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Evolving Role of Mass Spectrometry in Drug Discovery and Development

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1.1 ROUTE TO MARKET: DISCOVERY AND DEVELOPMENT OF NEW DRUGS

1.1.1 Industry Research and Development

The members of the modern biopharmaceutical industry are engaged in an on-going struggle to balance the needs of medicine and patient care with the demands of running a growing, profitable business. Moreover, new drugs must be proven to possess some combination of improved efficacy and safety compared with existing treatments. Success in drug research and development (R&D) is critical for meeting all of these objectives, and R&D efforts within the biopharmaceutical industry, as measured by spending, continue to grow steadily (Fig. 1.1). In recent years, the rate of annual growth in R&D spending has been between 5 and 10% in the United States, with the most recent data indicating that R&D spending in 2006 exceeded $50 billion (PhRMA, 2006).

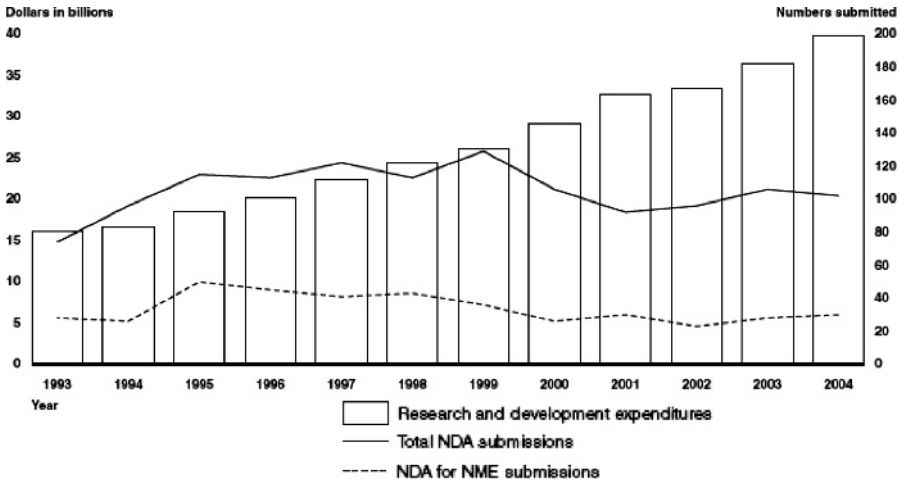


Figure 1.1. *1993–2004 Pharmaceutical R&D expenses, total new drug applications (NDAs), and NDAs for new molecular entity (NME) submission trends. [Reprinted with permission from the U.S. Government Accountability Office (GAO) 2006.]*

The many essential steps in the discovery and development of new drugs can be measured by two primary benchmarks. The first, the number of filed and approved investigational new drug (IND) applications, represents the threshold to human (clinical) testing. The second, the number of filed and approved new drug applications (NDAs), represents the threshold to marketing a drug. These numbers and their trends can represent the relative success of R&D efforts.

Given the typical 12–15 years required to discover, develop, and test a new drug (Fig. 1.2), the NDA submission and approval data will in part represent R&D

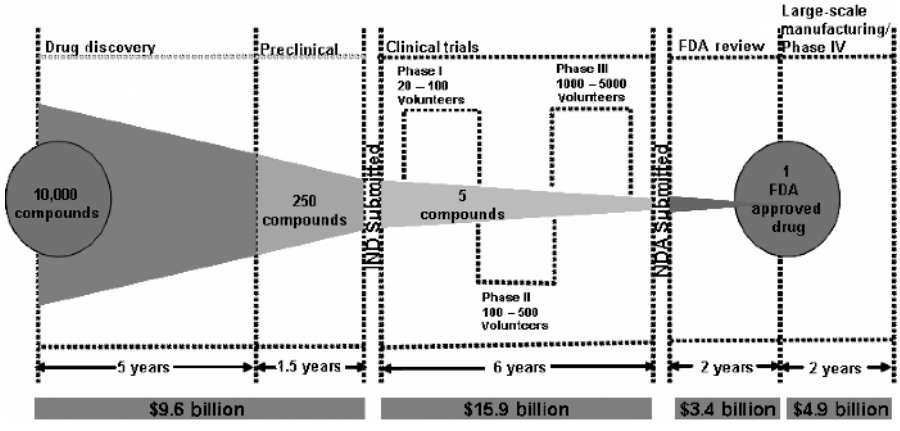


Figure 1.2. *Complex pathway of pharmaceutical R&D involved in bringing a new drug to the market. (Adapted from PhRMA, 2006.)*

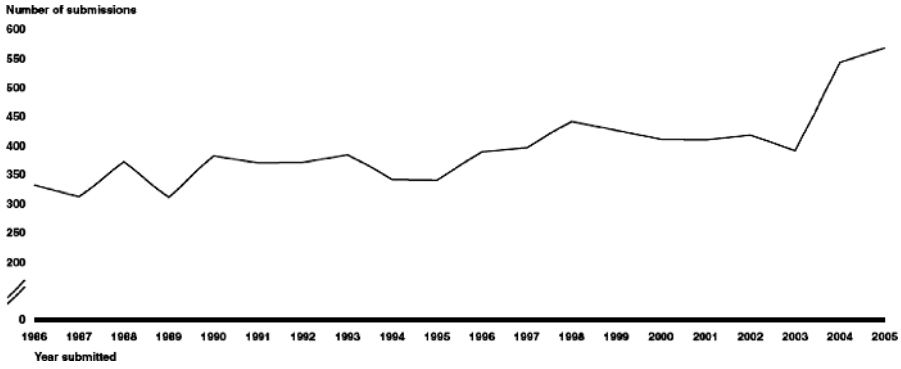


Figure 1.3. *Increase in INDs in recent years. Data are for commercial INDs. (Reprinted with permission from GAO, 2006.)*

progress from several years earlier. Since the late 1990s, the annual rate of NDA submissions and approvals has declined. A similar decline has been observed in the number of NMEs (GAO, 2006). Of the 93 NDA approvals for 2006, only 18 are considered to represent NMEs (*The Pink Sheet*, January 15, 2007). While both total NDAs and NMEs are important, the number of NMEs approved represents a particularly critical measure of overall R&D success.

The statistics of expenditure and NDA approvals can mask a major source of R&D cost and frustration in the industry: late-stage development and postmarketing failures. These types of failures attract significant unwanted publicity and only occur after hundreds of millions of dollars have been spent. Well-publicized examples have included the recent late-stage failure of torcetrapib (Tall et al., 2007) and the postmarketing withdrawals of fenfluramine-phentermine (Fen-Phen) and Vioxx (Embi et al., 2006).

Consideration of IND trends is more encouraging (Fig. 1.3). IND filings occur years before NDA filings and represent a more recent state of R&D success. The number of compounds in clinical testing has approximately doubled over the last decade to approximately 3000 compounds in 2005 in the United States alone. A recent tally of new treatments in clinical testing for various indications is summarized in Table 1.1 (PhRMA, 2006). It is encouraging to see this increase in clinical testing, but it is also important to remember that only about 8% of early-stage clinical testing drugs will produce an approved NDA (Caskey, 2007).

1.1.2 Drug Discovery and Development Process

The overall process of bringing a new drug to market is typically divided into two principal areas: drug discovery and drug development. Examples of summaries describing the entire process include the publication entitled "Drug Discovery and Development: Understanding the R&D Process" (PhRMA, February 2007) and a tutorial written by Jens Eckstein, recently available online at www.alzforurm.org/drg/tut/tutorial.asp.

TABLE 1.1. Treatments in Clinical Testing

Disease Area or Indication	Number of Compounds in Development
Oncology	682
Neurological disorders	531
Infectious diseases	341
Cardiovascular	404
Psychiatric	190
Human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS)	95
Arthritis	88
Asthma	60
Alzheimer/dementia	55

Source: PhRMA, 2006.

The following description very briefly summarizes some of the steps in drug discovery and development.

1.1.2.1 Drug Discovery The first step in discovering a new medicine is to identify a therapeutic target. Drugs in today's market as well as those in recent clinical testing target less than 500 biomolecules, with more than 10 times that many potential therapeutic targets waiting to be discovered and developed (Drews, 2000). More than 50% of the newly approved drugs result from R&D involving previously clinically tested and validated targets. Once a target has been validated (proven to be related to the disease process), high-throughput screening methods may be used to determine initial structural leads. Compounds are assessed for target affinity and for their "drug-like" properties, including absorption, distribution, metabolism, and excretion (ADME) using a series of in vivo and in vitro tests. The results of these tests are used to improve the structure and therefore the properties of the next round of test compounds, until ultimately one or more acceptable compounds are advanced forward in the process. This stage of discovery, which can be lengthy and difficult to predict, is generally referred to as lead optimization. The lead selection and lead optimization studies that are used to sift out the problematic compounds are summarized in Fig. 1.4.

Mass spectrometry enters into all phases of drug discovery (Feng, 2004; Lee, 2005). Early in the discovery, target proteins are identified and characterized by MS following LC or two-dimensional gel electrophoresis separation (Kopec et al., 2005; Deng and Sanyal, 2006). The make-up of an isolated protein is determined by enzymatically digesting the protein and then analyzing the peptides by MS (Link, 1999; Kopec et al., 2005; Köpke, 2006). Once a target is validated, compounds generated from any one of the following strategies are evaluated against the target: total synthetic process (33%), derivative of natural products (23%), total synthetic product with natural product mimic (20%), biological (12%), natural product (5%), total synthetic product based on a natural product (4%), and vaccine (3%) (Newman et al., 2003; Newman and Cragg, 2007). In almost all pharmaceutical

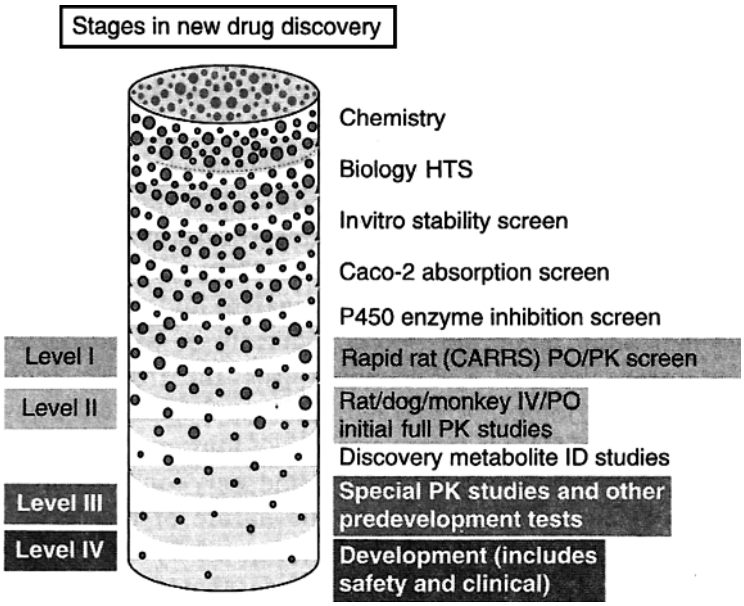


Figure 1.4. *NCE/NME progression scheme showing the various discovery stage liquid chromatography–mass spectrometry (LC–MS) and LC–tandern MS (LC–MS/MS) assays used for selecting NME/NCE to advance into development. (Reprinted with permission from Korfmacher, 2005.) (CARRS, Cassette accelerated rapid rat screening; IV, Intravenous administration; PO, Oral administration; NCE, New chemical entity)*

companies, open-access MS laboratories have been set up to allow medicinal chemists to confirm and assess the purity of their synthesis or isolated products (Chen et al., 2007). Once the compounds or compound series are confirmed, high-throughput screening (HTS) assays are used to weed out compounds that do not show any activity toward a host [protein, ribonucleic acid (RNA), deoxyribonucleic acid (DNA), etc.] (Fligge and Schuler, 2006). Mass spectrometric approaches also have been used to study noncovalent complexes involving protein–drug, DNA–drug and RNA–drug to identify structural details of the drug-binding sites (Benkestock et al., 2005; Siegel, 2005; Hofstadler and Sannes-Lowery, 2006; Jiang et al., 2007).

Compounds or compound series selected using HTS are further filtered using in-vitro-based solubility, chemical stability (Wilson et al., 2001), permeability (Bu et al., 2000a,b; 2001a–d; Mensch et al., 2007), and metabolic stability (Lipper, 1999; Thompson, 2000, 2005) assays before the lead selection/optimization stage (Lipper, 1999; Thompson, 2000, 2005). Most of these in vitro assays are faster, more efficient, and more sensitive due to unsurpassed involvement of the LC–MS (Thompson, 2001; Mandagere et al., 2002; Pelkonen and Raunio, 2005; Thompson, 2005). Results from such high-throughput in vitro assays are used to select compounds for additional in vitro tests and finally for in vivo testing in preclinical species (mouse, rat, dog, monkey, etc.). Similar to the early discovery stage high-throughput assays, LC–MS and LC–MS/MS assays are the methods of

choice for the late-stage discovery studies (lead optimization stage, levels II and III) because they are rapid, sensitive, easy to automate, and robust.

All the discovery stage quantitative and qualitative LC–MS assays (levels I, II, and III), which are used to select drug candidates for development, are not rigorously validated and are not required to satisfy any of the good laboratory practices (GLPs) guidelines set forth by the regulatory agencies (Shah et al., 2000; Hsieh and Korfmacher, 2006; Jemal and Xia, 2006).

1.1.2.2 Drug Development The preclinical testing represents the bridge between discovery and later clinical (human) testing. As shown (Fig. 1.2), if 10,000 compounds enter the screening stage, only about 250 will make it into the preclinical testing stage. During this stage, critical assessments of drug candidate safety are obtained in toxicology studies. Also essential understanding of the ADME, pharmacokinetic (PK), and pharmacodynamic (PD) properties of the drug is established.

1.1.2.2.1 The Drug Substance Before starting any long-term toxicological studies in rodent (rat or mouse) and nonrodent (dog or monkey) species, it is imperative to work out all the chemical, pharmaceutical, large-scale synthesis, purification, stability, and formulation issues associated with the drug substance (Smith et al., 1996; van De Waterbeemd et al., 2001).

For a drug substance to move further in the development pipeline, its physical and salt forms have to be optimized in pharmacokinetics studies often using quantitative LC–MS/MS assays. Pharmaceuticals can exist as either a crystalline form (which has long- and short-range order in three dimensions) or an amorphous form (which lacks the long-range order present in crystalline material). In the discovery stage, usually all ADME assays (levels I, II, and III) are conducted using laboratory-grade amorphous drug substance without optimizing for physical and pharmaceutical properties of the drug (Kerns, 2001). Although the stability of an amorphous drug substance is sufficient for short-term discovery studies and for making internal recommendations, a crystalline form is the preferred form for long-term toxicological and clinical studies due to its long-term stability. However, the ability of a drug (organic molecule) to exist in more than one crystalline form leads to polymorphism. Polymorphs (same chemical composition but different internal crystal structure) of a given drug can have widely different pharmacokinetic parameters (Chapter 2), especially bioavailability due to differences in physicochemical properties such as dissolution rate, density, and melting point (Kobayashi et al., 2000; Agrawal et al., 2004; Panchagnula and Agrawal, 2004).

Changes in the method of synthesis during the large-scale manufacturing phase of drug development can also lead to changes in the crystalline form (Perng et al., 2003; Huang and Tong, 2004). A well-documented example of crystalline form change was observed with ritonavir (Norvir), a protease inhibitor approved in 1996 for treatment of HIV infections. In mid-1998, sales of ritonavir were temporarily halted due to manufacturing difficulties associated with multiple polymorphs (Bauer et al., 2001; Van Arnum, 2007). Later, in 1999, reformulation and additional LC–MS/MS-based pharmacokinetic studies allowed Abbott Laboratories to bring ritonavir back

to the market. Today, the Food and Drug Administration (FDA) requires application of techniques such as X-ray diffraction and/or vibrational spectroscopic analysis [*Fourier transform infrared (FTIR)*, near infrared (NIR), Raman] to characterize polymorphic, hydrated, or amorphous forms of drug substances and for further evaluation of pharmacokinetic parameters using the final thermodynamically stable form of the drug.

Salt form selection/finalization is another crucial step in preclinical development (Engel et al., 2000; Furfine et al., 2004). Some of the common pharmaceutical salts include hydrochloride, sulfate, mesylate, succinate, tartrate, acetate, and phosphate. Similar to the changes that occur in the crystalline form, the changes that occur in the salt form also alter the oral bioavailability of a drug. When the salt form of a drug substance is changed, quantitative LC–MS/MS assays are used to reassess the key pharmacokinetic parameters as well as bridge the new parameters with the discovery stage data, if necessary. Along with physical and salt form optimization, the drug substance is also subjected to acid, base, and photostability tests, and when necessary, degradants are identified using LC–MS and nuclear magnetic resonance (NMR) techniques.

