications. Likewise, instruments will continue to become smaller, less expensive, and easier to use. Despite the recent advances, many opportunities still exist to improve the design and selection of compounds and to decrease the length of time required to bring new compounds to market. These efforts will continue because they are driven by understanding and treating diseases through pharmaceutical therapies, which are extremely complex, time-consuming and important endeavors, as the ultimate goals are improving the quality and duration of human life.

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