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Current Applications of Liquid Chromatography/Mass Spectrometry in Pharmaceutical Discovery After a Decade of Innovation

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Abstract

Current drug discovery involves a highly iterative process pertaining to three core disciplines: biology, chemistry, and drug disposition. For most pharmaceutical companies the path to a drug candidate comprises similar stages: target identification, biological screening, lead generation, lead optimization, and candidate selection. Over the past decade, the overall efficiency of drug discovery has been greatly improved by a single instrumental technique, liquid chromatography/mass spectrometry (LC/MS). Transformed by the commercial introduction of the atmospheric pressure ionization interface in the mid-1990s, LC/MS has expanded into almost every area of drug discovery. In many cases, drug discovery workflow has been changed owing to vastly improved efficiency. This review examines recent trends for these three core disciplines and presents seminal examples where LC/MS has altered the current approach to drug discovery.

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1. INTRODUCTION

LC/MS: liquid chromatography/mass spectrometry

Drug disposition: the study of the fate of drugs in living systems; typically involves the combined use of pharmacokinetics and drug metabolism

LG: lead generation

Proteomics (PTX): global analysis of protein mixtures used in target validation and biomarker discovery

Metabonomics (MTX): comprehensive analysis of endogenous small molecules, or specific molecular classes, used in the study of pharmacology and toxicology (synonymous with the term metabolomics)

Much has been written about the role of mass spectrometry (MS) in the pharmaceutical industry (1, 2). At present, there is hardly a discipline or laboratory engaged in drug discovery that has not benefited in some way from MS technology. The "hyphenated" technique liquid chromatography with MS (LC/MS) represents the most widely used tool in the MS arsenal. In many instances, it has dramatically altered the workflow of modern drug discovery owing to improved efficiency or the ability to provide essential data at earlier stages in discovery with rapid turnaround.

This article reviews advances in the three core disciplines of drug discovery: biology, chemistry, and drug disposition. In addition to recent trends, it cites key events over the past decade to provide an understanding of the impact of LC/MS on drug discovery. Prior to the aforementioned review, we provide background information in two areas. The following section provides an overview of the stages of drug discovery. This is followed by a brief discussion of LC/MS instrumentation currently used in drug discovery.

Drug discovery proceeds through a series of fairly well-defined stages: target identification, biochemical screening, lead generation (LG) (hit-to-lead), lead optimization, and candidate selection. **Table 1** lists representative activities associated with each stage, which are categorized according to the three core disciplines.

Drug discovery begins with the selection of a target. Most targets are proteins (e.g., enzymes, receptors) and are chosen based on available knowledge associating a target with a certain disease state. Drugs are agents designed to selectively regulate a biological pathway or process believed to be associated with the disease state in question. The goal of drug discovery is to identify a safe chemical entity that can be predictably delivered to the drug target to allow the hypothesized association between a target and a disease to be tested in the clinic.

Drug target validation often uses biological tools such as gene knockout animals or RNA interference to implicate the role of the target in the disease state. Investigators analyze specific biochemicals and study their perturbation in specific pharmacology or disease models. LC/MS has played a vital role in the analysis of the biomolecular classes listed in **Table 1**. Of particular significance is the field of proteomics (PTX), in which MS is the primary tool used. Metabonomics (MTX), the small-molecule counterpart to PTX, has also benefited from advances in MS technology. Both PTX and MTX have been used throughout drug discovery to assist in the identification of biomarkers for efficacy and/or toxicity. Owing to the cost and complexity of these methods, their deployment is reserved for specific applications (i.e., those not found on the critical path of project flow schemes).

In the next stage of drug discovery, high-throughout screening (HTS) identifies core structural motifs that bind to the target. Increasingly, LC/MS is being used as an alternative to ligand-binding methods for HTS, although it is not likely to supplant existing methods [e.g. enzyme-linked immunosorbent assay (ELISA), scintillation proximity assay, etc.], that are less expensive and have higher throughput. LC/MS is typically applied in cases in which selectivity and/or expedited method development is important.

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Table 1 Activities in the major stages of drug discovery supported by liquid chromatography/mass spectrometry

Stage	**Target ID**	**Biochemical screening**		**Lead generation (hit-to-lead)**			**Lead optimization**		
Function	**Biology**	**Biology**	**Chemistry**	**Biology**	**Chemistry**	**ADME**	**Biology**	**Chemistry**	**ADME**
Activities	PTX	HTS	Hit ID	In vitro pharmacology	Library QC	Physical properties	In vitro pharmacology	HTOS support	Physical properties
	MTX	Target validation	Library QC	In vivo model development	HTOS support	ADME screening	In vivo pharmacology	Med chem support	ADME screening
			HTOS support	BM discovery	COS	Exposure screening	BM discovery	Scale-up synthesis	In vitro assays
						PK	BM qualification	COS	Exposure screening
							PK/PD		PK
									TK

Abbreviations used: ADME, absorption, distribution, metabolism, and excretion; BM, biomarker; COS, confirmation of structure; HTOS, high-throughput organic synthesis; HTS, high-throughput screening; Med chem, medicinal chemistry; MTX, metabonomics; PD, pharmacodynamics; PK, pharmacokinetics; PTX, proteomics; QC, quality control; TK, toxicokinetics.

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HTOS: high-throughput organic synthesis

Structure-activity relationship (SAR): a correlation established between molecular structure and activity toward the target of interest

LO: lead optimization

ADME: absorption, distribution, metabolism, and excretion

Biotransformation: the official term used to describe the metabolism of drugs or other xenobiotics

The involvement of chemistry begins during the biochemical screening stage because of the need to deliver compounds for HTS. LC/MS is heavily involved in the characterization of the chemical libraries generated from high-throughput organic synthesis (HTOS) or from other sources, including natural products and corporate compound repositories. As discussed below, LC/MS can support HTOS in several ways, including library quality control, hit identification, and compound purity assessment.

As hits are identified, an expanded effort known as LG interrogates the chemical space around the identified hits and the influence of structural changes on biological activity. This activity leads to a correlation referred to as a structure-activity relationship (SAR). LC/MS is used to support chemical synthesis and to purify and register the compounds tested. Typically, a fraction of each compound is stored in a corporate repository. During LG, biological models follow a progression from in vitro assays to cell culture screens and ultimately to live-phase animal models. Much effort is expended to understand these models prior to lead optimization (LO). Because activity in more complex biological models relies on the delivery of the drug to the target, investigators direct additional effort in LG at understanding the ADME (absorption, distribution, metabolism, and excretion) characteristics of the molecules in the SAR. LC/MS has been extensively applied to create ADME-related screens and to assess relevant physical properties. Assessing drug exposure in the in vivo pharmacology models also begins in LG because of the throughput established by LC/MS methods. Initial investigation of the biotransformation of the lead molecules also occurs in LG to identify metabolically labile positions on molecules. In addition, chemical scaffolds found to generate reactive (potentially toxic) metabolites are also removed from active consideration.

The final stage leading to a selected candidate is LO. In many ways LO is an expanded version of LG that follows a specific testing cascade appropriate for the project. Although the previously developed in vitro methods are still used to screen compounds, LO places more emphasis on live-phase models for efficacy, as well as establishing pharmacokinetic (PK), pharmacodynamic, and toxicokinetic information for the lead compounds. ADME screening still occurs in LO and is used to more fully understand the factors that control drug clearance. In addition, researchers often conduct more definitive experiments on the compounds that advance to understand their potential for drug-drug interactions. LC/MS is also used in LO to study the major metabolic routes for leads that have the potential to become drug candidates. In addition, interspecies metabolism comparisons occur to guide toxicology assessment and to understand the disposition in live-phase pharmacology models.

Chemical supply is profoundly important in LO. Although HTOS still occurs in the early stages of LO, it is quickly supplanted by traditional medicinal chemistry so that more precise synthesis can occur and because larger amounts of highly purified material are required. In later-stage LO, process chemistry is used to produce sufficient material to perform toxicokinetic and other compound-intensive studies.

Investigators give increased attention in LO to identifying biomarkers that can be used in the clinic. Therefore, extensive effort occurs in parallel during the advancement of specific compounds to qualify biomarkers that can predict useful biological outcomes. This work coupled with the combined effort cited from the three core disciplines leads to the selection of a candidate for clinical development.

UHPLC: ultrahigh-pressure liquid chromatography

ESI: electrospray ionization

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2. LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY INSTRUMENTATION

The integration of LC/MS into the pharmaceutical industry has benefited from continuous innovation in instrumentation. The section below provides an overview of the major forms of LC/MS instrumentation. Further information on this topic is available for interested readers (3).

2.1. Liquid Chromatography

A decade ago, pharmaceutical applications of LC/MS primarily employed reversed-phase (RP) columns with 5-μm particles. As documented by a recent survey comparing high-pressure LC trends in 1997 and 2007 (4), RP-LC is still the dominant technique; however, there is a trend toward smaller particle sizes, driven by the current interest in sub-2-μm particles and ultrahigh-pressure LC (UHPLC). The survey also indicated that today's chromatographer has access to more tools, including monolithic phases, hydrophilic interaction liquid chromatography (HILIC), and supercritical fluid chromatography (SFC). In addition, stationary phases have improved significantly over the past decade (5).

For qualitative applications of LC/MS involving the analysis of complex mixtures, resolution and peak capacity are critical attributes. PTX (6), MTX (7), and drug biotransformation (8) have all benefited from UHPLC through improved resolution, shortened analysis times, and improved electrospray ionization (ESI) sensitivity. For example, using pellicular phases and UHPLC, Wang et al. (6) observed a 50% increase in peak capacity compared to a conventional porous phase for PTX analysis. Similarly, newer 1.7-μm RP materials were combined with UHPLC to improve resolution, speed, and sensitivity for metabolic profiling in urine (7). Superficially porous stationary phases offer another way to improve peak capacity using normal operating pressures and are used primarily for protein separations (9).