Once the salt and physical forms of a drug substance are finalized and large-scale manufacturing issues are addressed, the NCE/NMEs recommended for development and human testing is often referred to as the active pharmaceutical ingredient (API). Around this stage of the preclinical development, several kilograms of the API are manufactured under good manufacturing practices (GMP) guidelines established by the regulatory authorities (Webster et al., 2001). At this stage, LC–MS and MS/MS methods are used to fully characterize the API and to identify any major impurities and degradants present in the starting materials and/or formed during API processing (Kovaleski et al., 2007). Once all the API impurity issues are worked out, the certified API is used for toxicological studies conducted in support of first-in-human clinical studies. The International Conference on Harmonization (ICH) guidelines on the API suggest that impurities $>0.15\%$ and $>0.05\%$ respectively for ≤ 2 g and >2 g daily dose should be characterized and the impurity levels should be reduced if there are any known human risks.

Before the start of toxicological studies, an LC–MS/MS method to quantify the drug substance and/or its metabolites in plasma is developed using the certified API. This quantitative LC–MS/MS assay is developed under GLP guidance. Most often a stable isotope labeled form of the drug is used as the internal standard to correct for any experimental limitations. Upon completion of the rodent and nonrodent toxicological studies using the quantitative LC–MS/MS assays, safe human doses to be used in the first-in-human study come to light and the pharmaceutical company is ready to file for an IND. For perspective, the total testing regime up to this stage is estimated to consume about one-quarter of the total R&D expenditure in the industry (PhRMA, 2006). Of the 250 compounds that entered preclinical testing, only 5 on average will advance into human clinical testing.

1.1.2.2.2 Clinical Trials Once an IND is approved, clinical trials take place typically in three sequential phases, phases 1–3. However, based on the recent FDA guidelines, traditional phase 1 studies could be preceded by "phase 0" or "exploratory

IND" studies. These studies involve the administration of a single subtherapeutic dose of a radiolabeled NME to healthy adult volunteers to assess the human pharmacokinetics and/or metabolism (Lappin and Garner, 2005; Hill, 2007). Subtherapeutic doses are defined as the smaller of either 1/100 of the expected pharmacologically effective dose, or 100 μg. The FDA guidelines also require animal toxicity studies to be completed using doses above the human subtherapeutic doses to show no risk of toxicity before starting phase 0 clinical studies. Phase 0 studies may allow identification of "less promising" compounds earlier and at lower cost. According to a recent presentation, phase 0 studies can shorten the drug development process by 6–12 months (Kummar et al., 2007). However, most of the phase 0 studies cannot be completed using conventional LC–MS techniques because administered doses are around 100 μg and require the use of accelerator mass spectrometry (AMS), the only ultrasensitive technique capable of quantifying ^{14}C-labeled compounds with attomole ($10^{-18}\,M$) sensitivity (Chapters 2 and 7). However, several laboratories are hard at work developing ultrasensitive LC–MS techniques capable of detecting drugs and/or metabolites from microdosing studies (Lebre et al., 2007; Seto et al., 2007; Yamane et al., 2007).

Phase 1 clinical trials are conducted on a small number (20–100) of healthy adult volunteers to determine the potential toxicity of a drug, whether severe side effects can occur, and safe dosage ranges. An assessment of pharmacokinetics and drug metabolism is also included. For obtaining all the PK parameters, quantitative LC–MS/MS assays developed under GLP guidance are used. However, metabolism studies are conducted using non-GLP-based qualitative LC–MS and LC–MS/MS methods to get a glimpse of the metabolites present in human plasma and urine (Chowdhury, 2007; Ramanathan et al., 2007c; Ramanathan et al., 2007d). In specialized cases, phase 1 trials may include subjects with the targeted disease (e.g., oncology drugs). Overall, the critical criteria for phase 1 are the safety profile of the drug and determination of a safe dosage.

Phase 2 trials involve the administration of the potential drug to 100–500 volunteer patients to demonstrate the efficacy of the drug against the targeted disease or condition. A phase 2a trial is considered a relatively small, early study with a limited number of patients and may include both efficacy testing and refinement of the dosing regime. A successful phase 2a trial could be followed by a larger phase 2b trial to expand the available data, particularly on efficacy under the defined dosing regime. The first testing of efficacy in a patient population can also be called a proof-of-concept study.

Following a successful determination of safety and efficacy in phase 2, phase 3 trials are conducted on hundreds to thousands of volunteers suffering from the target disease or condition. The large size of phase 3 trials makes this by far the most expensive stage of clinical testing. Drugs that fail in phase 3 or later represent a significant cost without return and the industry as a whole has increased efforts to identify and terminate development investments in such compounds before the expense of phase 3 is incurred.

Upon completion of successful phase 3 clinical trials, a NDA is filed with the FDA for marketing approval of the new drug against a particular disease or condition.

NDA approval leads to large-scale manufacturing and marketing of the medicine. Clinical trials may continue to assess efficacy against different diseases or assess long-term safety in a larger population than was possible under phase 3 testing. As noted in Fig. 1.2, of the 5000–10,000 compounds that entered testing, approximately 1 will emerge as an approved drug.

1.2 DRUG METABOLISM AND PHARMACOKINETICS IN DRUG DISCOVERY AND DEVELOPMENT

Prior to the 1990s, the pharmaceutical lead finding activities were mainly driven by human diseases and dominated by chemistry and pharmacology ("disease-driven method," or "old paradigm"). During the 1990s, combinatorial chemistry, parallel chemical synthesis, and HTS revolutionized the drug discovery process and put forward a vastly increased number of biologically active NME/NCE leads. The increase in leads, the 50% success rate in Phase 3 for NME (PhRMA, 2006), and the increase in time required to complete clinical trials (3.1 years in the 1960s to 8.6 years in the 1990s (DiMasi, 2001b)); resulted in shifting to a new drug discovery and development paradigm. A new paradigm was also indicated by retrospective analysis that demonstrated the unacceptable pharmacokinetic (PK) characteristics, not identified in preclinical testing, was a significant cause of clinical failure (Prentis et al., 1988; Milne, 2003; Wahlstrom et al., 2006). Under the "new paradigm," or "target-driven method," pharmaceutical companies started to incorporate PK components early in the drug discovery process to generate more promising clinical candidates. A subsequent study 10 years later showed that the incorporation of PK early in the drug discovery process helped to reduce the clinical stage drug candidate failures associated with unacceptable PK characteristics to $<15\%$ (Hopkins and Groom, 2002; Kola and Landis, 2004).

Pharmacokinetics is the science that describes the movement of a drug in the body (Jang et al., 2001). In other words, PK is concerned with the time course of a drug's concentration in the body, mainly in the blood (plasma). The PK parameters are discussed in Chapter 2. Four separate but somewhat interrelated processes influence a drug's movement in the body: absorption (A), distribution (D), metabolism (M), and excretion (E). These four major components which influence a drug's level, its kinetics of exposure to tissues, and its performance as a drug are described in the following:

- *Absorption* The process by which a drug molecule moves from the site of administration into the systemic circulation (bloodstream). When a drug is administered intravenously (IV), the drug is 100% absorbed (bioavailability is 100%). However, when a drug is administered via other routes [such as orally (by mouth, PO, *per os*), subcutaneously (under the skin), intradermal (into the skin)], its absorption (bioavailability) is influenced by many factors, including the rate of dissolution, metabolism before absorption and the ability to cross the gastrointestinal tract (Martinez and Amidon, 2002). Therefore, bioavailability, as detailed in Chapter 2, is one of the essential tools in

pharmacokinetics, as bioavailability must be considered when determining dosing regimens and formulations for nonintravenous routes of administration.

- *Distribution* The process of a drug being carried via the bloodstream to its site of action, including extracellular fluids and/or cells of tissues and organs. Factors that affect a drug's distribution include blood flow, plasma protein binding, tissue binding, lipid solubility, pH/pK_a, and membrane permeability (Vesell, 1974). Although distribution is typically not the rate-limiting step, distribution to sites such as the central nervous system, bones, joints, and placenta could be slow, inefficient, and therefore the rate-limiting step (De Buck et al., 2007).
- *Metabolism* Metabolism or biotransformation is the process by which the body (human and animal) or a system (cell based or in vitro) breaks down and converts a drug generally via oxidation, reduction, hydrolysis, hydration, and/or conjugation reactions into an active, inactive, or toxic chemical substance. Enzymes (e.g., cytochrome P450s) present in the liver are responsible for metabolizing many drugs (Guengerich, 2006). When a drug is administered intravenously (or other nonoral routes such intramuscular and sublingual), some of these metabolism pathways are avoided.
- *Excretion/Elimination* The irreversible removal (elimination) of a drug and/or its metabolites from the systemic circulation or from the site of measurement. The process of elimination usually happens through the kidneys (urine) or the feces. Unless excretion is complete, accumulation of drugs and/or metabolites can lead to adverse affects. Other elimination routes include the lung (through exhalation), skin (through perspiration), saliva, and mammary glands.

Phermacodynamics (PD) is the relationship between a drug's concentration at the site of action and its pharmacological, therapeutic, or toxic response at the site of action. It is often difficult to measure a drug's concentration at the site of action. Therefore, the PK/PD relationship (Chapter 2) becomes essential to understand and relate a drug's concentration in the blood (plasma) or other biological fluids with its pharmacological, therapeutic, or toxic response at the site of action (Derendorf and Meibohm, 1999). In the pharmaceutical drug discovery and development arena, the parameters that define PK and/or PD are the primary drivers in the selection of a drug candidate to move forward to the clinic and finally to the patients. Therefore, for a NME/NCE to be an effective drug, it not only must be pharmacologically active against a target but must also possess the appropriate ADME properties necessary to make it suitable for use as a drug (Thompson, 2000).

1.3 MASS SPECTROMETRY FUNDAMENTALS

The dramatic increase in the complexity of the new drug discovery and development paradigm involving an evaluation of a vast number of leads for favorable activity, selectivity, and ADME properties in turn puts more pressure on the drug discovery

and early development teams. For drug metabolism and pharmacokinetics (DMPK) scientists, evaluating large numbers of compounds with limited supply meant creating high-throughput ADME assays that can provide answers quickly. The speed of analysis contributed directly to the discovery and development of optimized lead candidates, which in turn impacted the overall time required for developing new medicines. The inherent sensitivity, selectivity, and speed of MS turned out to be a superb solution for drug metabolism and pharmacokinetics applications, especially high-throughput ADME assays.

1.3.1 History

Mass spectrometry is an analytical technique that measures the mass-to-charge ratio (m/z) of gas-phase ions formed from molecules ranging from inorganic salts to proteins. The mass spectrometer is a device or instrument that measures the mass-to-charge ratio of gas-phase ions and provides a measure of the abundance of each ionic species. To measure the m/z of ions, the mass analyzer and detector must be maintained under high-vacuum conditions and calibrated using ions of known m/z. As explained in the following section, some ion sources can be maintained at atmospheric pressure, while others require vacuum conditions.

For excellent perspectives on the historical developments in MS, readers are directed to several outstanding books and reviews, including the American Society for Mass Spectrometry's 50th anniversary book (Grayson, 2002). Similar to any other field, the field of MS is laced with several Nobel laureates, including the father of modern MS, J. J. Thomson:

Scientist	Nobel Prize Year and Field	Contribution
Joseph J. Thomson	1906, Physics	Discovery of electrons
Francis W. Aston	1922, Chemistry	Stable isotopes
Wolfgang Paul	1989, Physics	Development of quadruplole and quadrupole ion trap
John B. Fenn	2002, Chemistry	Development of electrospray ionization (ESI)
Koichi Tanaka	2002, Chemistry	Development of matrix-assisted laser desorption ionization (MALDI)

The analytical capability of MS has been evolving at an astounding rate as Nobel laureates and developers push what is an inherently powerful analytical technique to even higher levels of capability. During the last decade, numerous ionization and analyzer configurations have been commercialized. Some of the most recent developments have made MS the gold standard for many pharmaceutical analyses, and has made the biopharmaceutical industry the major purchaser of mass spectrometers (Cudiamat, 2005).

1.3.2 Fundamental Concepts and Terms

For greater detail, the reader is referred to a comprehensive text on MS (Gross, 2004; Watson and Sparkman, 2007) or on terminology in MS (Sparkman, 2006). For brevity, a relatively simple list of definitions is provided here. For most mass spectrometry users, the concept of mass has been limited to the relatively simplistic integer mass level. The proliferation of high resolution and high mass accuracy instruments in the last decade, however, necessitates a brief consideration of the fundamentals of mass beyond the integer level. For beginners, the "mass" comes from protons and neutrons (and, marginally, electrons), and the "charge" comes from an excess of either protons (+ charge) or electrons (− charge). Mass spectrometers can only detect charged species. Finally, it is worth noting that the dominant focus of this book is on small molecules, where in general only a single charge resides during MS analysis. For these types of species, $z = 1$ and mathematically, $m/z = m$. The MS user community commonly discuss mass where mass-to-charge ratio would be accurate.

1.3.2.1 Mass Terminology

- *Mass Unit* The unified atomic mass unit, or u, is the fundamental unit of mass for most mass spectrometrists. The Dalton, or Da, is also generally accepted and is commonly used in descriptions of large, biological molecules. The mass unit is defined as one-twelfth of the mass of carbon-12. Atomic mass unit, or amu, is technically incorrect but still commonly used. The unit Thomson (Th) has been used as a unit of m/z. However, Th is not accepted by most mass spectrometry journals and the International Union of Pure and Applied Chemistry (IUPAC). Therefore, m/z used for labeling the x-axis of mass spectra is unit less.
- *Average Mass* Mass calculated using the weighted average atomic mass of each element. Average mass is not measured using a mass spectrometer; rather this is calculated using the values reported on the periodic table. For example, the average mass of dextromethorphan ($C_{18}H_{25}NO$) is 271.4 [$(18 \times 12.011) + (25 \times 1.0079) + (1 \times 14.0067) + (1 \times 15.9994)$].
- *Nominal Mass* The whole-number (nominal) mass of a molecule (or atom) is calculated from the integer mass of the most abundant, stable isotope of each constituent atom. For example, the nominal mass of protonated dextromethorphan ($C_{18}H_{25}NO + H^+$) is 272 [$(18 \times 12) + (26 \times 1) + (1 \times 14) + (1 \times 16)$].
- *Exact Mass* A calculated mass, and theoretically the mass (for $z = 1$) that should be observed on the mass spectrometer; sometimes also used to refer to a measured mass (see accurate mass below). The exact mass of a molecule is determined by adding the exact mass of a particular isotope for each constituent atom in the molecule. For example, the exact mass of protonated dextromethorphan ($C_{18}H_{25}NO + H^+$) is 272.2009 [$(18 \times 12.0000) + (25 \times 1.0078) + (1 \times 14.0031) + (1 \times 15.9949) + (1 \times 1.0073)$. The importance of the electron mass (0.00055 u) in the calculation of exact mass has been explained in detail by Ferrer and Thurman (2007).

- *Accurate Mass* A measured mass. Accurate mass is the observed mass to some specified number of decimal places of a molecule (or similar) as measured on the mass spectrometer. A so-called accurate mass measurement can be obtained on any mass analyzer, though it is generally assumed that the accuracy will be improved when the analysis is performed using high-resolution mass spectrometers (see below).
- *Monoisotopic Mass* An exact mass, derived from the mass of the most abundant, stable isotope of each constituent atom in the molecule. For example, the monoisotopic mass of protonated dextromethorphan containing one ^{13}C ($^{12}C_{17}$ ^{13}C $^{1}H_{25}$ ^{14}N $^{16}O + {}^{1}H^{+}$) is 273.2032 [$(17 \times 12.0000) + (1 \times 13.0034) + (25 \times 1.0078) + (1 \times 1.0073) + (1 \times 14.0031) + (1 \times 15.9949)$.
- *Mass Defect* The difference between the exact mass of an ion or molecule and the nominal (integer) mass. The mass defect can be highly characteristic of the constituent atoms and is useful in data handling (see below and Chapters 5 and 6).

1.3.2.2 Mass Calibration and Resolution

- *Mass Calibration* The process by which the mass analyzer is calibrated such that a measured and displayed m/z is accurate. Well-characterized calibration compounds are utilized, and measured m/z values for these compounds are compared to theoretical m/z values. Calibrants commonly used include various polymeric species (such as polypropylene glyol, or PPGs; polytyrosine (poly-t)) or fluorinated species (perfluorokerosene or PFK) but can be any compound or mixture (NaI/KI) of compounds properly characterized for MS.
- *Internal Calibration* The process by which one or more calibrant is introduced into the mass spectrometer simultaneously with the unknown sample, and the mass calibration is continuously updated during analysis. Considered the most effective means of obtaining highly accurate mass analysis (provided the calibrant does not interfere with the analysis of the unknown) (Herniman et al., 2004).
- *External Calibration* When mass calibration is conducted in an entirely separate exercise from analysis of an unknown. External calibration can be performed infrequently, avoiding the potential problem of simultaneous analysis of calibrant and unknown (direct interferences, suppression, etc.).
- *Lock Mass* Similar to internal calibration. The lock mass compound is monitored during analysis of the unknown, and the mass calibration is adjusted based on the comparison of the measured m/z and the theoretical m/z for the lock mass compound. If multiple lock mass compounds are used across the m/z range, the process effectively becomes internal calibration. Lock mass compound(s) can be introduced into the LC–MS source via a tee into the LC flow or sheath liquid inlet or dedicated sprayer.

- *Resolution* The width (in u) of a mass spectral peak at a given m/z value. Also frequently used interchangeably with resolving power below. Along with mass calibration, the mass resolution is the most essential parameter to control in the mass analysis.
- *Resolving Power* (RP) A measurement of how effectively a mass analyzer can distinguish between two peaks at different, but similar m/z. Mathematically, the formula $M/\Delta M$ is used, where M is the m/z value for one of the peaks and ΔM is the spacing, in unified atomic mass units, between the peaks. Most commonly, ΔM is the mass resolution, either via the 10% valley or FWHM definitions (see below). (Note that the definition used will affect the resolving power calculated.) Resolving power of 500–1000 approximately corresponds to unit resolution (e.g., at m/z 700 and FWHM resolution of 0.7, RP = 1000).
- *FWHM* Full width at half-maximum. Mass resolution is often difficult to determine at or near the base of a peak due to baseline noise and peak overlap. It is more common to measure the width of the peak halfway to the peak maximum, where a clean measurement is possible. The most common alternative to FWHM was the 10% valley definition, in which the peak width at 10% of height was examined. This latter definition is common in the literature, especially for magnetic sector mass spectrometers, but is currently used much less frequently than FWHM. The choice of FWHM or 10% valley has an impact on the calculation of resolving power.
- *Unit Resolution* Setting the resolution to produce a peak 1 mass unit wide at the base. For a Gaussian-shaped peak, the FWHM width for unit resolution is about 0.7 u.
- *High Resolution* There is no specific definition for high resolution, but it is generally accepted that a resolving power over 5000 or 10,000 represents the beginning of high resolution. For small molecules, this typically corresponds to a mass resolution of approximately 0.1 (FWHM) or below. The acronym HRMS (high-resolution mass spectrometry) is often used to describe analysis at a high resolving power.
- *Parts Per Million* The term parts per million (ppm) is a relative measure commonly used in discussing mass accuracy. One ppm is determined as the measured m/z divided by 10^6. For reference, accuracy within 1 ppm at m/z 500 would establish a yield of 500 ± 0.0005 u.
- mDa or mmu One mDa is 0.001 u. The millidalton (mDa) and the equivalent milli mass unit (mmu) are also used in describing small mass differences.

1.3.3 Mass Spectrometer Components

A mass spectrometer consists of a sample inlet, an ion source, a mass analyzer, and a detector (Fig. 1.5). Each component is described below.

1.3.3.1 Sample Inlet and Source A key component of any mass spectrometer is the mechanism of introducing the sample into the instrument. The first

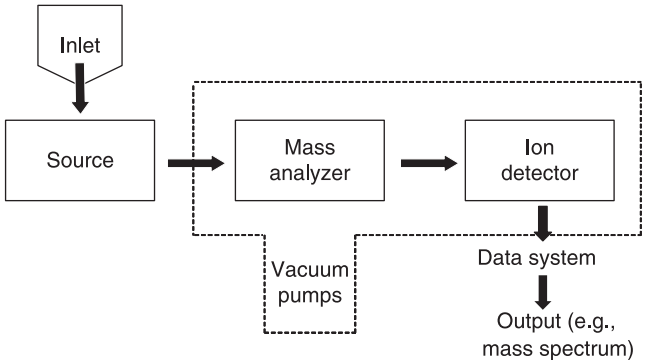


Figure 1.5. *Components of a mass spectrometer.*

component is the sample inlet. In many cases, this will be the liquid (or gas) chromatograph, which delivers the sample to the mass spectrometer source. Sources used with gas chromatography include electron impact ionization (EI) and chemical ionization (CI). Use of GC–MS has declined significantly due to improvements in LC–MS, and GC–MS sources are not described here. For MALDI systems, samples are typically "spotted" onto a surface (the target). The target is then physically placed in the source (Chapters 11 and 12). There are several common source types, as described below. For successful analysis, the sample introduced to the source must be converted from the liquid or solid phase to the gas phase and must be ionized before entering the mass analyzer.

- *API* The atmospheric pressure ionization (API) source is the most common category of source for LC–MS analysis, in which ionization is performed outside of the high-vacuum region of the mass spectrometer. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) sources are both examples of API sources.
- *ESI* A common LC–MS source in which the effluent from a liquid chromatograph is directed through a fine capillary to which a high electric field has been applied. Ions are formed in a solution via acid–base or redox chemistry and converted to the gas phase through some combination of ion evaporation or ion ejection mechanisms (Labowsky et al., 1984; Kebarle, 2000). ESI is considered a soft ionization technique, where little fragmentation of the analyte occurs. The technique is capable of creating multiple charges on a single molecule and is highly effective for analysis of large molecules such as peptides and proteins. ESI can also lead to a profusion of different ion types, such as $[M + H]^+$, $[M + Na]^+$, and $[M + NH_4]^+$, in the positive-ion mode and $[M - H]^-$ in the negative-ion mode.
- *APCI* Atmospheric pressure chemical ionization (APCI) is a source for LC–MS analysis in which the effluent from a liquid chromatograph is directed through a fine capillary and sprayed into a heated tube. The liquid is converted

to the gas phase through evaporation. Upon exiting the heated region, the gaseous effluent passes a high-voltage corona discharge needle, leading to the formation of reagent ions (typically derived from solvent species). Gas-phase proton transfer between reagent ions and analyte molecules ultimately leads to ionization of the analyte (Bruins, 1991; Duffin et al., 1992).

- *APPI* Atmospheric pressure photoionization (APPI) is an ionization source similar to APCI but the corona discharge needle is replaced with an irradiation source (e.g., krypton lamp). In comparison to ESI and APCI, APPI can be used to efficiently ionize broad classes of nonpolar compounds. In the bioanalytical tool box, APPI is an important complement to ESI and APCI (Hanold et al., 2004; Syage et al., 2004; Cai et al., 2005; Hsieh, 2005).
- *MALDI* A soft (gentle) method for creating gas-phase ions that utilizes energy from a laser targeted onto a mixture of analyte and a chemical matrix. Analyte ions can be formed from a combination of vaporization of existing ions and by vaporization of neutrals followed by ionization in the gas phase (Hillenkamp et al., 1990).
- *DESI* Desorption electrospray ionization (DESI) is a recently developed technique that permits formation of gas-phase ions at atmospheric pressure without requiring prior sample extraction or preparation. A solvent is electrosprayed at the surface of a condensed-phase target substance. Volatilized ions containing the electrosprayed droplets and the surface composition of the target are formed from the surface and subjected to mass analysis (Takats et al., 2005; Wiseman et al., 2005; Kauppila et al., 2006).
- *DART* Direct analysis in real time (DART) is an analogous technique to DESI that does not require the electrospray solvent (Cody et al., 2005; McEwen et al., 2005; Williams et al., 2006).
- *NSI* Nanospray ionization (NSI) is a low-flow (10–500-nL/min) ESI technique with many advantages over conventional-flow ESI ($\sim$200 μL/min) for the analysis of drugs, metabolites, peptides, proteins, and other macromolecules. Advantages of NSI over ESI include decreased sample consumption and increased sensitivity (Wilm and Mann, 1996; Corkery et al., 2005). NSI can be used for LC–MS or direct-infusion MS analysis of molecules (Wickremsinhe et al., 2006; Ramanathan et al., 2007c).

1.3.3.2 Mass Analyzers Analysis based on mass-to-charge ratio occurs within the mass analyzer of the instrument. The mass analyzer is often used as the basis for differentiating and discussing various types of mass spectrometers. Mass analyzers commonly considered to operate at a high resolving power are denoted by HRMS under the mass analyzer listing.

- *QMF* The quadrupole mass filter (QMF) or the transmission quadrupole is a mass analyzer that utilizes four parallel conducting rods arrayed such that a combination of two voltages permits the passage or filtering of only a single m/z value. Varying the amplitude of the fields permits a sequential range of

m/z ions to pass through the mass analyzer to create a mass spectrum. Low operating voltages (therefore tolerant of high operating pressures of $\sim 10^{-6}$ torr) and fast scanning capabilities make quadrupole analyzers ideal for coupling with LC systems (Dawson, 1986; Kero et al., 2005).

- *QIT* The quadrupole ion trap (QIT) utilizes a cylindrical ring and two end-cap electrodes to create a three-dimensional (3D) quadrupolar field for mass analysis. These instruments are capable of selectively trapping or ejecting ions and are often used for the sequential fragmentation and analysis experiments of product ion MS/MS. Also known as a 3D trap due to the configuration (March, 1997).
- *LIT* The linear ion trap (LIT) (also referred to as a two-dimensional, or 2D, trap) is a variation on the transmission quadrupole mass analyzer. In the LIT, the quadrupole is constructed such that either ions can be analyzed immediately or, ions can be trapped and held in the quadrupole region and then analyzed (Hager, 2002; Schwartz et al., 2002). Various types of MS/MS can be performed, as described in Chapter 3.
- *TOF* The time-of-flight (TOF) mass analyzer is conceptually the simplest of all. Ions are "gated" from the source region by an electrical field pulse and accelerated down the TOF flight tube. Low m/z ions travel at a higher velocity and reach the detector quicker than the slower ions with high m/z. Calibration of the accelerating field and resulting flight times permits mass analysis for unknowns. Hybrid instruments combining quadrupole and TOF mass analyzers (Q-TOF) have become common in recent years (Morris et al., 1997; Hopfgartner and Vilbois, 2000) (HRMS).
- *FTICR* The Fourier transform ion cyclotron resonance (FTICR) mass analyzer represents the highest performance in terms of resolving power. The FT (ICR) utilizes a strong magnetic field to store ions of various m/z in a cylindrical flight path (X and Y directions). An electric field is used to excite ions, which are detected when they pass near a detector plate. The frequency with which ions of a particular m/z pass the detector is recorded and fast Fourier transform is used to deconvolute the resulting data (Marshall et al., 1998). Hybrid FTICR often utilizes a quadrupole mass analyzer prior to the ICR cell (Patrie et al., 2004; P. O'Connor et al., 2006). Overall, these high-performance mass analyzers are the most expensive and massive of the common instruments and exist in relatively limited numbers compared to other instrument types (HRMS).
- *Orbitrap* The newest of the major mass analyzers, the Orbitrap is a hybrid MS consisting of a LIT mass analyzer, or transmission quadrupoles connected to the high-resolution Orbitrap mass analyzer. The Orbitrap utilizes electrical fields between sections of a roughly egg-shaped outer electrode and an inner (spindle) electrode (Chapter 5). Ions orbit between the inner and outer electrodes and their oscillation is recorded on detector plates (Hardman and Makarov, 2003; Hu et al., 2005). As with the FTICR, fast Fourier transform of the raw data is used to convert the data for mass analysis, making the Orbitrap the second major type of FTMS instrument. The resolving power of the Orbitrap is intermediate

between the TOF and FTICR, as is the price. Ease of ownership and use versus the hybrid FTICR instruments and the higher performance versus the Q-TOF instruments have both worked in favor of the Orbitrap (HRMS).

- *Tandem Mass Spectrometer* An instrument capable of performing multiple mass (m/z) analyses. There are two major categories: (1) tandem-in-space instruments (triple quadrupole and Q-TOF), (2) tandem-in-time instruments (QIT and FTICR).
- *Hybrid Mass Spectrometer* A tandem mass spectrometer comprised of multiple mass analyzers of different types. A Q-TOF is a hybrid, but a triple quadrupole is not. Ideally, a hybrid instrument harnesses the best features of each mass analyzer type to produce a system perhaps greater than the sum of the parts.
- *MS/MS* A process in which mass (m/z) selection or analysis is typically performed in two distinct serial steps. Operational examples include selected reaction monitoring or constant neutral loss scanning (see below).
- *MS^n* A series of n steps in which m/z selection is performed. MS^n can be conducted by linking a series of mass analyzers, each of which performs one selection step, or more commonly by using ion-trapping instruments such as QITs (2D or 3D) or FTICR.

Use of Mass Analyzer: Scan Types Depending on the configuration of the instrument, tandem and hybrid mass spectrometers are capable of far more than simply identifying the mass of a species that emerges from the source. The following is a brief list of relevant terminology and scan types that can be useful in generating additional information to support the identification of an unknown. Note that not all scan types are feasible on all types of instrument.

- *Full Scan* The mass analysis process by which a controlled series of m/z are allowed to be detected. The m/z range over which a mass analyzer can be used (e.g., m/z 20 to 4000) is one defining characteristic of the instrument.
- *Selected Ion Monitoring (SIM)* The mass analysis process in which only a single m/z value is selected by the mass analyzer and transmitted to the detector. Also referred to as the "single ion monitoring."
- *Precursor Ion (MS/MS)* Generally the ion selected by the first mass analysis of an MS/MS process. Also formerly referred to as the "parent ion."
- *Product Ion (MS/MS)* The species formed by fragmentation of the precursor ion. Also formerly referred to as the "daughter ion."
- *Product Ion Scan (MS/MS)* Determination of all possible product ions formed from a specific precursor ion. A key step in the characterization of an unknown species that can facilitate functional group and structure identification.
- *Precursor Ion Scan (MS/MS)* Determination of all possible precursor ions that form a specific product ion. Useful when a characteristic or significant product ion has been noted and the sources of that ion are sought. An

example would be the detection of structurally similar compounds (i.e., metabolites, degradants, etc.) by identifying all species that produce a common fragment. Also referred to as the "parent ion scan."

- *Constant Neutral Loss Scan* (*MS/MS*) Determination of precursor/product ion combinations that exhibit a specific, characteristic loss of a portion of a molecular ion. Particularly useful when the characteristic species (loss) is neutral and cannot be detected directly by the mass spectrometer. Analysis of glutathione conjugates via neutral loss of 129 is an example. For the purposes of this book, NLS is used to describe these types of MS/MS experiments.
- *Selected Reaction Monitoring* (*MS/MS*) Selected reaction monitoring (SRM) is the process by which the first mass analysis selects a specific m/z (the precursor ion) to be fragmented in the collision cell and the second mass analysis selects and detects a specific product ion. Most commonly used in the quantitative analysis of well-characterized, targeted species for which optimized precursor–product pairs can be established. In SRM-based LC–MS assays no qualitative information can be obtained. However, SRM can be used to trigger product ion, neutral loss, or precursor ion scans.

1.3.3.3 Detector The detector is the last major portion of the mass spectrometer, and it detects the presence, and preferably abundance, of ions after they have exited the mass analyzer. Examples include the electron multiplier, common on quadrupole instruments, and the microchannel plate (an array of electron multipliers), which have been common on TOF instruments. For most users, the actual detector is a relatively "invisible" portion of the instrument that needs little or no regular attention.

1.4 MASS SPECTROMETRY IN QUANTITATIVE ANALYSIS

Over the past 20 years, LC–MS-based quantitative bioanalysis has grown to replace every other quantitative analytical method, including LC–UV and GC–MS. As evidenced in Chapter 2 and a number of recent reviews, today, quantitative LC–MS/MS is the most important application area of MS (Hsieh and Korfmacher, 2006; Jemal and Xia, 2006). Routine quantification of drugs and metabolites is achieved using LC–MS run times of less than 5 min. Technological advances discussed in this book provide further evidence that LC–MS run times of less than 1 min are becoming standard practice in many laboratories. Quantitative LC–MS and LC–MS/MS assays (simplified as LC–MS except where differentiation is necessary) are required not only during the journey of a drug through discovery and development stages (ADME, toxicological and clinical studies), but also during the postapproval marketing period. Although quantitative LC–MS methods developed during the drug discovery stage may not be adequate to support the drug development stage studies, discovery stage assays may be improved and validated as necessary to satisfy the regulatory and the sensitivity requirements of development preclinical and clinical studies. The fundamental parameters for LC–MS method validation include

selectivity, sensitivity, linearity, precision, accuracy, matrix effects, recovery, stability, reproducibility, and dilution integrity (Jones, 2006; Shah, 2007). Components and criteria that define and/or impact a quantitative LC–MS assay are as follows:

- *Liquid Chromatography* The process by which the components of a liquid sample are physically separated based on their partitioning between a stationary phase and a moving (mobile) phase. Major modes include reverse phase, in which the stationary phase is non-polar, and normal phase, in which the stationary phase is polar. HILIC (Hydrophobic interaction chromatography) is a popular variant on the latter (Goodwin et al., 2007).