HILIC, which is an aqueous-containing format for normal phase separation, provides an excellent complement to RP-LC for highly polar molecules. It is also operationally simpler than conventional normal-phase LC for MS applications. Because HILIC uses high organic mobile phases, improved LC/MS sensitivity is obtained owing to improved desolvation. HILIC has been successfully applied to qualitative applications, such as MTX (10), and has provided improved quantification of polar analytes (11).

Quantitative LC/MS applications focus on sensitivity and throughput. Hence, LC formats such as monolithic columns (12) and ballistic gradients with short columns (13) have been widely used for screening applications and drug bioanalysis. Gradient elution has become the default for high-throughput applications because it combines fast run times with the flexibility to rapidly accommodate high chemical diversity. Investigators have also extended the cited benefits of UHPLC to

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quantitative applications (14). In addition, multidimensional LC applications linked via column-switching methods have been extensively deployed throughout drug discovery for automation and improved efficiency. Column switching is commonly used with PTX to permit large injections onto capillary columns and to carry out on-line two-dimensional (2D) separations coupling cation exchange and RP-LC (15). As discussed in Section 5, column switching has been applied extensively for on-line sample cleanup in bioanalytical applications. Information on this topic is available in a current review on bioanalysis (16).

Bioanalytical: pertains to quantitative analysis of drugs and their metabolites in biological matrices such as plasma

APCI: atmospheric pressure chemical ionization

Atmospheric pressure ionization (API): a mass spectrometer interface used in LC/MS that separates ion formation (atmospheric) from mass analysis (vacuum) via a pinhole orifice

2.2. Mass Spectrometric Ionization Methods

A vast majority of all LC/MS analyses use two ionization methods: ESI and atmospheric pressure chemical ionization (APCI). We can trace the robustness of these methods to the common use of atmospheric pressure ionization (API), meaning that spray initiation and desolvation occur at atmospheric pressure, separated from the high-vacuum internal region of the mass spectrometer by a pinhole or capillary orifice. Under ESI, droplets containing a charge excess are produced by the application of high electric fields to a needle-like sprayer. The resultant droplets contain a net charge having the same polarity as the voltage placed on the needle. As the droplets shrink from evaporation, they become unstable owing to excess charge, and they dissociate into droplets of smaller radius (Rayleigh dissociation). For small molecules, including peptides, gas-phase analyte ions are ultimately produced by ion desorption from solution driven by the intense electric fields (ion evaporation). Alternatively, ion formation for large molecules (e.g. proteins) is believed to occur by a competing mechanism (charged residue model) in which gas phase ions are produced by complete desolvation of the charged droplets. The popularity of ESI is attributed to its sensitivity, the wide range of molecules ionized, and the ease of interfacing to LC.

APCI involves ionization of gas-phase analyte molecules produced by heated nebulization of the LC effluent. Chemical ionization, typically by proton transfer from mobile phase ions, occurs in a plasma created by corona discharge. Because of the requirement for analyte volatilization before ionization, APCI is limited to smaller molecules than ESI. Nonetheless, APCI provides an excellent complement to ESI and has the advantage of being less influenced by sample matrix (17). More recently, a variant of APCI called atmospheric pressure photoionization was introduced that induces ionization by a xenon arc lamp instead of a corona discharge. A review by Hsieh (18) discusses further details about atmospheric pressure photoionization and other LC/MS ionization methods.

2.3. Mass Analyzers

One can differentiate mass analyzers by several attributes: scan speed, duty cycle, mass resolution, mass range, and cost. Quadrupole mass filters are the most common and cost-effective mass analyzer. Because of their durability and low cost, they are used for several routine LC/MS applications in chemistry and drug disposition. In comparison with other mass analyzers, quadrupoles have limited mass resolution and are less sensitive for full-scan work because they do not store ions that are not being detected. A tandem quadrupole instrument, known as the triple quadrupole MS, is the instrument of choice for many bioanalytical applications (2, 16). This instrument consists of two mass-filtering quads coaligned at opposite ends of a center, non-mass-filtering quad serving as a collision cell. Extremely high selectivity is derived by monitoring a characteristic fragment transition for a target analyte using the first and third quadrupoles. This technique, referred to as selected reaction monitoring (SRM), makes the triple quadrupole MS a powerful option for trace analysis in complex biological matrices (3).

Quadrupole ion-trapping devices provide an alternative to conventional quads and offer improved full-scan sensitivity with the ability to perform tandem mass spectrometry (MS/MS). Because ionization, collision-induced dissociation, and mass analysis occur within the filter, these devices utilize staged pulse sequences and have the ability to store ions. This latter feature gives ion traps improved sensitivity over quadrupoles for full-scan experiments. The recent introduction of linear 2D ion traps has led to improved overall performance (19, 20). These instruments have found extensive use in biotransformation studies and are among the most widely used tools for PTX experiments.

SRM: selected reaction monitoring (also known as multiple reaction monitoring)

Tandem mass spectrometry (MS/MS): the use of multiple, consecutive stages of mass analysis to derive structural information or to improve analytical selectivity

Time of flight (TOF) is another popular mass analyzer that, as its name implies, sorts ions according to their arrival time to the detector after receiving a common kinetic energy. TOF offers the advantage of higher mass resolution, allowing for exact mass determination. In addition, TOF is capable of extremely high acquisition rates (50 μs per mass spectrum). A hybrid known as the Q-TOF combines a quadrupole mass filter for mass selection, an intermediate collision cell, and TOF mass analysis (21). Fast acquisition and the ability to perform exact mass determination on both precursor and product ions make this instrument extremely effective for structural elucidation in complex mixtures. As a result, it has been extensively deployed for PTX, MTX, and drug biotransformation.

Recently, much interest has been generated by high-end ion-trapping devices capable of extremely high mass resolution, to go along with high duty cycle. Although Fourier transform MS (FT-MS) based on ion cyclotron resonance was commercially introduced in the 1980s, the unparalleled resolving power of this instrument was not compatible with the timescale of LC. This changed with the recent introduction of a hybrid instrument that employs dedicated ion-cyclotron-resonance detection at the back end of a linear ion-trap mass spectrometer that handles the bulk of the LC/MS duties (22). More recently, Markarov and colleagues (23) introduced a variation of high-resolution ion trapping called the orbitrap. Because this instrument traps ions by electrostatic fields, it is less expensive than FT-MS, which requires a superconducting magnet. Although the orbitrap has slightly lower performance specifications than FT-MS, it is finding increased utility in application space shared by Q-TOF and FT-MS instruments.

A current trend in LC/MS instrumentation involves the incorporation of gas-phase ion mobility as an orthogonal means for resolving ions. Clemmer and coworkers (24) pioneered the use of ion mobility for PTX, demonstrating its use for enhanced separation of protein digests. Additional applications of ion mobility are on the rise

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owing to the commercial introduction of two instrumental formats. High-field asymmetric waveform ion-mobility spectrometry uses ion mobility in the API ion source region to improve the signal-to-noise ratio of target analyte ions (25). In this method, ions drift through a field produced by a pair of electrodes to which a radio frequency voltage (alternating current) is applied. Application of a fixed supplemental voltage (direct current) selects for ions of a given mobility by allowing a stable trajectory through the electrodes. The second method, based on traveling-wave technology, sorts ions according to their mobility as they pass through a grid formed by a series of planar ring electrodes of alternating polarity (radio frequency). A voltage gradient (direct current) applied across the lens stack enables the ions to traverse through a series of potential wells created by the electrodes at a rate determined by their mobility. The incorporation of traveling-wave technology into the design of a Q-TOF mass spectrometer has been reported (26).

3. BIOLOGY

Of the three core disciplines, biology plays the most central role in the discovery of new pharmaceutical agents. Biology drives the initial stages of drug discovery through the identification and validation of drug targets (**Table 1**). LC/MS is used throughout discovery to assist biology, including the pivotal transition from in vitro to in vivo models. It is also used to support the increased effort now given to the discovery and qualification of biomarkers.

In contrast to chemistry and ADME applications (which analyze drugs or drug-related products), biological applications measure endogenous molecules. The applications discussed below are divided into two categories: profiling and targeted analysis. An example of the first category is the use of LC/MS for PTX and MTX analysis. These methods integrate large-scale profiling of endogenous molecules with bioinformatics to derive a more comprehensive understanding of the biology associated with the target and are applied later in drug discovery in the search for biomarkers.

The second category, targeted analysis, analyzes fewer molecules with higher throughput and is generally more quantitative. Targeted analysis by LC/MS is used to support in vitro screening, in vivo pharmacology, and ultimately biomarker qualification. Both categories of LC/MS for biological applications are addressed in the sections that follow.

3.1. Proteomics

PTX involves the large-scale analysis of protein mixtures usually by MS following multiple stages of off-line and/or on-line separation. Most PTX methods attempt a comprehensive analysis referred to as nonbiased. As discussed below, targeted approaches are also used and are becoming more common. To achieve greater sensitivity, investigators typically analyze proteins following enzymatic digestion to yield peptides. This approach is commonly referred to as bottom-up PTX because proteins are identified by the detection of one or more tryptic fragments using sophisticated algorithms to carry out searches of protein databases (**Figure 1**). Yates has pioneered

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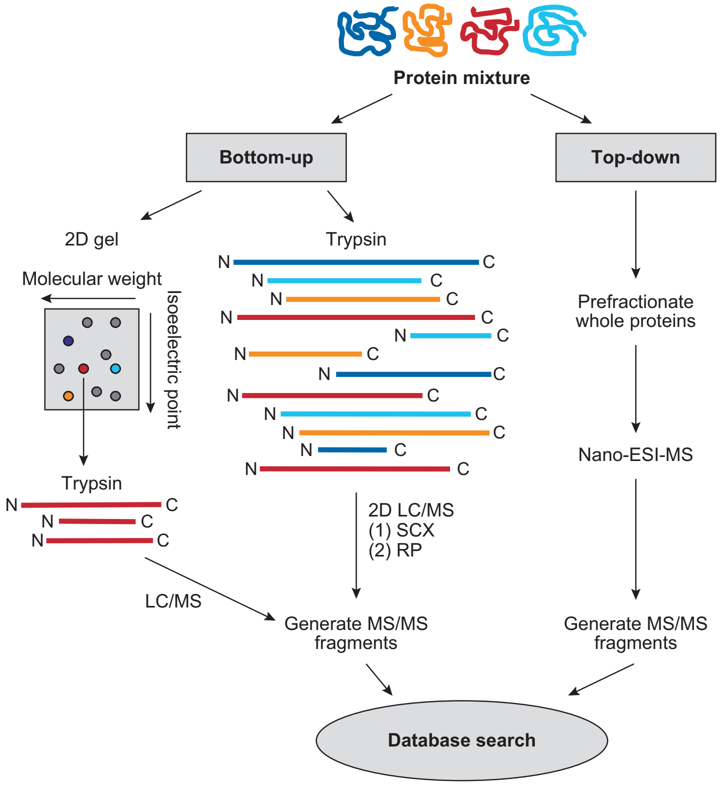