  For developing an LC method with high precision and accuracy, information about the sample/analyte such as number of possible analytes present, chemical structure of the analytes, molecular weight, concentration range, and solubility are crucial. LC separations are optimized by changing the following variables in the order listed: (1) mobile-phase composition/gradient, (2) column temperature, (3) solvent type, (4) additives, (5) pH, and (6) column type. For comprehensive description of HPLC systems, techniques, and method development, the readers are directed to specialized texts and review articles (Sadek, 2000; Tang et al., 2000; Tolley et al., 2001).
- *Mass Spectrometry* Mass spectrometer components, types of mass spectrometers, ionization sources, and scan types are described in Section 1.1.1.

  MS Dwell Time: Dwell time describes the time spent on a single step in a SRM or SIM analysis. Longer dwell time results in fewer data points but better signal-to-noise ratio, and should be optimized to produce acceptable data for each consideration. Common SRM dwell times in LC–MS would be 25–300 milliseconds (ms).

  MS Scan Time/MS Cycle Time: Scan time describes the time required to perform one complete MS data point for all targeted m/z. In SRM or SIM, this is the sum total time for each individual dwell, plus any additional time required by the system. In full scan or other scanning modes, scan time is the time required to complete one entire scan, e.g., from m/z 100 to m/z 1100 in one second.

  Run Time: The time for one complete injection and analysis, including any autosampler time required between injections. Run time is critical to determining the overall time required for analysis of a number of samples.
- *Sample Preparation/Extraction* The process of separating potentially interfering components from a sample prior to LC–MS analysis for the purposes of improving sensitivity, specificity, and/or method ruggedness. Variations include solid phase extraction (SPE), liquid–liquid extraction (LLE), and protein precipitation (PPT). Extraction may be performed off-line, in which the cleanup is completely independent from the LC–MS analysis, or on-line, in which the cleanup is integrated directly into the LC–MS analysis.
- *Method Validation* The procedure by which an LC–MS method (extraction, chromatographic separation, and MS detection) developed for quantitative

measurement of an analyte, in a given biological matrix, is demonstrated to be reliable and reproducible for the intended use. For analytes present in different biological matrices (plasma vs. urine), separate methods have to be validated. Cross-validation and/or partial validation experiments are required when changes (MS or LC instrument type, extraction methods, etc.) are made to a validated assay.

- *Standard Curve/Calibration Curve* The response from samples containing known, spiked quantities of analyte is mathematically regressed to create a calibration curve for each analyte. The response is usually peak area ratio (analyte area/internal standard area), but can be derived from area or height. The calculated curve is most commonly fit to the data using linear regression, but quadratic, power fit, and other models may be used. The variance observed across the assay range is often a function of concentration, and weighting such as $1/\text{concentration}^2$ (also known as $1/x^2$) is often used to improve the fit of the regression line to the data.
- *Internal Standard (IS)* The internal standard (IS) is a compound added in a fixed, known amount to every quantitation sample to serve as an internal control for the analysis. Most commonly, the IS is used to normalize response through determination of peak area ratio as described above. The ideal IS will track with the analyte(s) through the extraction, chromatography, and mass spectrometry to account for variable recovery, minor spills, and changes in response over time. Stable-isotope versions of the analytes are ideal IS for LC–MS quantitation, but in many cases structural analogs exhibit sufficiently similar chemistry to be useful in this role (Jemal et al., 2003; Wieling, 2002; Stokvis et al., 2005).
- *Quality Control (QC)* QC samples are used to check the performance of the bioanalytical method as well as to assess the precision and accuracy of the results of postdose samples. QC samples are prepared by spiking the analyte of interest and the IS into a blank/control matrix and processing similar to the postdose samples. QC samples cover the low ($3 \times$ LLOQ; LLOQ = lower limit of quantitation), medium, and high (70–85% of ULOQ; ULOQ = upper limit of quantitation) concentration ranges of the standard curve and are spaced across the standard curve and the postdose sample batch.
- *Matrix Effects* The suppression or enhancement of LC–MS response due to the presence of biological matrix components such as salts, proteins, metabolites, coadministered drugs, degradants, additives, impurities, and phospholipids (King et al., 2000; Avery, 2003; Weaver and Riley, 2006). Matrix effects may result in shifts in analyte retention times, poor chromatographic peak shapes, and inaccurate quantitative assessments. Although APCI is less susceptible to matrix effects in comparison to ESI, most of the pharmaceutical assays require the use of ESI due to thermal instability of the analyte (Matuszewski et al., 2003). Generally, matrix effects are examined by comparing the absolute LC–MS peak area for an analyte in neat solution with that of analyte spiked post-extraction into a blank matrix at the same concentration (Matuszewski et al., 1998,

2003). Alternatively, matrix effects can be evaluated using postcolumn infusion methods described in detail in this chapter and elsewhere (King et al., 2000; Weng and Halls, 2002; Mei, 2005).

- *Carryover* Analyte or IS response transferred from a previous analysis to a subsequent analysis. Carryover is classically considered to occur within the LC–MS system in the autosampler (syringe, injection canula, switching valve) or LC column, but can also occur in sample handling devices such as liquid handlers (pipets, robotic pipets) used during extraction. Carryover is assessed by injecting one or more control/blank matrix extracts and/or mobile-phase mixtures after a high-concentration QC, postdose sample, or standard. The typical benchmark for carryover of an analyte is a relative measure, with a target level of less than 20% of the LLOQ response measured following analysis of a ULOQ standard (Weng and Halls, 2002).
- *Crosstalk* An unwanted contribution to a LC–MS/MS transition from a previous LC–MS/MS transition. The potential for crosstalk is higher when multiple analytes with identical product ion mass-to-charge ratios are being monitored and when sufficient time is not provided for emptying the collision cell between MRM or SRM transitions. Crosstalk leads to over- or underestimation of an analyte of interest (Tong et al., 1999).
- *Acceptance Criteria* The acceptance criteria recommended by the current guidance calls for $\leq$15% for all the calibration curve standards and QCs with the exception of the LLOQ, where the acceptance criterion is increased to a 20% deviation. At least four of the six QCs must pass with $\leq$20% of the nominal value. In addition, at least one QC sample per concentration range must pass with this criterion. If additional QCs are used in a batch, at least 50% of the QCs need to be within each concentration range.
- *Lower Limit of Quantification (LLOQ)* The lowest concentration of the analyte of interest in a matrix that can be quantitatively determined using the standard curve with acceptable precision and accuracy. The LLOQ is usually defined as the lowest concentration at which the assay imprecision does not exceed 20%.
- *Upper Limit of Quantification (ULOQ)* The highest concentration of an analyte in a matrix that can be quantitatively determined using the standard curve with an acceptable precision and accuracy. If the analyte concentrations in the postdose samples are higher than the ULOQ, then a dilution QC is needed to cover the highest anticipated dilution.
- *Linear Range* The concentration range where increasing concentrations of an analyte have a proportional increase in LC–MS response. Overall QqQ-type mass spectrometers (triple quadrupoles, Q-TRAPS) are superior in terms of linearity. Most common causes for nonlinear response include MS detector saturation, dimmer/adduct formation, API droplet/vapor saturation at high concentrations, and space charge effects.
- *Analyte Stability* Analyte stability experiments are carried out mimicking the sample collection, storage, and processing conditions as closely as possible. Stability experiments are conducted for the assay duration in the same matrix

containing the same type of anticoagulant [Na–heparin, Li–heparin, Na_2–ethylenediaminetetraacetic acid (EDTA), etc]. Typical short-term stability evaluations include three freeze-and-thaw cycles, 4-hs at room temperature in matrix, and stability of final extracts (autosampler stability). Long-term stability experiments cover storage of unprocessed postdose samples at $-80^{\circ}C$, $-70^{\circ}C$, and/or $-20^{\circ}C$ for weeks and if necessary months or years.

- *Recovery/Extraction Efficiency* A ratio between the response of an analyte spiked into a blank matrix preextraction and the response of the same analyte spiked into a blank matrix postextraction. Although the recovery of an analyte need not be close to 100%, the extent of the recovery at all QC levels should be consistent, precise, and reproducible.
- *Dilution Integrity* To check dilution integrity, a QC sample prepared at a concentration greater than the ULOQ is analyzed using dilution in blank matrix. Acceptable assay precision and accuracy are required.
- *Inter- and Intra-Assay Precision* Intraassay precision and accuracy are assessed within one batch (QCs, standards, etc.), whereas interassay precision and accuracy are assessed using separate batches.

1.4.1 Applications in Pharmacokinetics

Quantitative analysis to track the concentration of one or more targeted species throughout the course of the drug discovery and development processes is broadly referred to as pharmacokinetic analysis. The data obtained permit critical determination of the movement and transformation of the initial drug, as described in Chapter 2. For a number of years, the quantitation required for pharmacokinetic studies was largely performed by LC with spectrophotometeric detection such as ultraviolet/visible (UV/Vis) absorbance, or occasionally fluorescence. While the latter technique offered good specificity, UV/Vis detection generally did not. This relative lack of specificity frequently necessitated careful sample-processing/extraction techniques and relatively long run times to minimize quantitation interferences.

Over the past two decades, QMF-based quantification assays have become the technique of choice for quantification of drug candidates and their metabolites. Combining a mass spectrometer with LC provides an additional degree of selectivity and makes the combined technique the method of choice for quantitative bioanalysis of drugs and metabolites. Among the mass spectrometer types, QMF are ideal for coupling with LC and atmospheric pressure ionization sources (ESI, APCI, APPI, DART, DESI, etc.) because QMFs have the lowest voltage requirements and vacuum requirements.

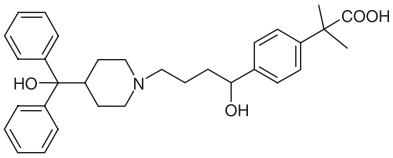
With the advent of the practical API-based LC–MS interfaces, the high specificity of mass spectral analysis permitted a radical decrease in the amount of analytical time invested (sample preparation, injection, chromatography) prior to final detection (Hsieh et al., 2006; Maurer, 2007). Although SRM detection as the final step in LC–MS analysis can incorporate several stages of specificity (Chapter 3), some form of sample preparation/extraction is still performed to remove unwanted

matrix components (proteins, phospholipids, salts, etc.). The extraction step can range from simple protein removal to highly specific solid-phase extraction (Kuhlenbeck et al., 2005; Chang et al., 2007a). Sample preparation is followed by chromatographic separation to resolve the analyte-like interferences from the peak of interest. In general, the combination of extraction and chromatography probably brings less specificity enhancement to LC–MS/MS analysis than it does to LC–UV analysis. But this is feasible because of the many levels of specificity in the final analytical step. To be detected in a SRM-based LC–MS/MS assay, an analyte must be eluting from the chromatography system at the correct retention time and be vaporized and ionized to the desired polarity under the conditions employed in the source. It must then have the correct m/z to transit Q1 successfully. In Q2 (sometimes the notation q2 is used because this set of quadrupoles cannot function as a mass analyzer and sometimes hexapoles and octapoles are used instead of quadrupole collision cells), the compound must fragment under the optimized conditions of gas pressure (argon, nitrogen, or helium) and energy employed, and only a fragment at the correct m/z can be transmitted through Q3 to reach the detector. The various drug discovery and development stages that require pharmacokinetic analysis are listed in Fig. 1.4.

1.4.2 LC–MS/MS in Pharmacokinetics: Example

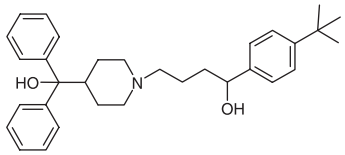
In a recent example, a sensitive LC–MS/MS method was successfully applied to assay for fexofenadine in plasma following a single oral administration of a microdose (100-μg solution) and a clinical dose (60-mg dose) to eight healthy volunteers (Yamane et al., 2007). Fexofenadine and terfenadine (IS) eluted at 0.95 and 2.07 min, respectively, and the correct m/z for the protonated precursor ions were observed at m/z 502.2 and 472.2. For SRM (or MRM) experiments, both precursor ions were fragmented separately in the collision cell and the fragment ions of m/z 466 and 436, respectively, were monitored for fexofenadine and terfenadine. The details of the fexofenadine assay are given in the following:

Analyte (drug)



Fexofenadine, $[M + H]^+$ at m/z 502.2

Internal standard



Terfenadine, $[M + H]^+$ at m/z 472.2

Mass spectrometer	Sciex API 5000, triple quadrupole
Ionization source/mode	Turbo IonSpray/positive
Scan type	SRM (MRM); transitions = 502.2 → 466.1 and 472.2 → 436.1
Sample preparation	SPE (Waters Oasis HLB)
LC system	Waters Acquity
Column	Waters XBridge C18 (2.1 x 100 mm, 3.5 μm)
Mobile phase	A: 2 m*M* ammonium acetate; B: Acetonitrile
LC flow rate	0.6 mL/min
Test system (species)	Human
Postdose blood sampling	0.5, 1, 2, 3, 4, 6, 8, and 12 h

Using the sensitive quantitative LC–MS/MS method described above, linear PK profiles between clinical dosing and microdosing were obtained. Furthermore, Yamane et al. (2007) demonstrated that concentrations in human plasma after an oral dose of 100 μg is quantifiable using LC–ESI–MS/MS (Fig. 1.6), similar to what can be achieved using AMS (Chapter 2).

1.4.3 Focus: Matrix Effects

In the above example, successful quantification of fexofenadine in the concentration ranges of 10–1000 pg/mL and 1–500 ng/mL required two standard curves because

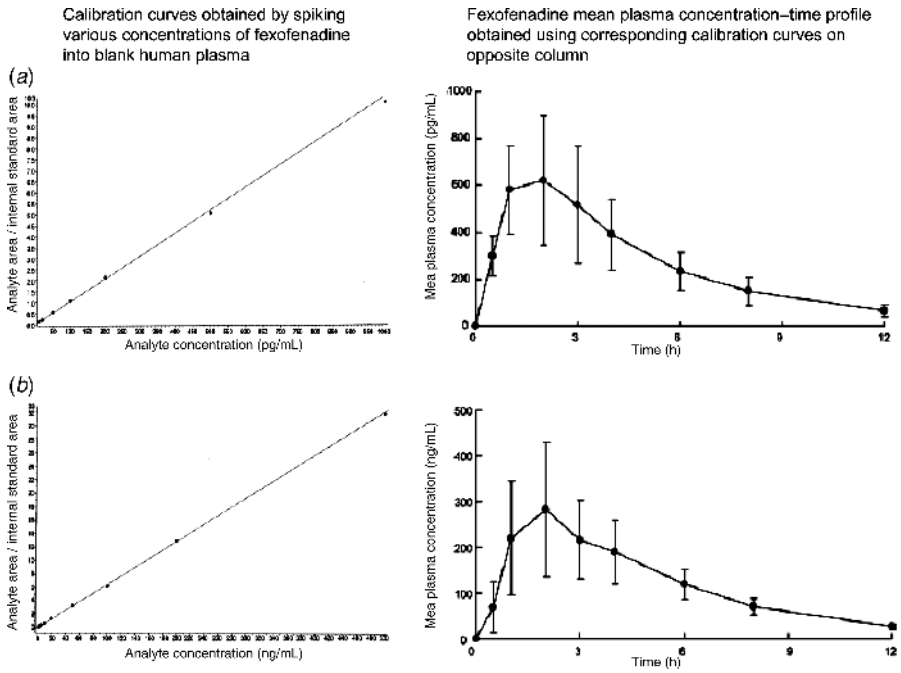


Figure 1.6. *Fexofenadine calibration curves and mean plasma concentration–time profiles following a single oral administration of (a) 100 μg (microdosing) or (b) 60 mg (clinical dosing) fexofenadine to healthy volunteers. (Reprinted with permission from Yamane et al., 2007.)*

often the linear dynamic range of an LC–MS or LC–MS/MS assay is limited due to calibration curve nonlinearity over wide concentration ranges. Calibration curve nonlinearity occurs due to detector saturation, adduct formation (dimers/multimer), and chromatographic carryover at higher concentrations as well as matrix effects (Matuszewski, 2006). Reduction (ion suppression) or enhancement of a MS signal caused by chromatographically coeluting matrix components was noted as a major issue in the 1990s (Matuszewski et al., 1998) and remains a significant issue in quantitation (Mei et al., 2003; Mei, 2005; Viswanathan et al., 2007). As far as the regulatory guidance is concerned, matrix effects are not required to be considered during a validation of a GLP assay. However, matrix effects can hamper assay reproducibility and/or linearity. Therefore, prior to validation and qualification of a quantitative LC–MS/MS method, matrix effects should be addressed. Figure 1.7 illustrates the steps necessary to evaluate a matrix effect (Bonfiglio et al., 1999; King et al., 2000).

As shown in Fig. 1.7, the method for evaluating ion suppression/enhancement encountered during a bioanalytical assay involves injection of a processed blank matrix sample on the column with continuous postcolumn infusion of a mixture of an analyte and an internal standard into the LC stream. The analyte and the internal standard are monitored (MRM or SRM scan) throughout the entire LC run time while the matrix components are eluting from the column. Data from a matrix effect experiment obtained using the postcolumn addition method are given in Fig. 1.8.

Extensive studies performed by several leading quantitative bioanalytical laboratories indicate that matrix effects can be limited by selecting the appropriate sample preparation techniques (Muller et al., 2002) and selecting the appropriate internal standard (Matuszewski, 2006).

Occasionally, interfering peaks are observed from metabolites, dosing vehicles, or the sample matrix itself. Suppression and interfering peaks can often be eliminated by changing the MS conditions, including the source type and resolving power

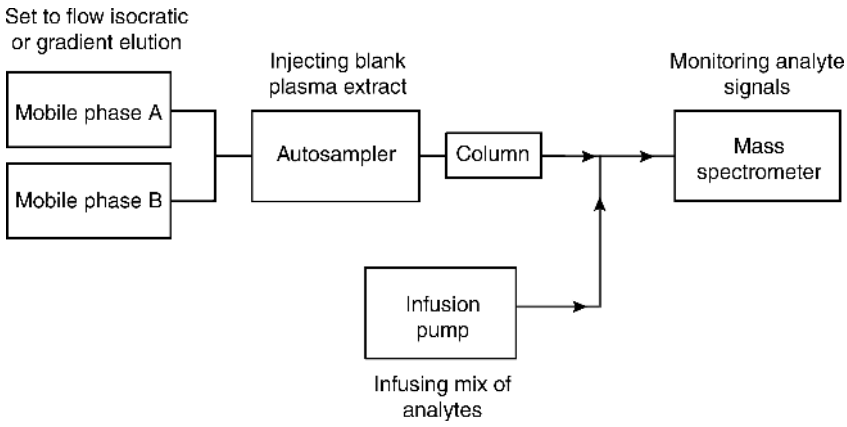


Figure 1.7. *Postcolumn infusion method to evaluate matrix effect originally described by Bonfiglio et al. (1999) and King et al. (2000). (Reprinted with permission from Bakhtiar and Majumdar, 2007.)*

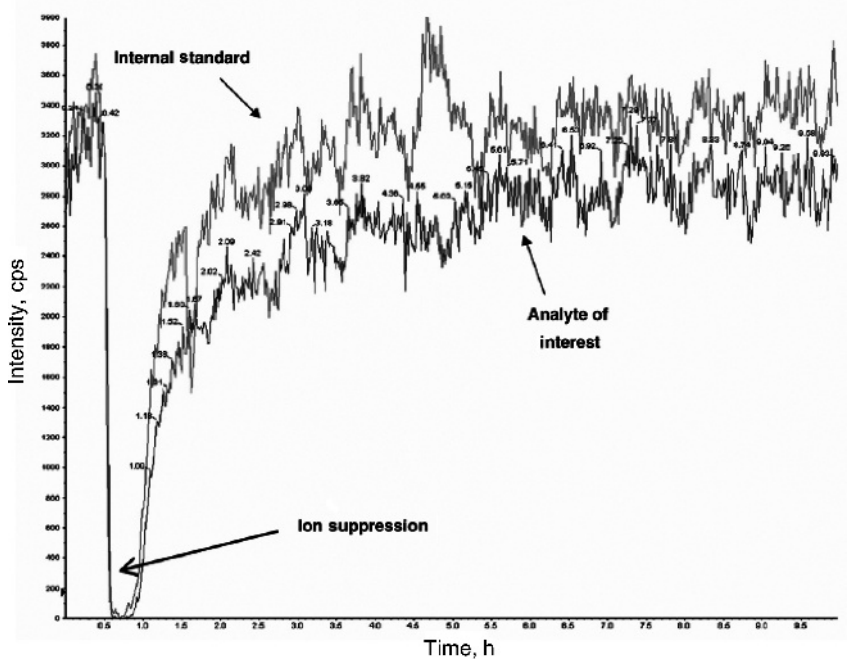


Figure 1.8. *MRM scans for analyte and internal standard obtained using ion suppression experiment described by Bonfiglio et al. (1999); and King et al. (2000). (Reprinted with permission from Bakhtiar and Majumdar, 2007.)*

(Xu, 2005), or by improving the sample preparation/extraction or chromatography. The latter approach, while powerful, is often seen as time-consuming in both the development of the required technique and the operation during sample analysis.

1.4.4 Applications in Toxicokinetics

The goal of toxicology experiments in drug discovery and development is to find out a drug's human health risks from the results in toxicological species. A recent survey (Lasser et al., 2002) reports that, among the drugs approved from 1975 through 2000, 45 drugs received one or more black-box warnings, and 16 were withdrawn from the market. Although a limited patient population and incomplete patient representation (ethnic, age, gender differences, etc.) are the main reasons for the failures of early clinical detection of drug toxicity, pharmaceutical scientists strive to conduct a well-designed preclinical toxicological study to avoid any human ethical issues. Toxicokinetic (TK) studies are required in the course of conducting toxicological experiments. In TK studies, PK parameters (Chapter 2) are applied to understand the relationship between a drug's exposure and its toxicity. Since a drug's exposure is a function of dose and time, historically, toxicology studies have been conducted using much higher doses than those which are pharmacologically relevant for a

drug's action. However, over the years, the science of toxicology has changed from very high doses and adverse events such as death and changes in organ size/weight to more relevant doses and more sensitive endpoints such as biochemical and functional changes in the immune system, endocrine system, and neurological system. As a result, analytical techniques used for TK studies were also shifted from less sensitive LC–UV and GC–MS assays to more sensitive LC–MS/MS assays.

Regulatory guidelines issued by the FDA and the ICH dictate the required drug toxicity studies in preclinical species for supporting the start of phase I, II, and III clinical studies. Drug TK assessments may be performed in the following preclinical toxicity studies: (1) safety pharmacology, (2) single dose and rising single dose (1–2 weeks), (3) repeat dose (1–4 weeks), (4) longer repeat dose (6–12 months), (5) reproduction, (6) genotoxicity, and (7) carcinogenicity. In all preclinical in vivo studies, blood draw (sample size) is limited to less than 10% of the circulating blood in rodents (rat/mouse) and nonrodents (dog/monkey). Regular blood draws, over several days, for clinical chemistry and hematology changes limit the blood draws for TK analysis. Therefore, TK studies involve a sparse sampling of blood with five or six sampling time points. Sample limitations in TK studies further require the application of rugged and sensitive LC–MS methodologies for quantitative monitoring of drugs in blood and plasma. All development stage TK study samples are analyzed using quantitative LC–MS or LC–MS/MS bioanalytical methods developed under GLP guidance. Most often, GLP bioanalytical assays developed for TK studies are further validated and used in clinical trials and the postmarketing period (Srinivas, 2007).

1.4.5 Special Techniques in LC–MS/MS Quantitation

1.4.5.1 Quantitative Bioanalysis with High Mass Resolution Prior to the introduction of the API sources for LC–MS, GC–MS was the dominant format for mass spectrometry. Within GC–MS, mass analysis at high resolution using magnetic sector instruments was relatively common, especially in the central mass spectrometry facilities of major corporations and universities. Uses of these instruments included quantitation by GC-HRMS for improved specificity and sensitivity.

Quantitation using high resolution mass spectrometry faded with the shift to MS/MS, and was particularly driven by the combination of the electrospray source and triple quadrupole mass spectrometer. Significant efforts continue however to reintroduce HRMS for quantitation using LC–MS on time of flight, quadrupole, or Orbitrap mass analyzers. As described above in Section 1.3.2.2, high resolution is a broad term, and the instrumentation cited here reflects that. The typical maximum resolving power at m/z 400 is about 4000 for the TSQ Quantum (high resolution triple quadrupole; HRMS on both mass analyzers), about 10–20,000 for a modern time of flight (HRMS on TOF only if Q-TOF hybrid), and 15–60,000 for the Orbitrap (HRMS on Orbitrap only if LTQ-Orbitrap hybrid).

A number of authors have discussed the utility of a high resolution triple quadrupole such as the TSQ Quantum (Fig. 1.9) (Jemal and Ouyang, 2003; Xu et al., 2003; Hughes et al., 2003; Paul et al., 2003). As shown in Fig. 1.10, nominally isobaric

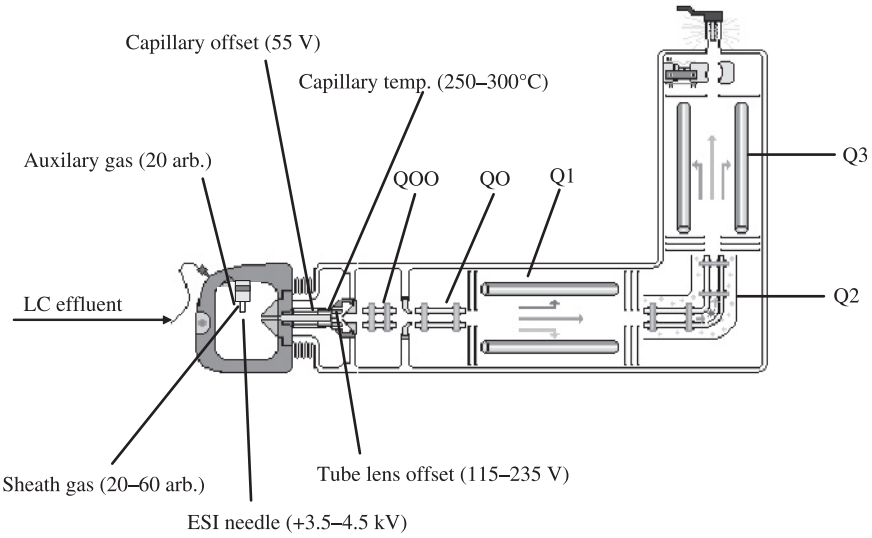


Figure 1.9. *Triple-stage quadrupole Quantum mass spectrometer capable of operating under enhanced resolution conditions. To reduce the chemical noise and to improve the sensitivity, the mass analyzers were oriented in an "L" shape rather than the conventional "straight" design. An additional benefit of the "L" shape orientation is a smaller foot print. (Courtesy of ThermoFisher Scientific.)*

PPG ions were resolved from both the 35Cl and 37Cl isotopic peaks of mometasone through the use of high resolution in Q1, Q3, or in both mass analyzers (Yang et al., 2002). Transmission losses on increased resolution, which are traditionally high for most quadrupole mass analyzers, were examined by Yang et al. (2002) for SRM quantitation. These results (Fig. 1.11 and Fig. 1.12) demonstrated that while absolute signal was reduced by approximately a factor of three, the true sensitivity as signal : noise ratio was maintained or possibly improved. In cases where interfering peaks or high background noise levels are problematic in SRM quantitation, a quick examination of the utility of enhanced resolution would potentially be far more time efficient than redevelopment of chromatography or extraction conditions.

Time of flight instruments, and perhaps especially hybrid Q-TOF systems (Fig. 1.13), have also been examined as quantitation systems (Zhang and Henion 2001; Yang et al., 2001b; O'Connor and Mortishire-Smith, 2006; D. O'Connor et al., 2006). While most testing has successfully demonstrated the concept, comparisons of sensitivity between conventional unit mass triple quadrupole and Q-TOF systems (Fig. 1.14) have shown that the quadrupoles generally produce greater sensitivity (Yang et al., 2001b). However, the rate of change and improvement in TOF systems has been extremely rapid in the last ten years, and such platform comparisons need to be revisited frequently to reflect the state of current commercial systems (Hashimoto et al., 2005; Weaver et al., 2007; De vlieger et al., 2007; Hopfgartner et al., 2007; Inohana et al., 2007).

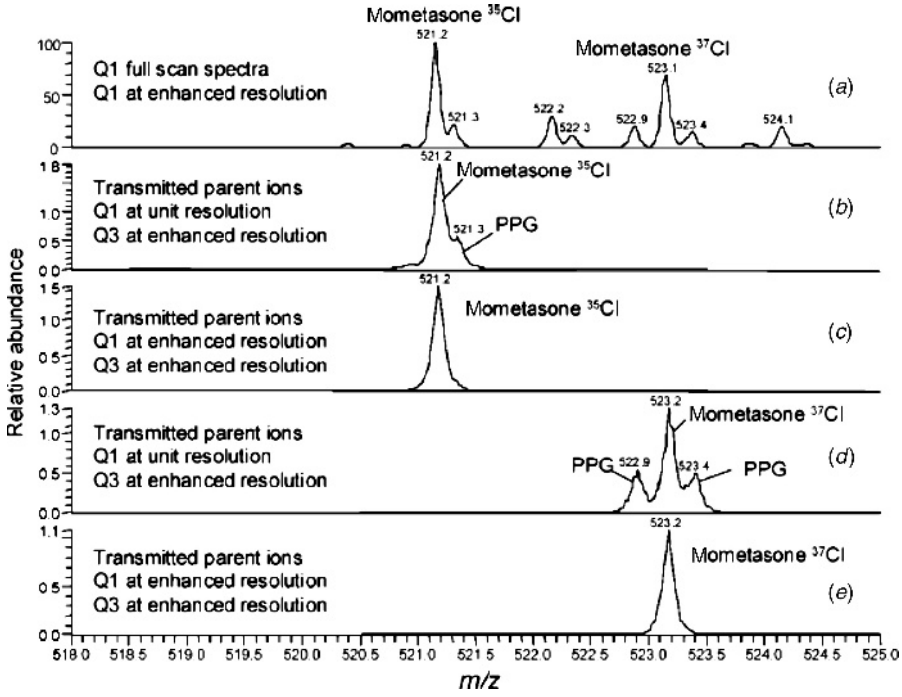


Figure 1.10. *LC–MS spectra of mometasone in the presence of PPG interferences obtained under unit- and enhanced-resolution settings showing the minimum loss in ion transmission under enhanced-resolution settings. (Reprinted with permission from Yang et al., 2002.)*

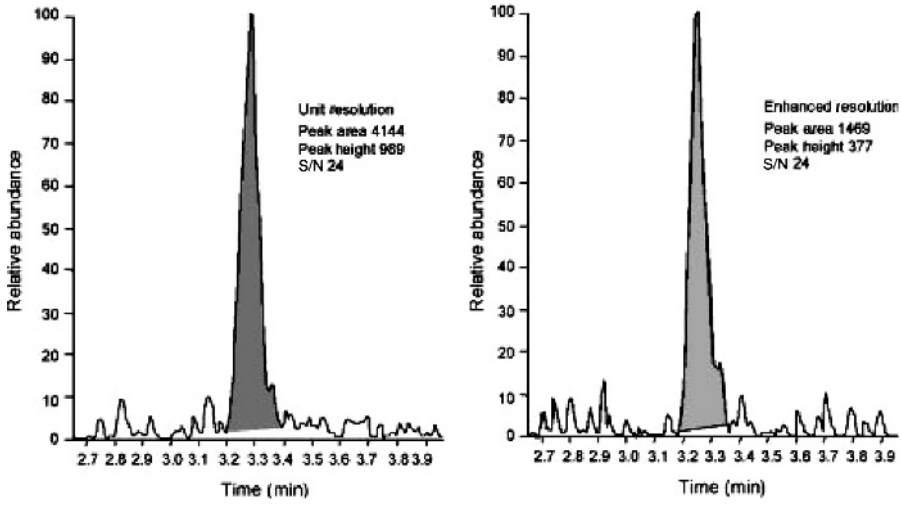


Figure 1.11. *LC–MS/MS chromatograms of desloratadine (SCH 34117) (fortified into plasma) obtained under unit- and enhanced-mass-resolution conditions. (Reprinted with permission from Yang et al., 2002.)*