Figure 1

Schematic diagram comparing bottom-up and top-down proteomics. In bottom-up methods, protein identification occurs through the detection of predicted tryptic fragments. In contrast, top-down methods measure intact proteins. Although the top-down approach adds a useful complement to the more common bottom-up approach, it has comparatively lower sensitivity. Two bottom-up methods are compared in this figure. One method is based on two-dimensional gel electrophoresis, whereas the second method uses two-dimensional liquid chromatography (LC) for separation. The latter method, often referred to as shotgun proteomics, has become popular owing to its higher automation and throughput. Abbreviations: ESI, electrospray ionization; MS, mass spectrometry; RP, reversed phase; SCX, strong cation exchange.

many developments in the bottom-up approach, including a commonly used 2D LC approach called MudPIT (multidimensional protein identification technology), which fractionates complex peptide mixtures on-line using cation exchange prior to RP-LC. Yates and coworkers (27) recently published the latest version of this approach. Once sorted, the peptides are sprayed directly into the mass spectrometer (Q-TOF or quadrupole ion trap) to generate MS/MS spectra. These spectra are then searched against a database generated in silico using search algorithms (e.g., SEQUEST, MASCOT, X-Tandem). More recently, FT-MS and orbitrap mass analyzers have provided powerful interrogation of complex peptide mixtures under high mass resolution (22, 28).

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We can trace the widespread use of bottom-up PTX directly to the automation provided by on-line LC/MS/MS, representing a great savings in labor relative to older methodologies involving 2D gel electrophoresis followed by MS analysis of excised spots. A disadvantage to the bottom-up approach is that information about post-translational modifications (PTMs) is lost. As a complement to the bottom-up approach, Kelleher (29) has advocated the use of top-down PTX (**Figure 1**), whereby intact proteins are analyzed directly by MS and MS/MS under high mass resolution following chromatographic fractionation. Although top-down methods permit PTM detection, sensitivity currently limits the use of this technique.

One chief concern about PTX is the ability to derive quantitative information. Reported approaches to quantification are divided into label and label-free methods. Label strategies draw on the well-established precedent of using stable isotope-labeled (SIL) molecules to improve MS precision. Label-free methods, conversely, also give acceptable precision and have the advantages of lower complexity and cost. Higgs and coworkers (22) demonstrated that high reproducibility ($<10\%$ relative standard deviation) can be obtained using label-free quantitation employing a linear ion trap and bottom-up methodology. More recently, Weiner et al. (30) reported a different label-free approach called differential MS. This method identifies ions that have differential intensity in samples compared by LC-FT/MS under high resolution. Ions that show statistically significant differences are further interrogated by MS/MS for sequence identification. Differential MS offers streamlined computation because only the statistically different products are targeted for identification.

Quantitative strategies incorporating SIL peptides are largely represented by three main approaches: stable isotope labeling by amino acids in cell culture (SILAC), isotope-coded affinity tags (ICAT), and isobaric tagging for relative and absolute quantitation (iTRAQ). The SILAC approach introduces isotope labels into proteins by growing mammalian cells in media containing an SIL version of an essential amino acid (31). Differential protein expression is quantified by comparing normal cells with cells grown using SILAC. Because of the *m/z* shift introduced by the SIL, the samples can be combined and analyzed in a single run. A limitation of SILAC is that it is restricted to tissue culture applications.

ICAT, introduced in 1999 by Gygi et al. (32), was the first application of chemical labeling for quantitative PTX. ICAT reagents insert stable isotopes into proteins through the derivatization of specific amino acids (e.g., Cys) in protein mixtures with a biotin-containing structure to facilitate affinity cleanup. Differential protein expression is observed by derivatizing samples with alternate versions of the ICAT reagent (with and without SIL). As with SILAC, the samples are combined prior to analysis. The use of ICAT with MS/MS allows sequence identification and accurate quantification of proteins in complex mixtures, and it has been applied to the analysis of global protein expression changes, protein changes in subcellular fractions, components of protein complexes, and protein secretion in body fluids.

iTRAQ is a newer labeling approach that allows the simultaneous comparison of up to eight samples. Under iTRAQ, the N-terminus and lysine residues are derivatized with a reagent that introduces a common mass shift to each free amino group.

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Differential analysis occurs by the appearance of different product ions that range from *m/z* 114 to 121 according to the reagent used (33). Samples to be compared are each derivatized with a different iTRAQ reagent, allowing differential expression to be assessed by the relative intensities of the reporter ions.

One of the most important applications of PTX technology is the study of PTMs. Although several known PTMs exist, the most widely investigated are phosphorylation (34) and glycosylation (35). Phosphorylation is the more relevant PTM to drug discovery because of its importance to cell signaling and the high interest in kinase targets. Carr et al. (34) provide a recent thorough review of phosphoproteomics.

Because the ability to detect PTMs can be compromised by the liability of the phosphopeptide (or sugar-peptide) linkage under collision-induced dissociation, extensive interest has been generated by two related techniques that selectively fragment the peptide backbone leaving the PTM intact. Electron capture dissociation (36) and electron transfer dissociation (37) use electrons and negatively charged reagent ions, respectively, to induce c- and z-type cleavage. Although these techniques were originally introduced using FT-MS, capabilities are now being implemented on lower-cost mass spectrometers.

3.2 Metabonomics

In the simplest sense, one can think of MTX as the small-molecule counterpart to PTX. This fast-growing area has been the subject of previous reviews (38, 39) and has advantages over PTX owing to the comparative simplicity of the metabolome and the fact that metabolites are highly conserved between species. A limitation to MTX is that the changes observed, particularly in homeostatic fluids, are often displaced from the biological target or stimulus. Hence, researchers commonly employ sophisticated bioinformatic tools to map observed changes in metabolite flux to specific pathways.

The original applications of MTX in the pharmaceutical industry involved the use of nuclear magnetic resonance (39), although LC/MS, along with other MS techniques, has rapidly increased in popularity owing to the need to analyze complex mixtures and perform structural analysis. LC/MS offers several advantages, including sensitivity, selectivity, and dynamic range. Several LC formats, including HILIC (10) and UHPLC (7), in addition to RP-LC, can maximize the information obtained by MTX. Kind et al. (40) provide an excellent recent example. This group used each of these tools in conjunction with gas chromatography TOF-MS to detect signals of renal cell carcinoma in urine from affected patients.

Along with multiple LC formats, researchers have employed several mass analyzers for MTX, including quadrupole (41), quadrupole ion trap (42), Q-TOF (7), FT-MS (43), and orbitrap (44). Owing to the complexity of the mixtures analyzed, high-resolution mass analyzers are generally favored. The ability to identify unknown metabolites using MS/MS is also an important consideration.

MTX, similar to PTX, is divided into nonbiased and targeted methods. Nonbiased methods attempt to analyze as many molecules as possible in a single injection. Targeted methods, discussed below, profile a specific molecular class. Multivariate

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NCE: new chemical entity

statistical tools, such as principal components analysis (PCA), are typically used to analyze the complex data sets acquired by nonbiased methods. Although such methods can differentiate experimental groups and identify the spectral features responsible for clustering, they do not directly result in identified metabolites. Hence molecular libraries, based on retention time and mass spectral information, are often used to facilitate metabolite identification.

Plumb and coworkers (45) reported the first pharmaceutical application of MS-based MTX, using negative-ion RP-LC-ESI/MS with TOF mass analysis to compare the urinary metabolome of rats following a single oral dose of one of three new chemical entities (NCEs). Using PCA, they showed clear differences between each drug and a vehicle control. Five unique *m/z* values were identified from the PCA loadings plot as strong contributors to the observed clustering, although specific metabolites were not assigned.

Another representative illustration of nonbiased MTX is the investigation of drug-induced phospholipidosis by the antidepressant citalopram (41). In this study, rats were given 125 mg kg^{-1} day^{-1} citalopram by oral gavage for 14 days. **Figure 2*a*** displays the PCA scores plot constructed from the LC-ESI/MS data acquired from

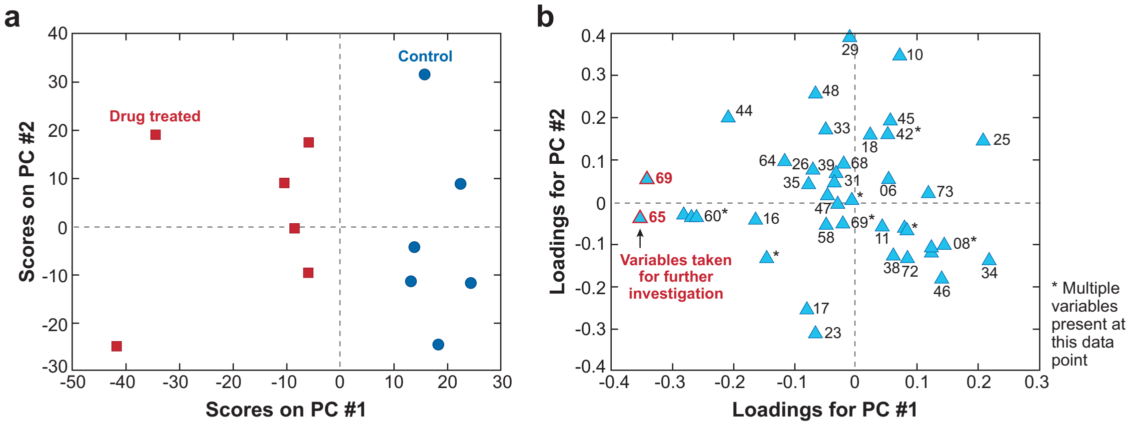

Figure 2

Principal component analysis (PCA) plots from a nonbiased metabonomics investigation of drug-induced phospholipidosis. In this study 12 male Wistar rats were dosed daily for 2 weeks by oral gavage with either tap water or citalopram (125 mg kg^{-1} day^{-1}). Urine collected prior to dosing (day –5) and on days 1, 3, 7, 10, and 14 was analyzed by single quadrupole liquid chromatography–electrospray ionization/mass spectrometry with alternating positive- and negative-ion detection. (*a*) The PCA scores plot obtained at day 7 clearly shows separation of the six drug-treated rats (*squares*) from the six control animals (*circles*) when the data are plotted along axes defined by principal components 1 and 2. (*b*) The loadings plot for PCA of day 7 displays the spectral features most responsible for the observed differentiation, as indicated by displacement from the origin. The two highlighted variables (65 and 69) were taken for further structural investigation using tandem mass spectrometry. Figure reproduced from Reference 42 with permission from the American Chemical Society.