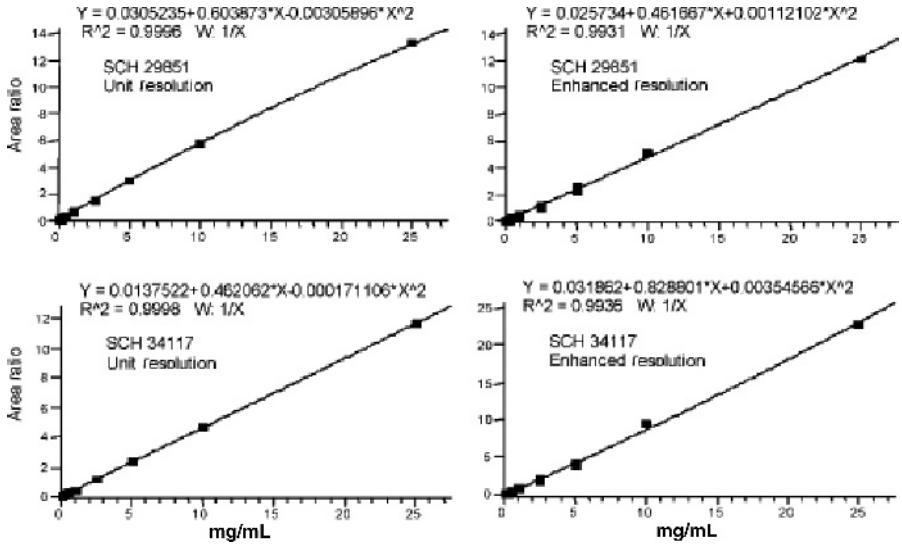


Figure 1.12. *Calibration curves for loratadine (SCH 29851) and desloratadine (SCH 34117) obtained under unit- and enhanced-resolution conditions. The precision and accuracy under both unit- and enhanced-resolution conditions met the assay acceptance criteria, correlation coefficients at enhanced resolution (0.993) were lower than those obtained at unit resolution (0.999). The lower correlation coefficients under enhanced-resolution conditions might have resulted from a slight mass window shift during the long overnight 17-h run. (Reprinted with permission from Yang et al., 2002.)*

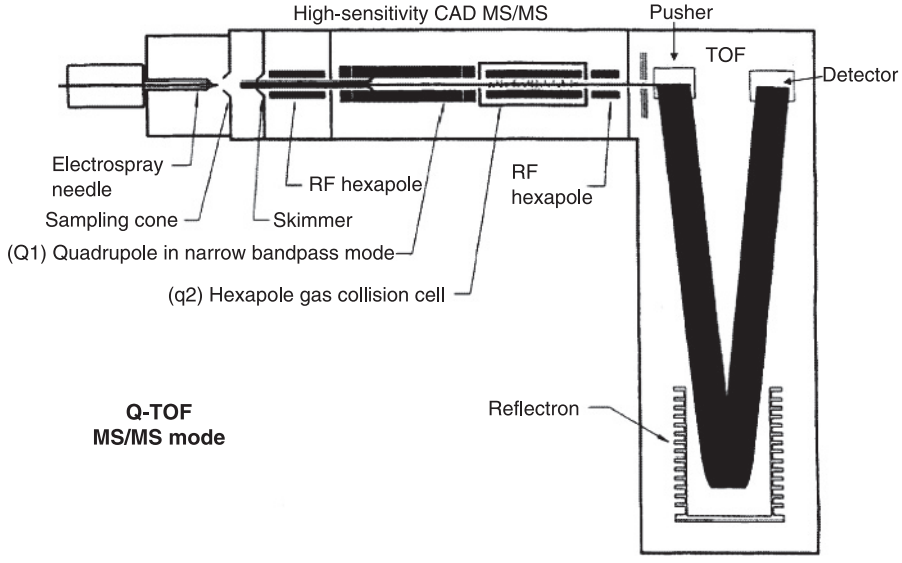


Figure 1.13. *Schematic of Q-TOF mass spectrometer. An updated Q-TOF schematic is presented in Chapter 4. (Reprinted with permission from Morris et al., 1996.)*

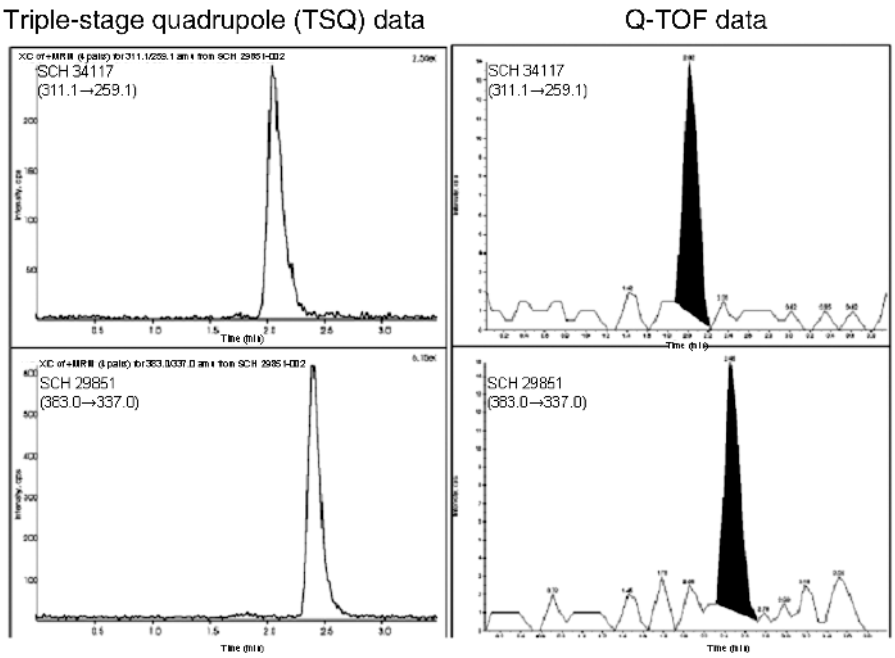


Figure 1.14. *MRM chromatograms of SCH 29851 (383.0→337.0) and SCH 34117 (311.1→259.1) obtained using Sciex API 3000 (triple-stage quadrupole) and Sciex QSTAR pulsar (Q-TOF). Comparison of MRM chromatograms of SCH 29851 and SCH 34117 obtained at the LOQ (1 ng/mL) using the API 3000 mass spectrometer with those from the Q-TOF mass spectrometer indicated that the S/N ratio is at least 10–20 times better on the API 3000 mass spectrometer. However, the MRM chromatograms from the API 3000 mass spectrometer do not provide the option to further examine the MS/MS spectra whereas the full-scan MS/MS spectra from a Q-TOF based quantitative bioanalysis assay allows one to easily eliminate any questions about false-positive data. (Reprinted with permission from Yang et al., 2001b.)*

The Orbitrap-based systems have emerged as the newest option for LC-HRMS. When configured as hybrid linear trap-Orbitrap (LTQ-Orbitrap), the systems are conceptually similar to Q-TOF in that mass analyzer 1 is nominally a unit mass analyzer, and mass analyzer 2 is capable of high resolution. These systems are capable of either LC-HRMS or LC–MS/HRMS operation. A new variant on the commercial Orbitrap, the Exactive, is expected to be released in late 2008. This system, which consists only of the single mass analyzer, has shown promising results in early assessment of quantitation by LC-HRMS (Bateman et al., 2008).

Finally, one concept that must be included in assessing quantitation by HRMS is the effective scan rate of the system. Quadrupole and time of flight mass analyzer are capable of rapid scan rates for SRM-type quantitation, with individual dwell times (quad) or scans (TOF) at 10–50 milliseconds possible. This permits acquisition of numerous data points across a chromatographic peak, which is critical for accurate and precise quantitation. Mass resolution is unaffected by changes in dwell time/scan

rate, though signal : noise usually decreases with faster scanning. Resolution on Fourier-transform based mass analyzers is linked to scan rate however. The early LTQ-Orbitrap instruments could achieve resolving power 60,000 with a scan rate of about 1 second/scan. Resolving power dropped to 15,000 when a scan rate of say 300 milliseconds was used. This trade-off must be considered when matching chromatographic performance with mass analysis, and is critical if ultra-high performance liquid chromatography (Section 1.4.5.2 below and Chapter 4) are considered (De vlieger et al., 2007; Hopfgartner et al., 2007; Inohana et al., 2007).

1.4.5.2 Quantitative Bioanalysis with Enhanced Chromatographic Resolution The majority of quantitative bioanalytical assays today involve the use of reverse-phase HPLC separation before MS detection. To improve upon conventional HPLC with respect to sample throughput/run time, chromatographic resolution, analyte sensitivity, and solvent usage, several laboratories are evaluating ultrahigh-pressure liquid chromatography (UHPLC) (Swartz, 2005a,b; Dong, 2007; Messina et al., 2007) or, as one vendor calls it, ultraperformance liquid chromatography (UPLC). For the purpose of this chapter, UHPLC and its variants are referred to as the UPLC. As detailed in Chapter 4, both HPLC and UPLC are governed by the van Deemter equation, which describes the relationship between plate height (N) and linear velocity (van Deemter et al., 1956; Wren, 2005; Wang et al., 2006). Based on this equation, as the particle size (d_p) decreases to less than 2 μm, there is significant gain in efficiency that does not diminish significantly at higher flow rates (Jerkovich et al., 2005; Swartz, 2005*a*,*b*; Wang et al., 2006). This creates an opportunity to optimize time efficiency while simultaneously improving chromatographic resolution and sensitivity (Jerkovich et al., 2005; Swartz, 2005*a*,*b*; Wang et al., 2006; Gritti et al., 2005; Martin and Guiochon, 2005; Plumb et al., 2004).

Most UPLC setups utilize columns of conventional LC–MS dimensions (length 30–50 mm, diameter about 2 mm), and operate at flow rates from 0.2–0.6 mL/min. As the particle size of the packing material decreases towards and below 2 μm, as is common in UPLC, the backpressure generated by resistance to flow increases rapidly to 10–15,000 psi (Plumb et al., 2004; Gritti et al., 2005; Martin and Guiochon, 2005; Swartz, 2005a; Swartz, 2005b). As most conventional HPLC systems are not designed for pressures greater than approximately 5000 psi, UPLC necessitated the development of new hardware prior to widespread commercialization.

The utility of UPLC has been demonstrated for both qualitative and quantitative analyses. In 2005, Castro-Perez et al. (2005) compared the performance of a HPLC with that of a UPLC and showed that improved chromatographic resolution and peak capacity attained with UPLC lead to reduction in ion suppression and increased MS sensitivity. Comparison of the mass spectrum obtained using HPLC with that from UPLC (Fig. 1.15) revealed that the higher resolving power of the UPLC–MS system resulted in a much cleaner mass spectrum than that obtained using the HPLC–MS system. The sensitivity improvement directly resulted in a higher ion count in the UPLC mass spectrum (855 vs. 176).

The additional sensitivity attainable with the UPLC approach is again demonstrated in Fig 1.16. While HPLC–MS resulted in signal-to-noise (S : N)

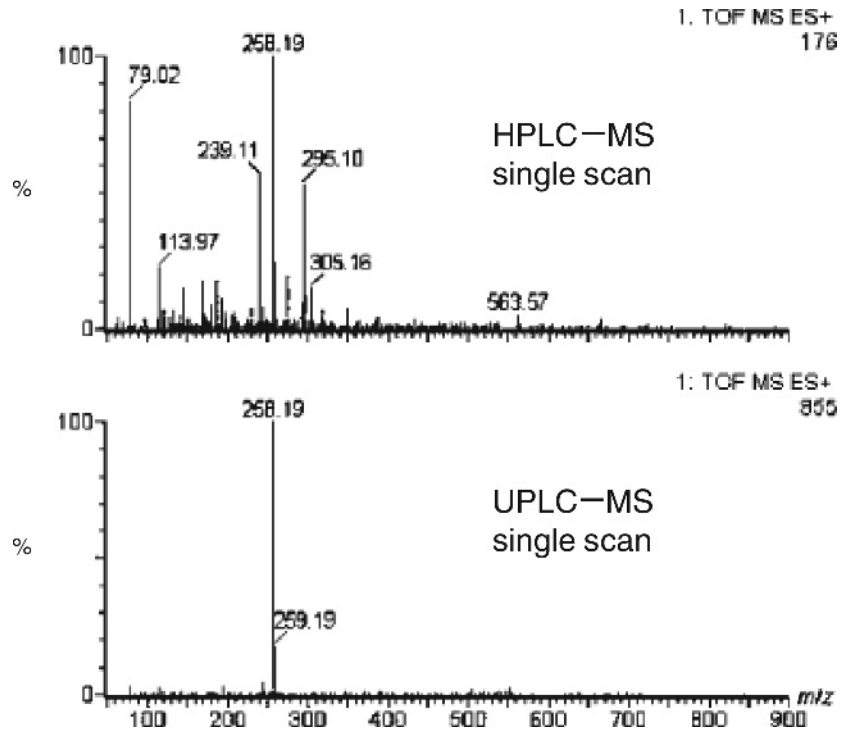


Figure 1.15. *LC–MS spectra of* N*-desmethyl metabolite of dextromethorphan (*m/z *258.19) following incubation of dextromethorphan with rat liver microsomes. (Reprinted with permission from Castro-Perez et al., 2005.)*

ratio of 25 : 1 for desmethyl-dextromethrophan-glucuronide metabolite (m/z 434), UPLC–MS provided a S : N ratio of 115 : 1 for the same peak. The increased S : N ratio achieved using the UPLC was attributed to improved peak resolution (sharper peak) and a reduction in ion suppression resulting from other co-eluting metabolites and endogenous compounds. The sensitivity gain was utilized by Pedraglio et al. (2007) in re-validation of a quantitative bioanalysis assay for NiK-12192, an antitumor candidate, using UPLC–MS. The UPLC–MS assay provided an LOQ of 0.1 ng/mL, whereas the previous HPLC–MS assay resulted in an LOQ of 0.5 ng/mL. Sensitivity improvements achieved with the UPLC–MS assay allowed the quantification of the 24 hour plasma samples that was previously not possible using the HPLC–MS assay (Fig. 1.17).

To highlight the advantages of UPLC for quantitative bioanalysis, Yu et al. (2006) enriched rat plasma with alprazolam, ibuprofen, diphenhydramine, naproxen, and prednisolone and compared HPLC–MS/MS and UPLC–MS/MS approaches for quantification of all five compounds. Apart from the particles that were used to pack the columns, all other separation and mass spectrometry methods were kept as similar as possible.