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urine collected at day 7. This plot clearly shows that the treated rats were shifted to the left of the control animals. The PCA loadings plot (**Figure 2*b***) identifies spectral features having the largest contribution to the observed separation. Two variables were selected for further analysis with MS/MS; however, structural assignments for the metabolites were not provided.

Additional examples of nonbiased MTX have been reported for the study of toxicity (42, 46), including a novel approach by Gamache et al. (47) that provided structural insight for redox-active metabolites in rat urine using parallel electrochemical array detection on-line with LC-ESI-TOF/MS. Nonbiased MTX has also been used to study pharmacology models and disease states with examples involving cancer (48), clinical depression (49), and diabetes (50).

The most active area for targeted MTX is lipid profiling, often referred to as lipidomics. Generally, nonbiased approaches are not viable for lipidomics owing to the prevalence of isomers and the lack of universal analysis conditions for structurally divergent lipid classes. Information about the field of lipidomics has been summarized in recent reviews (51, 52).

LC/MS is one of the main tools used to profile lipids. One can find a representative pharmaceutical application in the work of Mortuza et al. (53) who profiled phospholipids in rat urine after exposure to the phospholipidosis-inducing compound amiodarone. Investigators have also used LC/MS to profile other lipid classes including steroids, eicosanoids, and fatty acids. Because of the limited resolution provided by RP-LC for lipid mixtures, UHPLC has been applied to improve peak capacity for lipid profiling (54). Ion mobility offers another option, as demonstrated by Kapron and coworkers (25), who used high-field asymmetric waveform ion-mobility spectrometry with RP-LC-ESI/MS to improve the sensitivity and selectivity of eicosanoid profiling. Lee et al. (55) introduced the most selective and sensitive LC/MS method for eicosanoid profiling with a novel methodology based on negative-ion APCI and normal-phase chiral LC. In addition to offering superior chromatographic resolution, low picogram-per-milliliter detection was obtained by enhancing electron capture through chemical derivatization.

3.3. Pharmacology Screening

MS is increasingly being incorporated into strategies for pharmacology screening. Despite the inherent throughput limitation compared to traditional ligand-binding methods, which are parallel in nature, LC/MS is being considered for select applications. Typically LC/MS warrants investigation for cases in which reagents are rate limiting (e.g., antibodies), fast method development is needed, and/or medium throughput is required. In addition, LC/MS offers a label-free method that accommodates several molecular classes and allows for the simultaneous detection of substrate and product. The selectivity of LC/MS is another important consideration and is vital when screening involves the detection of multiple chemical structures, a topic addressed in greater detail in Section 5.1.

LC/MS has been used in several applications of pharmacology screening, beginning with hit identification and extending through the support of cellular-based

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screens. A comprehensive review by Siegel (56) serves as an excellent resource on this subject. Additional information is available in a review by Geoghegan & Kelly (57).

A variety of LC/MS methods assess ligand binding by capture and release. Although direct information about ligand binding can be obtained by the analysis of gas-phase noncovalent interactions observed by ESI-MS (58), this topic is beyond the scope of this review. A common method for capture and release involves on-line affinity capture. After washing to remove weak binders, the affinity interaction is disrupted and captured ligands are measured by LC/MS. Numerous formats have embraced this general concept, including affinity chromatography, frontal chromatography, affinity ultrafiltration, pulsed ultrafiltration, and gel-permeation spin columns. Although MS can directly identify compounds that bind in such formats, the more common approach involves repetitive detection of a single molecular entity to maximize throughput.

Enzyme targets often lend themselves to LC/MS-based screening because it is possible to monitor the appearance of a small-molecule product in the presence of chemicals being screened as potential inhibitors. A representative example is the cellular assay reported by Xu and coworkers (59), in which they screened inhibitors of the metabolic syndrome target 11-β hydroxysteroid dehydrogenase, measuring the ratio of cortisol (product) to cortisone (substrate). In this screen, they achieved a throughput of 1 min per sample. Other examples in the literature have been produced using an LC/MS/MS screening technology introduced by a commercial vendor. In one such application, LC/MS/MS compared favorably with the scintillation proximity assay for the cancer target AKT1/PKB alpha (60).

One of the most novel applications of LC/MS for pharmacology is the assessment of receptor occupancy. Recently, Chernet and coworkers (61) demonstrated the ability to measure the receptor occupancy of neurochemical drugs by displacing a selective, nonlabeled tracer from the receptor of interest. Tracer measurements in brain tissue offer a viable alternative to radiolabeling and can assist in the development of positron-emission-tomography ligands for clinical use.

3.4. Biomarker Quantification

LC/MS/MS is frequently used to quantify endogenous molecules to assess their ability to serve as useful predictors of biological outcomes. We recently reviewed this subject, covering both large- and small-molecule applications (62). The use of quantitative LC/MS for small molecules pulls from a rich tradition of using MS to screen for inborn errors in metabolism (63). To date, investigators have used LC/MS to profile or quantify almost every conceivable small-molecule class, including amino acids, organic acids, sugars, nucleotides, neurotransmitters, and lipids. Unfortunately, the breadth of this topic exceeds the capacity of this review.

Because of the need to qualify protein biomarkers, intense interest has been focused on the application of LC/MS/MS for protein quantification. To facilitate analysis, a peptide surrogate generated by enzymatic cleavage is quantified using SRM detection. In addition, SIL peptide standards are used to improve assay precision and to provide an absolute rather than a relative measurement. Triple quadrupoles are commonly

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used because they provide the best balance of selectivity and sensitivity; however, quadrupole ion traps (64) and FT-MS instruments (65) have also been used owing to their prevalence in PTX laboratories. Although applications of peptide quantification using stable isotope dilution data date back to the late 1980s, the resurgence has been traced to a 2003 paper by Gerber et al. (66), in which the authors used SIL peptides to quantify target proteins with a method referred to as AQUA, standing for absolute quantification. They used LC/MS/MS with SRM detection to quantify the native and phosphorylated forms of a tryptic fragment, derived from a protein isolated from cell lysate by sodium dodecyl sulfate polyacrylamide gel electrophoresis. An overview of AQUA appears in **Figure 3**.

A common application of AQUA is the interrogation of biomarker leads identified using nonbiased PTX. Because of the number of proteins involved, methods with multiple SRM transitions are employed, hence the frequently used term multiple reaction monitoring. In one example, Anderson & Hunter (67) used 137 multiple reaction monitoring channels to monitor 53 medium-to-high-abundance proteins in human plasma. In this example, they prepared a concatenated protein made from all the individual SIL peptide standards using recombinant methods.

One fundamental limitation of nonbiased PTX is that the identified proteins are of extremely high abundance (microgram per milliliter). Although examples of AQUA-type analysis of serological biomarkers have been published (68, 69), abundant proteins are unlikely candidates for disease biomarkers (70). In a recent review, Rifai et al. (70) covered this issue and other challenges involved in using MS to discover, qualify, and ultimately verify protein biomarkers. One solution to address the need to access lower-abundance proteins is to incorporate immunoaffinity isolation. Anderson and colleagues were the first to apply immunoaffinity enrichment by a technique named SISCAPA (stable isotope standards and capture by antipeptide antibodies). They successfully applied this method, which allowed for an enrichment of greater than 100-fold, to lower-abundance proteins in plasma (71). We recently published a complementary strategy that uses antiprotein antibodies or antibodies against a target peptide (72). This strategy is outlined in the flow scheme displayed in **Figure 4**. By simple analogy, one can think of this scheme as a sandwich ELISA in which MS replaces the detection antibody. We have used this methodology to expedite the qualification of protein biomarkers with lower quantification limits in the range of 100 pg ml^{-1} (73–75). Whiteaker et al. (76) recently published a similar approach, using immunoprecipitation on magnetic beads to assay for TNF-α.