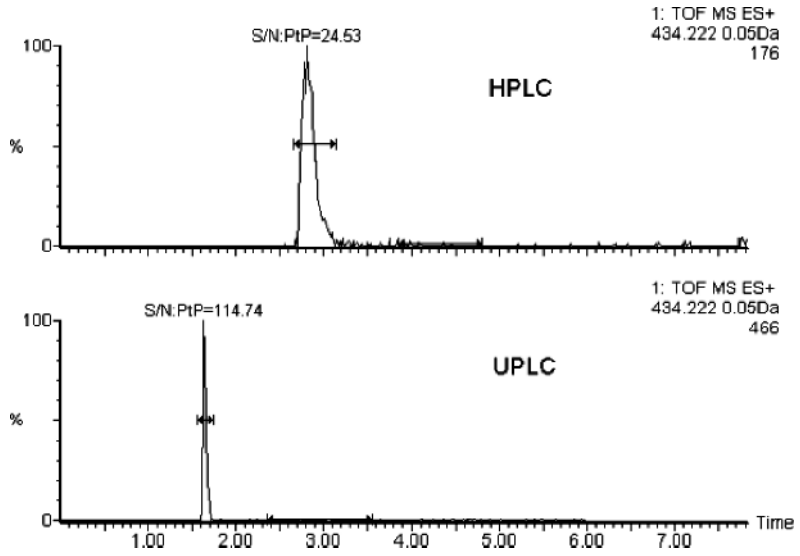


Figure 1.16. *Comparison of peak-to-peak (PtP) S/N ratio for the desmethyl-dextromethorphan-glucuronide (*m/z *434.222) obtained using HPLC–MS (top trace) and UPLC–MS (bottom trace). (Reprinted with permission from Castro-Perez et al., 2005.)*

The results demonstrated some of the characteristics of UPLC–MS. Shorter retention times were observed, along with reduced chromatographic peak width. This in turn leads to less analyte dilution and improved S : N. With the HPLC approach, the alprazolam peak is about 4.8 seconds wide (Fig. 1.18) and the same peak

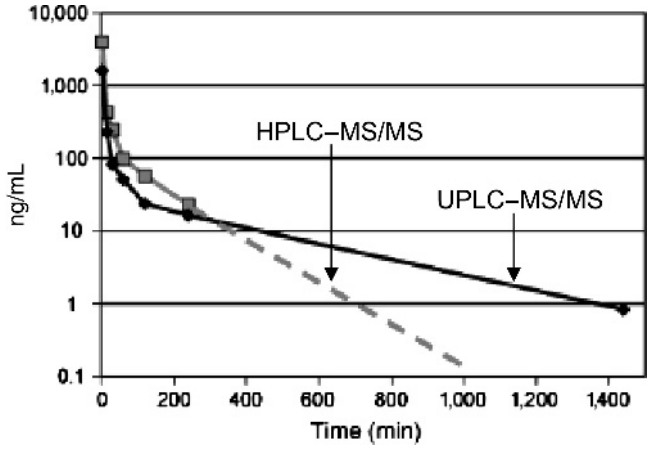


Figure 1.17. *Concentration–time plot following 3-mg/kg IV administration of NiK-12192 to mice. The dashed line is an extrapolation of the plasma sample concentration. The elimination phase determined using HPLC–MS is clearly different from that obtained using UPLC–MS. (Reprinted with permission from Pedraglio et al., 2007.)*

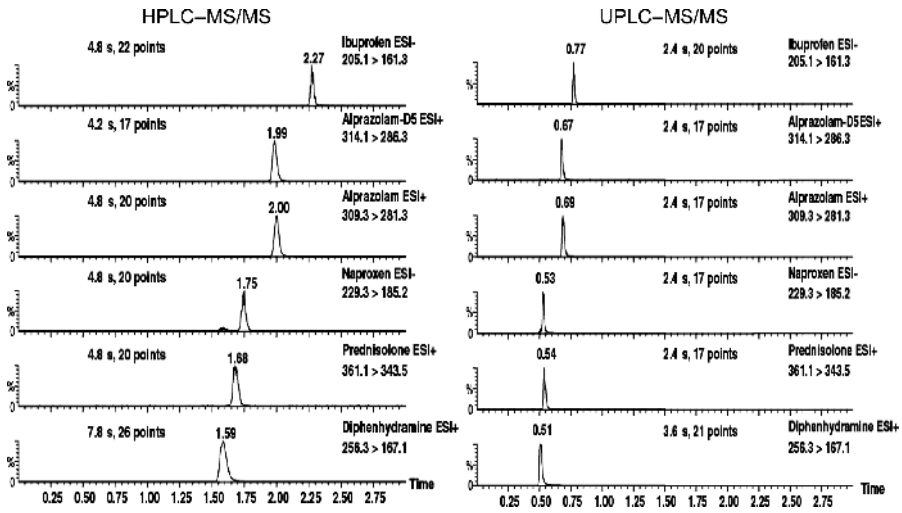


Figure 1.18. *Comparison of MRM chromatograms following HPLC–MS/MS and UPLC–MS/MS analysis of a mixture containing alprazolam, ibuprofen, d5-alprazolam, diphenhydramine, naproxen, and prednisolone. Each set of chromatograms was obtained from a single 100-ng/mL injection of rat plasma. d5-Alprazolam was used as the internal standard for quantification of alprazolam. (Reprinted with permission from Yu et al., 2006.)*

under the UPLC approach is about 2.4 seconds wide. In order to achieve the same number of data points ($\sim$20) across the peak, faster scanning using shorter dwell times was required for the UPLC method (Churchwell et al., 2005).

The overall benefits of going from HPLC to UPLC include increased separation efficiency, improved chromatographic resolution, and reduced analysis time (Shen et al., 2006; New et al., 2007). Although some reports suggest that the analysis time is reduced by ten-fold, a realistic, average estimate in reduction in analysis time is anywhere from three- to ten-fold. Overall, the higher chromatographic resolving power and increased separation efficiency of the UPLC result in improved MS sensitivity and a reduction in ion suppression. UPLC has proven to be one of the most promising developments in the area of LC–MS.

1.4.5.3 Quantitative Bioanalysis with Increased Selectivity: Application of FAIMS High-field assymetric waveform ion mobility spectrometry (FAIMS) is an atmospheric pressure ion separation technique introduced to the MS community in 1998 (Purves et al., 1998). A recent review by Guevremont (2004) introduces the reader to fundamentals of FAIMS and describes its application to small- and large-molecule separation and detection. Typically, FAIMS is used in conjunction with ESI to improve the analytical selectivity of the conventional ESI–MS quantifications assays. As shown in Fig. 1.19, FAIMS is placed in between an ESI source and a mass spectrometer skimmer/orifice entrance. During the operation of FAIMS, a high-voltage asymmetric waveform is applied to the inner and outer electrodes. A time-dependent voltage difference between the

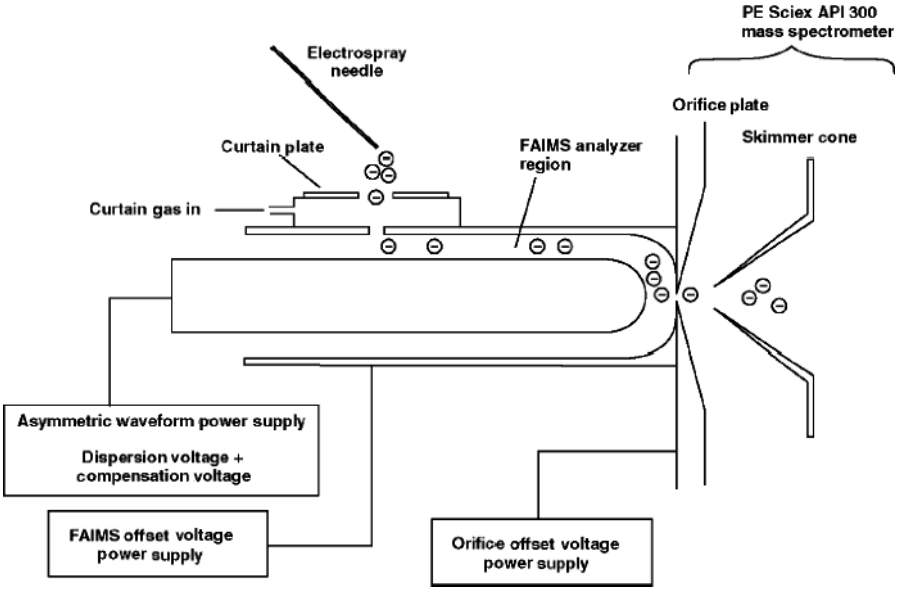


Figure 1.19. *Schematic of ESI–FAIMS–MS instrument. (Reprinted with permission from Guevremont, 2004.)*

electrodes causes the ions introduced via a carrier gas (helium, nitrogen, oxygen, carbon dioxide, or a mixture of gases) to oscillate and drift (ion mobility) toward one of the two electrodes. A compensation voltage (CV) with a correct magnitude and polarity is required to successfully transmit an ion through the electrodes. Since the CV is analyte (m/z) and temperature (Purves et al., 1998; Purves and Guevremont, 1999; Wu et al., 2007) dependent, it can be used to selectively transmit an ion of interest in the presence of other matrix or interfering ions. This unique CV-dependent selectivity feature of FAIMS allows the separation of isobaric drugs and/or metabolites as well as the separation of components that are difficult to separate under the fast LC conditions used for quantification of drugs.

Kapron et al. (2005) showed that FAIMS can be used to selectively quantify an amine compound in the presence of an interfering N-oxide metabolite (Fig. 1.20). Under the conventional LC–MS/MS settings, a SRM based precursor/fragment

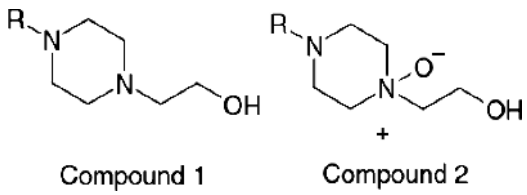


Figure 1.20. *Partial structures of the amine compound (Compound 1) and its N-oxide (Compound 2) analyzed using LC–FAIMS–MS/MS. (Reprinted with permission from Kapron et al., 2005.)*

ion pair (488/401) assay resulted in over estimation of the amine compound (compound A) (Fig. 1.21*b*) due to the N-oxide metabolite (m/z 506) undergoing fragmentation in the ESI source. In-source fragmentation of the N-oxide (compound B) resulted in the formation of m/z 488 ions through loss of an oxygen atom, which in turn contributed to Compound A transition of 488 → 401. The on-line FAIMS set-up allowed the metabolite interference to be removed before the entrance to the mass spectrometer.

Hatsis et al. (2007) showed that FAIMS can be used to increase quantitation throughput by eliminating chromatography altogether. To limit the impact from ion suppression, FAIMS was used in conjunction with a nano-flow ESI source rather than the conventional flow ESI source. As shown in Fig 1.22, the three minute LC–ESI–MS run time required to separate compound MLN A from the endogenous interference was reduced to 30 seconds by using FAIMS, although manual loading of the sample syringe added to the effective analysis time. Overall data quality was considered appropriate for the targeted discovery quantitation application.

A number of additional examples of quantitation using FAIMS have been published (McCooeye et al., 2002; McCooeye et al., 2003; Kolakowski et al., 2004; McCooeye and Mester, 2006). FAIMS also has been used to separate analytes of interest from endogenous matrices, metabolites, and other sample components (Ells et al., 2000; Venne et al., 2004; Venne et al., 2005). However, FAIMS is yet to be developed as a routine technique for the separation of complex biological samples.

1.4.5.4 Quantitative Bioanalysis with Ion Traps (3D versus 2D)

Although the pharmaceutical industry has long recognized the conventional three-dimensional (3D) ion trap as a powerful tool for structural elucidation of metabolites,

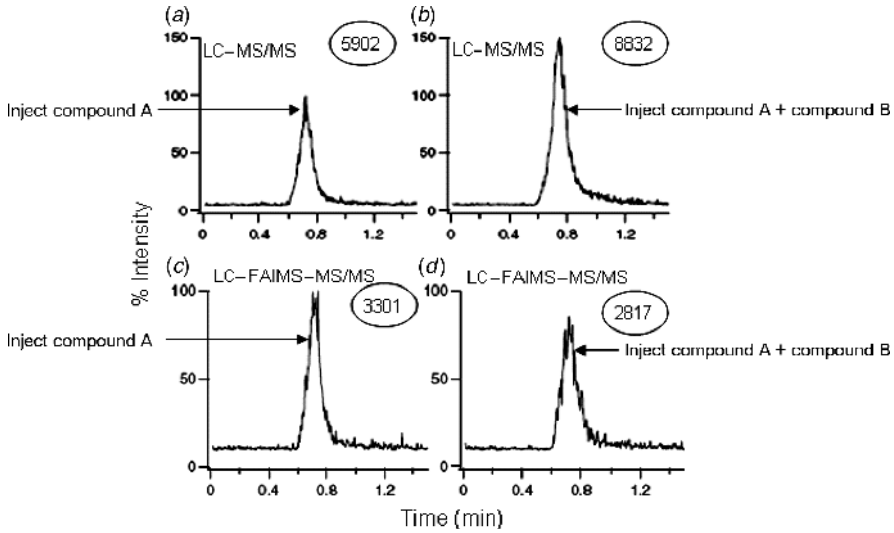


Figure 1.21. *Representative SRM (488→401) chromatograms obtained using LC–MS/MS (a,b) without and (c,d) with FAIMS. (Reprinted with permission from Kapron et al., 2005.)*

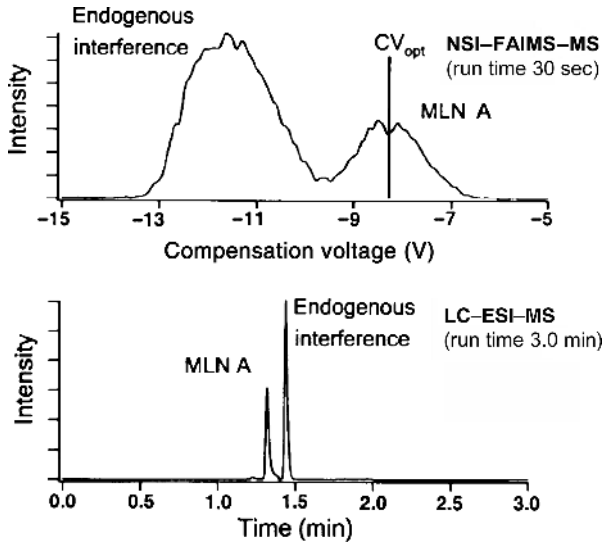


Figure 1.22. *FAIMS is used to increase the analytical throughput by eliminating the LC component altogether from quantification assays. The 3-min LC–ESI–MS run time required to separate compound MLN A from the endogenous interference was reduced to 30 s by using FAIMS. (Reprinted with permission from Hatsis et al., 2007.)*

unknowns, and degradants, due to inherent limitations, 3D ion traps (Fig. 1.23) were never accepted as the analytical technique of choice for quantification of drugs and metabolites in biological matrices. The limitations of the 3D trap as the desired quantification tool stems from its limited linear dynamic range and the extensive sample-processing requirements for achieving acceptable precision and accuracy.

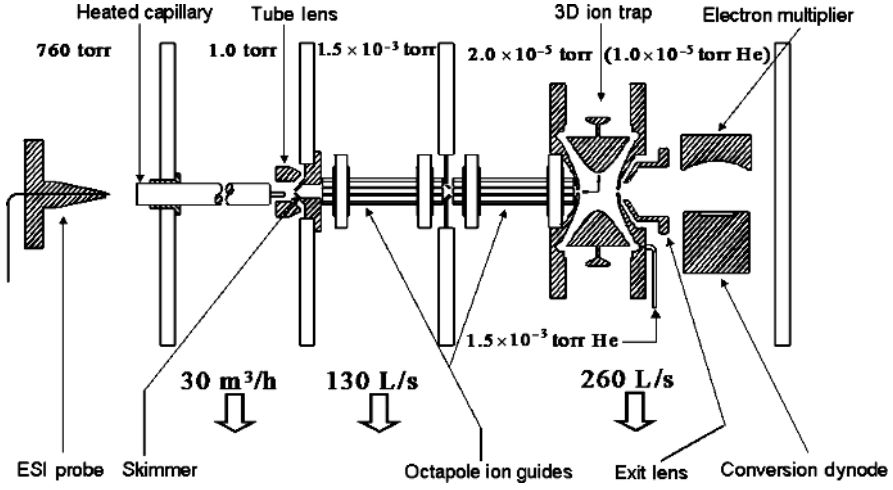


Figure 1.23. *Ion path and differentially pumped regions of LCQ mass spectrometer. (Courtesy of ThermoFisher.)*

Nevertheless, several groups demonstrated successful use of quadrupole ion trap mass spectrometers for quantification of drugs (Wieboldt et al., 1998; Chavez-Eng et al., 2000; Abdel-Hamid et al., 2001; Werner et al., 2001; Yang et al., 2001a; Naidong et al., 2002a; Werner et al. 2002; Yang et al., 2003; Salem et al., 2004; Sun et al., 2005; Vlase et al., 2007). More commonly however, QIT were the mass spectrometers of choice in the 1990s and early 2000s for performing quantification of drugs and simultaneous identification of their metabolites (Fig. 1.24) (Cai et al., 2000; Decaestecker et al., 2000; Cai et al., 2002; Kantharaj et al., 2003; Kantharaj et al., 2005a; Kantharaj et al., 2005b).

The two-dimensional (2-D) or linear ion trap (LIT) emerged in the 2000s as an effective alternative to the 3-D trap. Before 1995, linear traps were used primarily as ion storage/transfer/ion-molecule reaction devices in combination with FTICR (Senko et al., 1997; Belov et al., 2001), TOF (Collings et al., 2001), 3D ion trap (Cha et al., 2000), and triple-quadrupole (Dolnikowski et al., 1988) mass spectrometers because LITs offer better ion storage efficiencies in comparison to 3D quadrupole ion traps of the same dimensions (Hager, 2002; Schwartz et al., 2002). In 2002, commercial LITs were introduced as either stand-alone mass spectrometers (Schwartz et al., 2002) or as part of a triple quadrupole mass spectrometer (Hager, 2002).

The commercially available stand-alone LITs, marketed under the name LTQ, are made of four hyperbolic cross-sectional rods (Fig. 1.25). Since ions are trapped in an axial mode as opposed to central trapping on 3D ion traps, LTQs have been successfully coupled with Orbitrap and FTICR for achieving high-resolution capabilities (Peterman et al., 2005; Sanders et al., 2006) (Chapter 5). Functional improvements in 2D traps over 3D traps include 15 times increase in ion storage capacity, 3 times faster scanning, and over 50% improvement in detection efficiency and trapping efficiency.

The LIT introduced as part of a triple-quadrupole mass spectrometer is marketed under the name QTRAP. As shown in Fig. 1.26, the ion path and the differentially pumped region of QTRAP are similar to a triple quadrupole (API 3000, API 4000, and API 5000), except the Q3 is capable of functioning as a linear trap. QTRAP and its capabilities are described in detail in Chapter 3. Table 1.2 compares some of the advantages and limitations of QTRAP and LTQ mass spectrometers.