4. CHEMISTRY

In many ways, one can think of medicinal chemistry as the engine that drives small-molecule drug discovery. Although the goal of pharmaceutical discovery is indeed biological in nature, the flow of drug discovery is built around the need to explore diverse chemical space coupled with the need to synthesize increasingly larger amounts of material, with requisite purity, to allow stage-specific experiments to be conducted enroute to a drug candidate. Over the past decade, MS has greatly impacted all facets of chemical synthesis involved in drug discovery. This section reviews key

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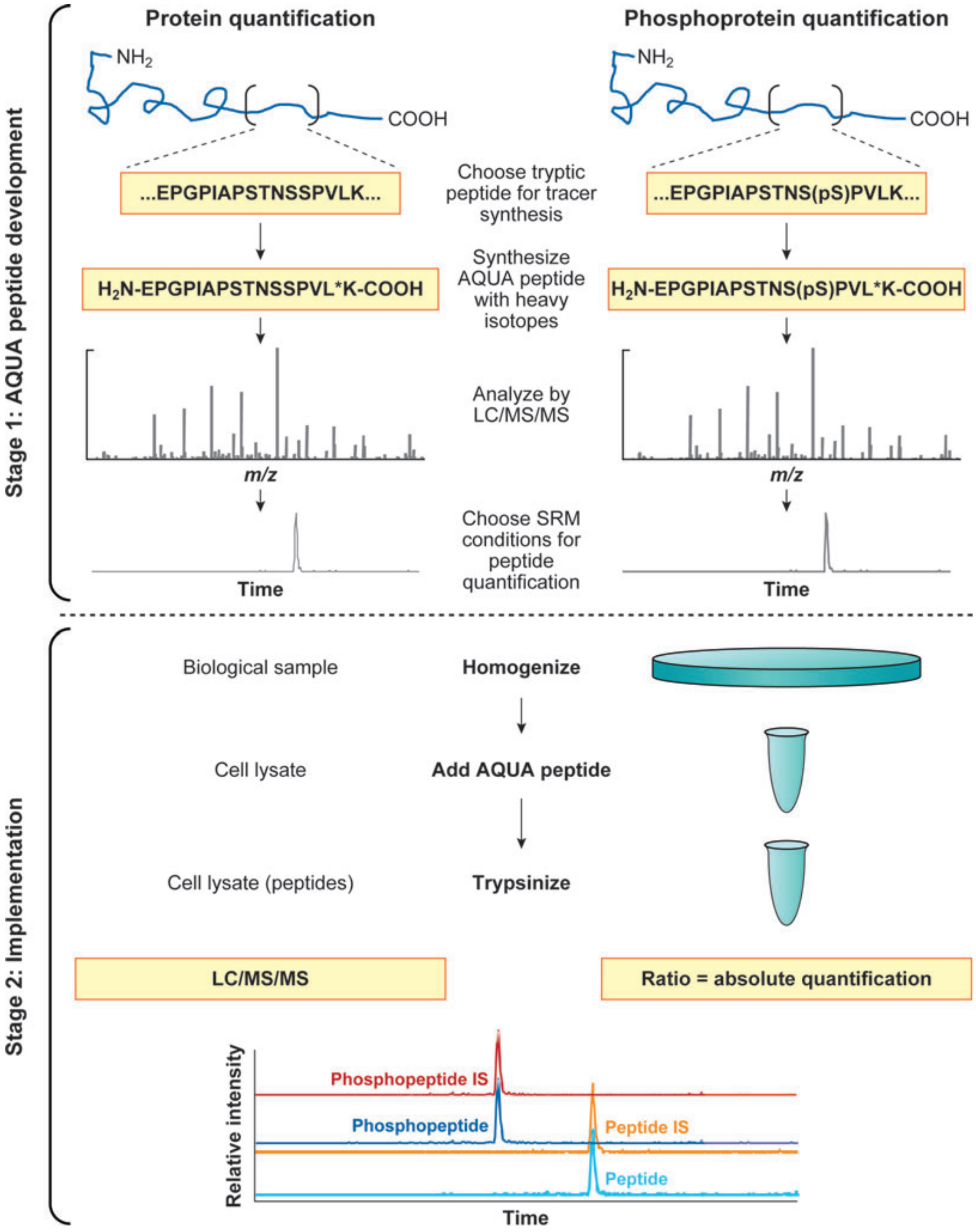

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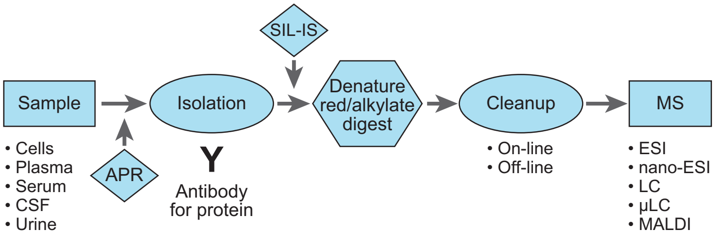

Figure 4

Flow scheme for liquid chromatography/tandem mass spectrometry (LC/MS/MS)-based protein quantification using immunoaffinity capture via an antiprotein antibody. As with the AQUA method, quantification occurs using a peptide surrogate with a stable isotope-labeled peptide internal standard. This strategy allows for expedited qualification of low-abundance protein biomarkers due to a 1000-fold enrichment that occurs through the use of immunoaffinity isolation. This strategy also is useful in guiding the development of sandwich ELISA methods and verifying their selectivity. Figure adapted from Reference 78. Abbreviations: APR, abundant protein removal; CSF, cerebrospinal fluid; ESI, electrospray ionization; MALDI, matrix-assisted laser desorption/ionization; SIL-IS, stable isotope-labeled internal standard.

contributions, organized according to the primary ways LC/MS can support organic synthesis.

For effective small-molecule drug discovery, it is essential to have access to large numbers of structurally diverse compounds for both the generation and optimization of leads. Pharmaceutical companies rely on two primary sources for molecular libraries: corporate compound repositories and HTOS. Although natural products have long been a source for molecular diversity (77), we do not cover this topic in this review.

All major pharmaceutical companies maintain corporate collections of registered compounds and use these collections to screen new targets for hits. Because corporate compound collections are stored in dimethyl sulfoxide (DMSO) over extended periods, significant effort has gone into understanding the storage stability of

←

Figure 3

Absolute quantification of proteins and phosphoproteins using the AQUA strategy. In this strategy protein quantification occurs through the analysis of surrogate peptides produced from enzymatic digestion. Selective analysis occurs via liquid chromatography/tandem mass spectrometry (LC/MS/MS) in the selected reaction monitoring (SRM) mode, typically performed using triple quadrupole MS. The AQUA strategy is illustrated for the quantification of a native protein and its phosphorylated counterpart. In the first stage of the strategy, surrogate peptides are identified, standards are synthesized, and LC/MS/MS conditions are obtained. In addition, stable isotope-labeled forms of the peptides are also prepared for use as internal standards. The second stage of the process involves the actual isolation of the target protein followed by digestion to liberate the peptides of interest. Quantitative estimates of protein concentration are obtained from peak area ratios of the target peptides to their corresponding internal standards spiked during the digestion step. Figure reproduced from Reference 71 with permission from the National Academy of Sciences.

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compounds in corporate compound collections (78, 79). For more detail on this and other related topics, we refer the reader to a review by Cheng & Hochlowski (80).

HTOS is used both to generate libraries for initial screening and to explore chemical space around distinct chemical scaffolds identified from HTS. HTOS consists of two basic formats: combinatorial chemistry and parallel synthesis, both using solid-phase and solution techniques. Combinatorial chemistry, popular in the mid-1990s, has largely given way to the more focused parallel synthesis. Combinatorial chemistry uses variations on a split-couple-recombine strategy to allow almost unlimited permutations of known building blocks to be coupled (81). Although such approaches can yield unparalleled diversity, combinatorial chemistry suffers from a number of logistical problems, including the need to identify hits to obtain structure [it often requires a chemical decoding strategy (82)] and difficulty in controlling reaction yield and purity. Despite these drawbacks, the fundamental limitation of combinatorial chemistry is that it does not yield sufficient material to permit follow-up on hits, making resynthesis necessary. Today, most HTOS involves parallel synthesis whereby discrete chemical reactions occur simultaneously in an array format. Parallel synthesis has become the default approach because it allows for more controlled synthesis and generates larger quantities for testing. As leads progress through discovery, HTOS gives way to traditional organic synthesis to enable targeted synthesis and the production of larger quantities. More in-depth information about the role of MS in HTOS can be obtained from several reviews (80, 83, 84).

4.1. Strategies for Increased Throughput

The ruggedness of the API interface combined with the encompassing scope of ESI transformed organic synthesis in many ways. The first significant impact occurred in the form of open-access (OA) analysis. Beginning in the mid-1990s, chemists started analyzing their own samples, either using flow injection analysis (FIA) with ESI (85) or APCI (86). LC/MS was soon added to the repertoire (87), and most organizations today still have a blend of FIA and LC/MS for their OA needs. High-resolution mass analysis has also been offered for OA analysis (88). Current trends in OA along with other areas pharmaceutical synthesis support can be found in a recent survey (89).

With the uptake of HTS fueled by HTOS methods, the serial nature of MS made analytical characterization rate limiting in the generation of leads. Over the past decade, significant innovation has occurred to rectify this problem. As one might expect, the highest attainable throughput is obtained by FIA. Rapid methods for FIA characterization of compound libraries were introduced using modified autosamplers and multiport injectors. The best example is work by Morand et al. (90), who used a modified eight-port Gilson 215 autosampler to permit a throughput of <4 s per sample, allowing an entire 96-well plate to be analyzed in approximately 5 min. Although FIA is often used to analyze LC fractions or the products of a discrete chemical reaction, Yates and coworkers (91) demonstrated that FIA can also be used for expedient quality control of entire compound libraries.

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Despite the speed of FIA, LC/MS is still the workhorse for synthesis support because it can provide a more detailed assessment of complex mixtures, particularly when combined with other forms of detection. Because of the need for generic methods that accommodate wide structural diversity, gradient elution has become the default approach. Several methods can increase the throughput of LC/MS. For rapid LC/MS analysis, most laboratories use short columns (2–3 cm), high flow rates (2–5 ml min^{-1}), small particles (<4 μm), and generic gradients (10%–90% acetonitrile) to achieve full analysis in 3–5 min per sample. As early as 1997, Weller et al. (92) demonstrated the ability to perform 300 LC/MS analyses per day on a single instrument. More recently, Kyranos and coworkers (93) demonstrated the ability to do full-gradient characterization in 1 min using LC/MS interfaced to ultraviolet (UV) and evaporative light scattering detection (ELSD) for the rapid characterization of high-throughput parallel synthesis. A discussion of the theory and application of fast gradient elution LC/MS has been published (94).

SFC offers a viable alternative to LC/MS and is capable of even higher throughput (approximately threefold) owing to lower viscosity (reduced back pressure). The most frequently used mobile phase composition incorporates carbon dioxide and methanol, which exhibits behavior similar to normal-phase LC. Wang et al. (95) first reported the use of packed SFC/MS for HTOS. Since this time, there have been a number of improvements, particularly with regard to mobile phase additives to permit the analysis of more polar compounds. Recently, Pinkston and coworkers (96) performed a detailed comparison of 2266 compounds using LC/MS and SFC/MS and found both methods to be very comparable with respect to compound coverage. These results were consistent with a previous comparison by Searle et al. (97).

To achieve the next level in throughput, investigators needed to find ways to interface parallel LC methodologies to MS. Given the relative expense of a mass spectrometer, multiplexed methods were introduced to enable the effluent from multiple LC columns to be sequentially sampled by a single mass spectrometer. An approach taken by several laboratories is to employ multi-injector autosamplers, permitting the operation of as many as eight columns in parallel. This mode uses a single LC system to provide a common gradient that is split prior to the injectors. The effluent from each column is sent to a single mass spectrometer in which rapid sampling occurs in succession via a multiport switching valve. Because this approach introduces significant intersample carryover, two groups simultaneously introduced indexed ESI ion sources that use a dedicated ESI sprayer for each column (98, 99). In these designs, discrete sampling of individual effluent streams occurs by using a notched rotating plate assembly that at any instant occludes all but a single sprayer. Micromass introduced a commercial form of this design known as MUX™. **Figure 5*a*** displays a schematic representation of the MUX™ interface. **Figure 5*b*** shows a diagram of the four-column parallel LC/MS system with MUX™ detection used by Xu et al. (100) for compound analysis and purification. This system also incorporated multiple UV and ELSD detectors for purity estimation and automated fraction collection (we review these topics below).

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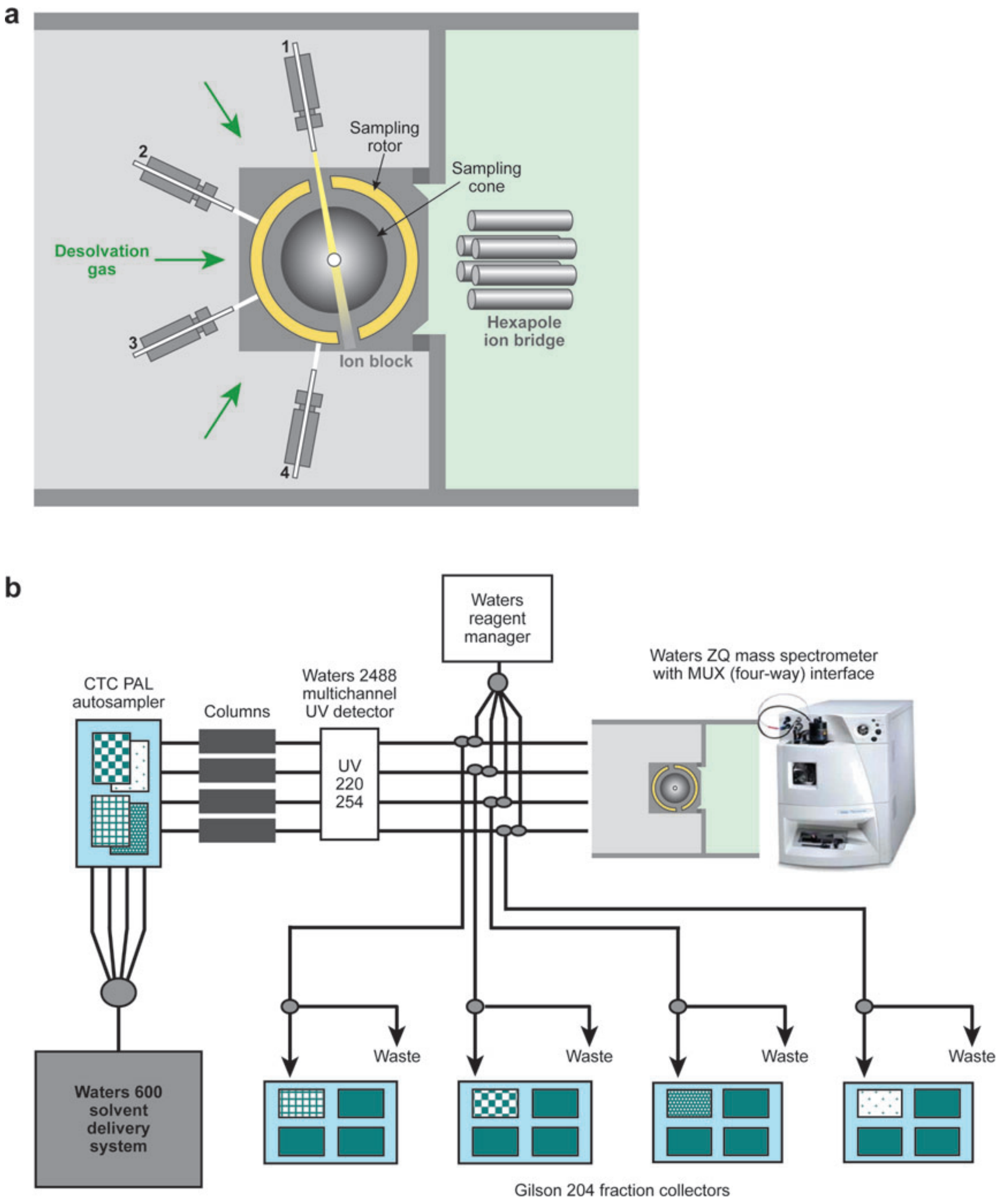

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Although MUX^{TM} can provide up to an eightfold increase in throughput, drawbacks include slight intersprayer cross-talk and the intersprayer dwell time (50 ms), which makes the system incompatible with ultrafast LC methods. Some practitioners have employed multiple LC systems to avoid the deleterious effect from having a single column plug.

4.2. Purity Assessment via Hyphenated Techniques

The issue of compound purity is paramount to successful LG and optimization. During LG, a detailed assessment of the influence of chemical structure on biological activity occurs, known as an SAR. From this perspective, it is easy to see how an SAR could be confounded by the introduction of impure compounds. This issue becomes even more important in LO in which a higher utilization of animal models occurs, including preliminary toxicological assessment. As a result, most pharmaceutical companies have adopted guidelines to govern the level of compound purity required as a function of stage in drug discovery. However, because of the nonuniform nature of MS response for different compounds, other forms of detection are required to provide purity assessment. Several detectors have been used on-line with LC/MS for this purpose, including UV (83), ELSD (101), and chemiluminescence nitrogen detection (CLND) (102). Yurek et al. (103) provide a powerful illustration of the utilization of alternative detectors by coupling LC-ESI-MS with TOF detection with UV (diode array detection), ELSD, and CLND. The authors used this combination of detectors to simultaneously provide compound identification, purity assessment, and an estimate of abundance for compounds prepared by parallel synthesis. In addition, they used the exact mass capability of the system with information obtained from UV and CLND to determine the structure of side products.

In addition to purity, solubility is another important factor that influences screening results derived from compounds synthesized by HTOS. Issues can arise both from insolubility in DMSO upon freeze-thaw cycles and from incomplete dissolution during dilution for HTS. To address this concern, one group routinely uses UV and CLND data, acquired as part of an overall LC/MS-based strategy for library analysis and purification, to provide direct estimates of compound abundance presented for HTS (104).

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Figure 5

(*a*) Schematic diagram of the MUX^{TM} electrospray ionization (ESI) source (Waters Corp., Milford, MA) showing four ESI sprayers positioned around an indexed sample rotor. At any given instant, the rotor occludes all but a single sprayer, allowing for sequential sampling of each effluent stream. Figure 5*a* used with permission from Waters Corp. (*b*) Diagram of the mass-triggered purification system designed by Xu and coworkers, incorporating the MUX^{TM} ESI source. Figure 5*b* reproduced from Reference 107 with permission from the American Chemical Society.

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4.3. Compound Purification

As molecules progress through the testing cascades used in drug discovery, increasing amounts of material are needed for testing. In the early stages of LG, semipreparative columns [10-mm internal diameter (i.d.)] are used to purify low-milligram quantities. Eventually, during LO, preparative LC (50-mm i.d.) is used to purify hundreds of milligrams to gram quantities to fuel compound-intensive in vivo experiments. Over the years, sophisticated systems have been built and are now commercially available for automated purification (105). Weller and coworkers (92) are credited with the first published system that used UV triggering to perform automated fraction collection to purify products from parallel synthesis. Unfortunately, systems using UV triggering collect an inordinate number of fractions that must be analyzed by FIA-MS. Later, Kibbey (106) interfaced LC/MS in parallel with UV-guided fraction collection to avoid this problem and also introduced the idea of using an analytical LC/MS reconnaissance run prior to prep-LC to optimize collection. Cheng & Hochlowski (80) published a variation of this theme, using a single LC/MS equipped with ELSD and UV to perform the reconnaissance to feed four prep-LC systems using either UV or ELSD triggering. The system described had the capacity to purify 200 compounds per day.

In subsequent years, researchers introduced several methods based on MS-triggered fraction collection. Zeng et al. (107) described the first such system, referred to as parallel analyt/prepLC/MS. This system used a valve to switch between dual parallel analytical columns with UV detection and dual parallel preparative columns connected to fraction collectors. In either mode of operation, the effluent streams were coupled to a dual-sprayer ESI interface for MS detection. Initial analysis by analytical LC/MS was used for structural confirmation with on-line UV detection (220 and 254 nm) to estimate purity. Compounds less than 85% pure were scheduled for preparative LC and MS-triggered fraction collection.

Increasingly, several laboratories are using SFC for mass-guided fraction collection (97, 108, 109). In addition to improved separation speed, SFC significantly reduces mobile phase cost and expedites solvent stripping from collected fractions because of the use of carbon dioxide. SFC has also become the mainstay for chiral purification because most chiral columns operate under normal phase conditions (110). Ultimately, because there are advantages to both LC and SFC, many laboratories utilize a blend of these techniques. For example, Ventura and colleagues (109) reported a highly automated strategy for optimal use of the two methods.

In an effort to maximize throughput for compound purification, Xu and coworkers (100) applied parallel LC with MUX^{TM} detection (**Figure 5*b***). In this example, they used mass triggering with semipreparative LC to purify up to 10 mg of material. Interestingly, although this application used mass triggering, there is still no consensus on the preferred mode for triggering fraction collection. Mass triggering has been suggested as being preferred for early discovery applications to minimize the number of collected fractions given the need to purify larger numbers of compounds (83). Regardless of the method used, the current utilization of LC/MS for compound purification represents a blend of walk-up use by organic chemists and dedicated core laboratories.

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5. DRUG DISPOSITION

According to Kola & Landis's (111) 2004 report on pharmaceutical attrition rates, clinical attrition due to poor PK/bioavailability dropped from just over 40% to under 10% in the period from 1991 to 2000. The reason for this dramatic decline is straightforward: ADME properties are investigated far earlier in the drug-discovery process. In the past, ADME properties were not fully investigated until after candidate selection largely owing to an analytical bottleneck. More than any other single factor, LC/MS is responsible for this profound improvement. Using LC/MS, routine methods can be rapidly developed and executed for diverse structural sets within the cycle time mandated by LG and LO. This section is organized according to quantitative and qualitative applications, the latter represented by drug metabolite identification and profiling.

5.1. Quantitative In Vitro Applications

Because ADME applications trace the fate of NCEs in biological systems, tools are needed that can rapidly dial in analytical conditions specific for each compound studied. This situation is further complicated by the frequent need for low-level detection in a variety of biological matrices. LC/MS relieves this bottleneck in two ways. First, the implementation of fast gradient elution methods with on-line sample cleanup by one of several column-switching formats (16, 112) provides a means for rapid method development along with high injection-to-injection throughput. Secondly, the versatility of ESI combined with the analytical selectivity of MS/MS delivers nearly universal detection when judged by historical standards. Amazingly, in what has become a landmark paper, Janiszewski and coworkers (113) demonstrated the ability to analyze 2000 samples per day per mass spectrometer for routine hepatic metabolic stability assessment. This same group also developed methods for automated instrument tuning to optimize the conditions for each NCE tested, a process that eventually became rate limiting (114). Since this time, a variety of instrumental variations have been introduced to improve throughput dealing with methods for sample preparation, injection, chromatography, and detection. Two reviews on ADME screening contain accounts of these advances (115, 116).

The current mandate across the pharmaceutical industry is to factor drug-like properties into the development of chemical leads. Medicinal chemists have become increasingly educated about the need to incorporate favorable ADME characteristics into molecules (117) and routinely utilize a variety of medium-throughput ADME screens in the process of refining an SAR. Although such screens may be run at any time during drug discovery, the limiting factor with respect to starting early is the need for low-milligram quantities of purified compounds. To address this constraint and to reduce costs, researchers have found increased use for in silico models built from empirical data sets in guiding early SAR development (118).

Typically, ADME screens are run by highly automated centralized laboratories. A number of integrated strategies have appeared in the literature (119–121) using a variety of mass analyzers, including single (119) and triple quadrupole (120) and

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quadrupole ion traps (121). Because the bioavailability of small molecules largely depends on absorption and hepatic metabolism, most primary screens are aimed at understanding these two attributes. Tools used to examine absorption range from an assessment of physical properties [e.g., solubility, log D (122)] to transport studies across intestinal cell monolayers (i.e., Caco-2). The technique known as PAMPA (parallel artificial membrane permeability assay) has become popular because it provides an estimate of passive diffusion across cell membranes without incurring the overhead of tissue culture (123).

Active transport of drugs across membranes (e.g., intestinal, hepatocyte, blood-brain barrier) often affects drug absorption, metabolism, and distribution and has become an active area of research (124). Although several important transporters have been identified, the efflux protein p-glycoprotein is the most commonly studied. Smalley and colleagues (125) developed an LC/MS/MS assay for p-glycoprotein inhibition by measuring the influence of NCEs on the bidirectional transport of the known p-glycoprotein substrate digoxin in Caco-2 cells.

The most widely used ADME screen is metabolic stability, which measures the extent or rate of disappearance for NCEs incubated in hepatic media (e.g., microsomes, hepatocytes). Since the introduction of LC/MS-based metabolic stability screening in the late 1990s (126), several variations and refinements have been reported (119, 121). An important secondary screen, useful for assessing the potential for drug-drug interactions, is cytochrome P450 (CYP) inhibition. During these studies, NCEs are coincubated with chemical probes known to be selectively metabolized by a single CYP isoform. Inhibition is indicated by the decreased production of a specific metabolite for the probe in the presence of the NCE. Dierks et al. (127) demonstrated that increased throughput can be obtained by simultaneous incubation of seven probe substrates, each selective for a different CYP isoform. They monitored metabolite formation for each probe in a single LC/MS/MS run. More recently, Kerns and colleagues (128) reviewed common methods used for CYP inhibition and advocated the use of a double cocktail system.

Distribution refers to a drug's ability to spread beyond the vasculature into various tissues and organs. Because distribution often depends on protein binding, several methods in discovery can estimate the binding of NCEs to plasma proteins. The most popular screening methods use either ultrafiltration or equilibrium dialysis with detection by LC/MS. An interesting alternative is to estimate protein binding from retention on columns prepared using immobilized human serum albumin. Cheng et al. (129) described this approach, including favorable comparisons with the other methods mentioned above. Estimating penetration across the blood-brain barrier is another important issue related to distribution for which LC/MS has had profound impact. We refer readers interested in this topic to a comprehensive review of in vitro and in vivo models to assess brain penetration (130).

5.2. Quantitative In Vivo Applications

The monitoring of drugs in biological fluids and tissues is important during both preclinical and clinical development and is typically referred to as bioanalysis. The

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selectivity and sensitivity of LC/MS/MS have significantly transformed the practice of bioanalysis by allowing methods to be quickly developed and executed in a time frame consistent with drug discovery. By far the most common fluid analyzed is plasma as it is the accepted surrogate for modeling drug exposure. Today, exposure determination starts in LG and continues through candidate selection. This topic has been the subject of prior reviews (16, 112).

As mentioned above, bioanalysis has benefited from several advances in LC, including monolithic columns (12), UHPLC (14), and HILIC (11). Moreover, these methodologies are used in conjunction with fast gradient elution methods to achieve run times often under 1 min (13). Whereas LC-ESI-MS/MS using triple quadrupole MS is the default tool for bioanalysis, APCI and atmospheric pressure photoionization are also commonly used and are less prone to matrix effects (17, 18, 131). Various instrumental techniques have also been applied to increase the selectivity of LC/MS/MS, including a high mass resolution triple quadrupole (132) and high-field asymmetric waveform ion-mobility spectrometry (133).

Investigators usually extract analytes from a biological matrix using traditional off-line techniques such as protein precipitation (PP), liquid-liquid extraction, or solid-phase extraction. PP using organic solvents is the most common approach in drug discovery because it is the least expensive and the most universal. Although PP yields dirtier samples than the other methods, low nanogram-per-milliliter levels can be achieved routinely for most drugs.

With shortened LC/MS/MS run times, sample preparation has become more of a bottleneck for high-throughput bioanalysis. Most laboratories employ semiautomated approaches to sample preparation using robotic liquid-handling systems in conjunction with a 96-well plate format to increase the speed of bioanalysis. Automation using on-line extraction techniques is also widely applied. A common theme to all on-line methods is the application of column switching to couple the extraction and analysis steps. Column switching can provide additional cleanup for samples prepared by PP and can be viewed as on-line solid-phase extraction. Indeed, commercial versions of on-line solid-phase extraction have been available for several years (134). Other on-line methods permit direct plasma injection, allowing sample preparation to be even further minimized. The most common methods are turbulent flow chromatography (135) and the use of restricted access media (136). Recently, monolithic columns have been shown to be another choice for direct plasma injection (137).

One inefficiency of LC/MS is that actual MS detection occupies only a small fraction of the total LC duty cycle. Numerous instrumental variations take advantage of this dead time. One of the simplest variations is to use parallel extraction columns with a single analytical column in alternate-regenerate mode (**Figure 6**). Other variations include dual extraction and two analytical columns (138), four columns with staggered injections (139, 140), and the four-channel MUX^{TM} system introduced above (**Figure 5*a***) (141).

Noninstrumental strategies have also increased bioanalytical capacity, including cassette dosing (142) and sample pooling (143). [We refer interested readers to White & Manitpisitkul's (144) discussion of PK considerations for cassette dosing.]

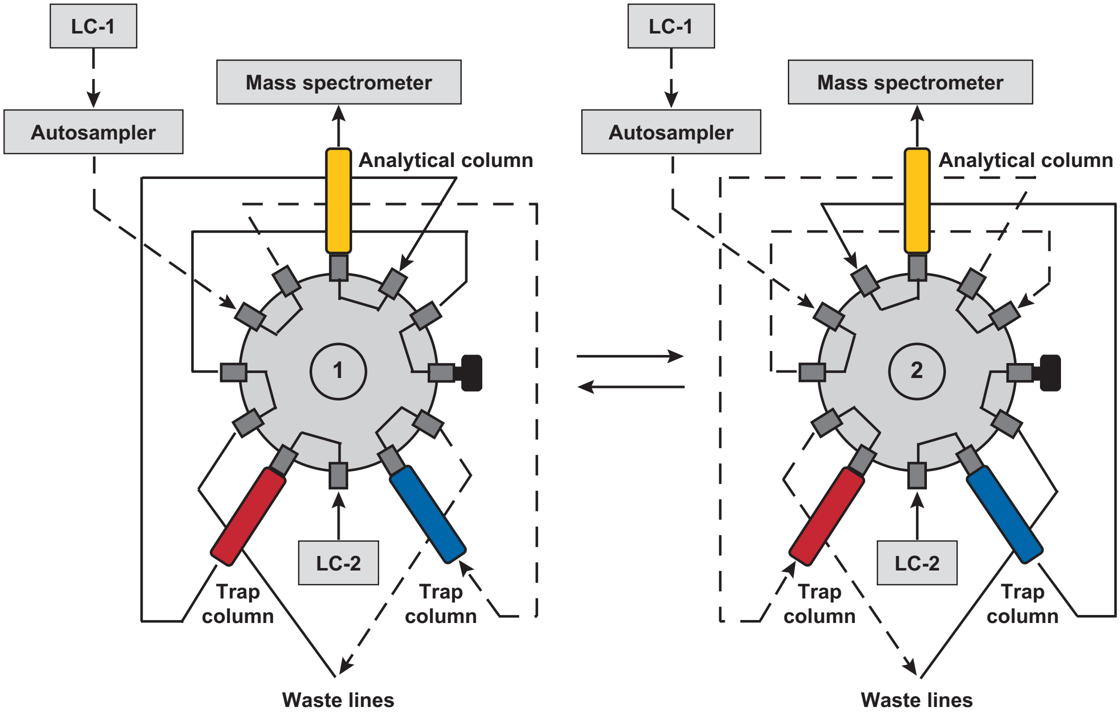

Figure 6

Schematic illustration of a liquid chromatography/mass spectrometry (LC/MS) system configured to perform alternate-regenerate column switching using a 12-port valve. This system uses two LC systems. LC-1 is connected to the autosampler and is responsible for sample loading (*dotted line*). The second system (LC-2) performs gradient elution for sample analysis (*solid line*). In valve position 1, the blue trap column is being loaded and washed, while the red trap column is back-flushed and its contents sent to the analytical column (*yellow*) for gradient elution LC/MS/MS analysis. As the valve switches to position 2, the red column is loaded from LC-1, while the blue column is eluted for analysis. When short columns and fast gradient elution are used with the configuration shown, an injection-to-injection duty cycle of less than 1 min is easily obtained. Alternate-regenerate column switching has been employed with several column formats for on-line sample cleanup and offers a simple means of doubling LC/MS throughput.

One key limitation of quantitative LC/MS is the issue of matrix effects caused by the competition for ionization that occurs when coeluting molecules are simultaneously ionized. Beginning with the publication of landmark papers in the late 1990s that identified and characterized the issue of ionization suppression (145, 146), extensive research has occurred and is the subject of a recent review by Mei (147). To date, several sources for ion suppression have been identified, including plasma phospholipids, sample containers, anticoagulants, and the dosing vehicle (147).

Using current-generation instruments, quantification in the low picogram-per-milliliter range in plasma is not uncommon. Owing to this exquisite sensitivity, investigators have now demonstrated the use of LC/MS/MS to support PK assessment for

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microdosing studies (148). A microdose has been defined as 1/100th of the proposed pharmacological dose or less than 100 μg, whichever is lower (148). Microdosing has been proposed as a means for accelerated clinical assessment of PK behavior. Another study achieved a lower limit of quantitation of 0.1 nM for fluconazole, tolbutamide, and an investigational drug (MLNX) using 100-μL plasma (149). This allowed PK parameters to be calculated in rats for doses as low as 0.001 mg kg^{-1} using current-generation LC/MS/MS instrumentation.

5.3. Drug Metabolite Profiling and Identification

A number of comprehensive reviews dealing with the use of MS to study drug metabolism have appeared in the literature (150–152). LC/MS is the primary analytical tool used to study drug biotransformation, which is frequently categorized into phase I– and phase II–type reactions. In phase I, metabolizing enzymes modify the NCE through hydroxylation, epoxidation, dealkylation, deamination, N- or S-oxidation, reduction, and hydrolysis. In phase II, polar groups are added to the parent drug itself or to one of the phase I metabolites (152). Some examples of phase II reactions include glucuronidation, sulfation, methylation, acetylation, and conjugation with glutathione (GSH) or amino acids such as glycine, glutamic acid, and taurine (152). These reactions result in either the addition or subtraction of known mass increments that can be easily monitored by MS.

A common approach to study drug metabolism involves the use of a radiolabeled drug (e.g., ^{14}C, ^{3}H) to facilitate metabolite detection in biomatrices using LC/MS coupled with radioactive detection. Because radiolabeled drugs are typically not available during discovery, LC/MS/MS techniques coupled with additional forms of detection (e.g., UV) are used to study the metabolic fate of NCEs in drug discovery. In LG and early LO, metabolic hot spots are identified to guide the SAR toward more metabolically stable molecules. This occurs by incubating NCEs with in vitro metabolizing systems (e.g., recombinant enzymes, liver microsomes, hepatocytes, liver slices, or liver S9 fractions) followed by metabolite profiling and identification by LC/MS/MS. MS detection is frequently carried out in both the positive and negative mode to cover the range of basic and acidic metabolites. Relevant metabolite peaks identified by full-scan MS are subsequently interrogated by MS/MS experiments (often conducted via automated data-dependent analysis) to obtain structural information. Common MS/MS scans include product ion, MS^n product ion (quadrupole ion trap), precursor ion, and constant neutral loss scans (triple quadrupole). A representative example is the use of neutral loss scanning to detect glucuronide conjugates, in which a loss of 176 mass units is monitored.

A prerequisite for successful LC/MS/MS metabolite identification is good chromatographic separation. Although significant advances have occurred in LC modes and stationary phases, the separation of isobaric metabolites, such as different hydroxylated forms, can require long run times. UHPLC has enabled these analyses to be carried out at higher speed and with better sensitivity (8).

One limitation of LC/MS for metabolite profiling is that different ESI response factors are observed for metabolites, making LC/MS at best a semiquantitative

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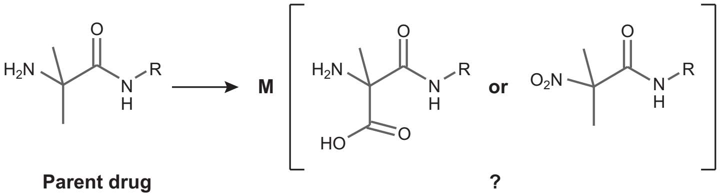

Figure 7

Isobaric chemical structures showing two possibilities for metabolite M, a biotransformation product observed in a recent metabolism study. These two structures that differ by 0.0125 mass units could not be distinguished using nominal mass analyzers such as a quadrupole or an ion trap. Accurate mass–product ion data acquired using a Q-TOF mass analyzer confirmed that the nitro metabolite was the correct structure.

tool. This problem is further influenced by gradient elution because the desolvation efficiency increases during the run due to increased organic solvent content. In 2005, Hop et al. (153) demonstrated that ESI response factors could be normalized using nanoelectrospray (ESI at 200–1000 nL min^{-1} using 10–20-μm i.d. emitters). Building on this work, Ramanathan and coworkers (154) recently published a method that efficiently normalizes ESI response for metabolite profiling. Their method utilizes nanoelectrospray in combination with a postcolumn makeup solution having the inverse composition to the analytical gradient. Their examples showed excellent agreement between radioactivity and LC/MS data by this method (154).

Accurate mass measurements have significantly impacted the study of biotransformation. Today, instruments such as Q-TOF, FT-MS, and the orbitrap are used routinely to solve structural problems that are refractory to LC/MS instruments with unit mass resolution. **Figure 7** illustrates a representative example of the power of accurate mass. In this example, a product ion mass spectrum for a metabolite exhibited as loss of 46 mass units. Because this loss could correspond to the removal of H_2CO_2 (accurate mass = 46.0054) or NO_2 (accurate mass = 45.9929), accurate mass–product ion data were acquired using a Q-TOF mass analyzer. Accurate mass MS/MS indicated a neutral loss of 45.9908 mass units confirming the structure of the metabolite as a nitro compound instead of a carboxylic acid. This example illustrates the power of accurate mass to quickly solve structural problems without resorting to metabolite isolation and nuclear magnetic resonance analysis.

Drugs can undergo metabolic activation to form reactive metabolites that bind to endogenous nucleophiles (e.g., protein, DNA), potentially causing adverse drug reactions. Molecules in drug discovery are routinely screened for their propensity to form reactive metabolites, by the detection of drug-related GSH adducts. LC/MS/MS analysis of reactive metabolites trapped by GSH is now a common practice (155–156). Typically, the signature neutral loss of 129 Da (pyroglutamic acid) is used to detect GSH adducts. GSH derivatives can be used to enhance the detection of trapped adducts. Examples include dansyl-GSH (157), as well as trapping with stable isotope-labeled GSH (158). In the latter method, isotopic doublets differing by 3 Da signal

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the presence of GSH adducts. In addition to these methods, a linear quadrupole ion trap MS was used to screen GSH metabolites in a recent publication that emphasized the selectivity and sensitivity of multiple reaction monitoring detection (159).

6. CONCLUSIONS

This review discusses both recent and historic advances regarding the use of LC/MS for drug discovery for three core functional areas involved in drug discovery: biology, chemistry, and drug disposition. LC/MS clearly has transformed the way drug discovery is conducted in each of these components.

Now that commercial LC/MS instruments based on the API interface have a history spanning more than a decade, it is not surprising that innovation has slowed for applications that have become fully reduced to practice. Several medicinal chemistry applications now fall into this category along with selected applications in drug disposition and biology. At the same time, other areas are still finding fertile ground for research. For instance, the use of LC/MS to discover and qualify novel biomarkers is rapidly expanding. Other applications that push the limits of MS technology for complex mixture analysis (e.g. biotransformation), sensitivity (e.g. microdosing), or throughput (e.g. pharmacology screening) are also receiving attention. Irrespective of the applications that lie ahead, the path forward will continue to rely on sustained improvements in LC/MS instrumentation. For example, a reduction in cost (and perhaps size) is clearly needed for LC/MS to be more broadly introduced into pharmaceutical applications. Unfortunately, despite the introduction of microfluidics and chip-based MS (160, 161), the impact of the nanorevolution has yet to fully materialize in the modern pharmaceutical laboratory. This represents an obvious opportunity. The need for multiplexed MS detection can be cited as another gap, although there has been some pioneering work (162). Lastly, there is always a need for improved ionization techniques. In this sense, the recent development of in situ ionization methods warrants further attention (163).

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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