Most often simultaneous parent drug quantification and metabolite identification experiments are performed during early stages of drug discovery to conserve resources and expedite the lead selection process. To highlight the utility of the QTRAP mass spectrometer in this endeavor, King et al. (2003) demonstrated that information about circulating metabolites, dosing vehicle, interfering matrix components, and coeluting metabolites can be obtained by collecting full-scan data during quantification of drugs and metabolites in biological matrices. The QTRAP software controls were set up to perform a combination of SRM transitions and full scans (QTRAP scan functions are explained in detail in Chapter 3). While SRM transitions for urapidil (analyte) and labetalol (IS) were performed in the triple-quadrupole mode, full-scan spectra were acquired using the LIT mode. The LIT was operated in the enhanced MS (EMS) mode with a scan speed of 4000 daltons/sec. The cycle time for the combined scan function was 0.31 s. To

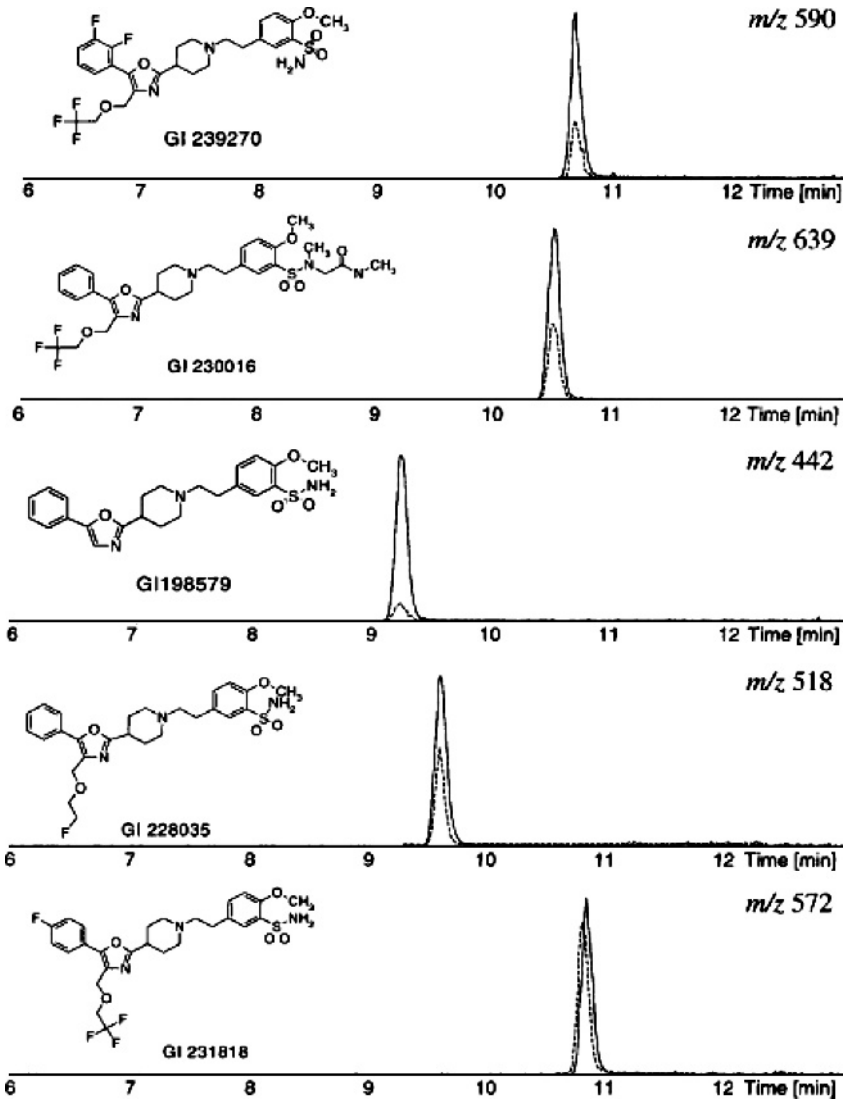


Figure 1.24. *Comparison of parent ion extracted ion chromatograms at* t = *0 min (solid line) with those at* t = *60 min (dotted line) following incubation of a five-compound cassette with dog microsomes. The high sensitivity of the QIT (3D) in the full-scan mode prompted Cai et al. (2000, 2001) to push the limits of QIT by simultaneously quantifying multiple drugs from cassette incubations and quantitatively studying metabolits from each drug. In an effort to increase the analytical throughput of the metabolic stability assays, compounds were individually incubated with dog microsomes for 60 min, quenched using acetonitrile, and the supernatants from four to five compounds were pooled to form cassette groups before LC–MS analysis. The percent metabolized for each compound in a cassette was calculated by measuring the extracted ion chromatogram (XIC) peak intensities for the parent analyte from each 0- and 60-min incubation. (Reprinted with permission from Cai et al., 2000.)*

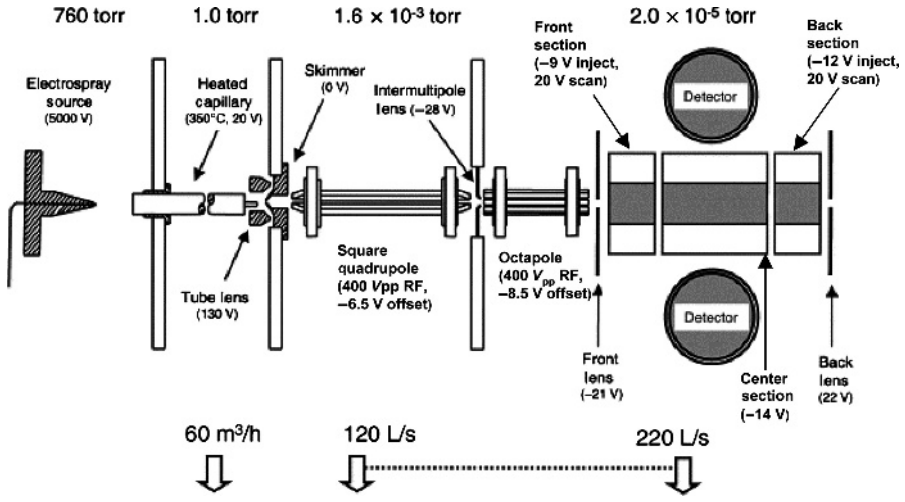


Figure 1.25. *Ion path and differentially pumped regions of LTQ mass spectrometer. (Reprinted with permission from Schwartz et al., 2002.)*

validate the quantification data from the alternating full-scan and SRM analysis (SRM/EMS), the data from the SRM-only analysis were compared with those acquired using the alternating method. The quantification data from both methods were generally acceptable in terms of sensitivity, accuracy, and precision. However, some loss of precision was observed at the lowest concentrations (Table 1.3).

The SRM/EMS mode of operation provided information on the coeluting glucuronide conjugates and the PEG dosing vehicle. Information about the coeluting

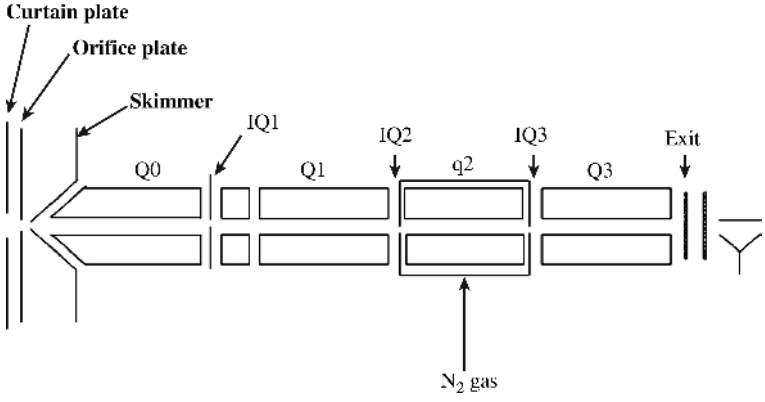


Figure 1.26. *Ion path and differentially pumped regions of QTRAP mass spectrometer. (Reprinted with permission from Hager, 2002.)*

TABLE 1.2. Comparison of LTQ and QTRAP Functionalities

LTQ	QTRAP
Coupled to Orbitrap, FTICR, etc.	Not practical to couple with other MS
Quantification possible with some limitations	Quantification similar to a triple quadrupole
MS^n ($n = 1, \ldots, 9$)	MS2 and MS3
Helium is used as the collision gas	Nitrogen is used as the collision gas
Inherent low-mass cutoff limits structural information form MS/MS experiments (pulsed-Q capability, however, allows one to get around this limitation)	Information rich tandem mass spectra similar to that from triple quadrupole are available
Two detectors lead to improved sensitivity	One detector
Radial ejection of ions to detectors	Axial ejection of ions to the detector
Neutral loss scan (NLS) and precursor ion scan (PIS) data obtained postacquisition by filtering MS/MS data	True NLS and PIS data can be acquired on the fly

material allowed the modification of the quantification method to improve the quality of the assay. Additionally, application of the SRM/EMS method to the in vivo PK samples provided information about circulating metabolites from early PK studies. Such information from early PK studies provided insight into the metabolic hot spots and allowed the medicinal chemist to modify the structure to optimize the PK of lead compound.

Since King et al. (2003) used SRM-triggered EMS for acquiring quantitative and qualitative data, characterization of the metabolites involved separate MS/MS acquisitions. To avoid analyzing the samples for the second time and to improve upon King et al. (2003), Li et al. (2005b) demonstrated the possibility of acquiring both parent drug quantification data and qualitative metabolite MS/MS data using the SRM-triggered information-dependent acquisition (IDA). Li et al. (2005b) tested the SRM-triggered IDA MS/MS experiments in both the conventional triple-quadrupole mode and the ion trap mode and showed that the cycle time decreased from 2.78 to 1.14 s with the latter technique. The longer cycle time in the triple-quadrupole mode of operation would have resulted in possibly missing some of the metabolites.

As shown in Fig. 1.27, a concentration–time profile for the parent molecule of compound A determined by the SRM-only method correlated very well with the SRM-triggered IDA method. Furthermore, the SRM-triggered IDA approach not only allowed Li et al. (2005b) to quantify the drug molecules, but also metabolites were quantified relative to the parent drug. As shown in Fig. 1.27, the authors were able to generate peak area–time profiles for two dioxy (M1A, M3A) metabolites and compare the PK parameters (e.g., half-life) of the metabolites with those of the parent compound. Understanding the PK parameters of the metabolites allowed the group to redesign the molecules with desired PK and metabolism profiles.

TABLE 1.3. Comparison of Accuracy and Precision from SRM-Only Mode with Those from Alternating SRM/EMS Mode (Reprinted with permission from King et al., 2003)

SRM[a]					SRM/EMS[b]				
Expected Value	Avg.	Std. Dev.	%CV	%Accuracy	Expected Value	Avg.	Std. Dev.	%CV	%Accuracy
5.0	5.1	0.2	4.4	101.5	5.0	5.1	0.8	15.2	102.7
10.0	9.9	0.5	5.0	98.7	10.0	9.5	1.4	14.3	94.7
20.0	19.6	1.6	8.1	98.0	20.0	20.5	1.6	8.0	102.5
50.0	49.7	3.1	6.3	99.3	50.0	47.7	4.7	9.9	95.4
100.0	95.2	4.8	5.0	95.2	100.0	97.4	5.9	6.1	97.4
200.0	197.3	8.2	4.2	98.6	200.0	188.2	15.0	8.0	94.1
500.0	517.3	41.6	8.0	103.5	500.0	509.7	52.3	10.3	101.9
1000.0	1020.3	61.2	6.0	102.0	1000.0	1081.6	88.0	8.1	108.2
2500.0	2581.2	222.5	8.6	103.2	2500.0	2576.9	98.7	3.8	103.1

Note: $n = 5$ replicates. Urapidil standard curve was prepared in rat plasma and processed before analysis by QTRAP mass spectrometer.
[a] $R^2 = 0.99592$.
[b] $R^2 = 0.99042$.

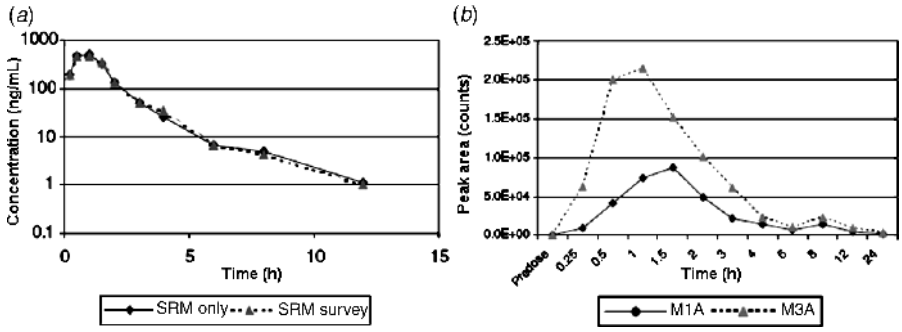


Figure 1.27. *(a) Comparison of concentration–time profile of compound A acquired using SRM-only and SRM-triggered IDA methods and (b) relative peak area–time profiles of dioxy metabolites (M1A, M3A) of compound A. (Reprinted with permission from Li et al., 2005b.)*

1.5 ADVANCES IN SAMPLE PREPARATION/CLEANUP AND COLUMN TECHNOLOGY

As summarized in Table 1.4 by Chang et al. (2007a), advances in sample preparation/clean-up and column technologies have been synonymous with improvements in analyte detection technologies over the past several decades. Improperly prepared samples for LC–MS analysis can very easily clog the LC system, associated tubing, or an API source as well as diminish the sensitivity of a mass spectrometer. On the other hand, sample preparation can not take hours and days in a high throughput bioanalytical laboratory where thousands of samples are analyzed per week. Therefore, to develop fast, sensitive and robust LC–MS and/or LC–MS/MS assays, one needs to select an appropriate and efficient sample preparation/clean-up technique and a suitable LC column to achieve adequate chromatographic separation.

1.5.1 Sample Preparation/Cleanup

Regardless of whether an assay is being developed to support a regulated (GLP) or discovery (non-GLP) study, common goals of sample preparation/cleanup include the following: (1) obtain a representative sample in solution, (2) remove coeluting/interfering matrix components with minimum analyte/drug-derived material losses, (3) achieve/maintain sufficient concentration for MS detection, (4) limit the number of steps, and (5) maintain ruggedness and reproducibility.

Some of the sample preparation/clean-up strategies used in high throughput LC–MS and LC–MS/MS quantitative bioanalytical analyses have been described in detail (Weng and Halls, 2001; Weng and Halls, 2002; Souverain et al., 2004a; Souverain et al., 2004b; Chang et al., 2007a), and a general approach is described in Fig. 1.28. While the 96-well format is now common, use of the 384-well format may permit further efficiency gains, provided that the most time-consuming steps of selecting, thawing, and aliquoting samples can be streamlined sufficiently (Chang et al., 2007a; Chang et al., 2007b).

TABLE 1.4. Advances in Sample Preparation Techniques Compared with Advances in Detection Technologies

Era	PK Requirement	Detection Technologies	New Goals of Sample Preparation	Major Sample Preparation Technology
1950–1975	Detection of metabolites, estimate exposure	Colorimetry, radioimmunoassay (RIA), GC	Bring the analyte concentration to assay range; remove interference; make analyte volatile	Dilution, LLE, PPE, TLC, GC (normal phase and ion exchange); derivatization
1975–1985	Determination of exposure	RIA, enzyme-linked immunoassay (ELISA), HPLC with visible UV detection	Bring analyte concentration to assay range; protein removal; remove interferences	Dilution, use of internal standard. LLE with back extraction; silica-based reverse chromatography with intention for fractional; commercial SPE cartridge
1985–1995	GLP bioanalytical	RIA, ELISA, HPLC, GC, GC–MS, capillary zone electrophoresis	Reliability of quantitative data; validated assay with proven sample history and stability	Automation, online elution of SPE, online SPE, use of analogue internal standard
1995–2000	Guidance for industry	ELISA, HPLC, GC, GC–MS, and HPLC–MS	Validated assay with proven specificity; cost reduction to compete with contract research organization	Commercial automation, high-throughput (high-density) assay based on 96-well SBS format; pre- and postextraction techniques in SBS format
2000 to	current	Biomarker and large-molecule determination	HPLC–MS/MS, HPLC–MS, Biacore, Mesoscare	Reduce matrix effect and improve incurred sample repeatability; reduce manual labor to compete with off-shoring Integrated process in SBS format; time sharing of MS by multiplex of HPLCs using multiple sprayers or stream selection valves; online SPE

Source: From Chang et al., 2007a.

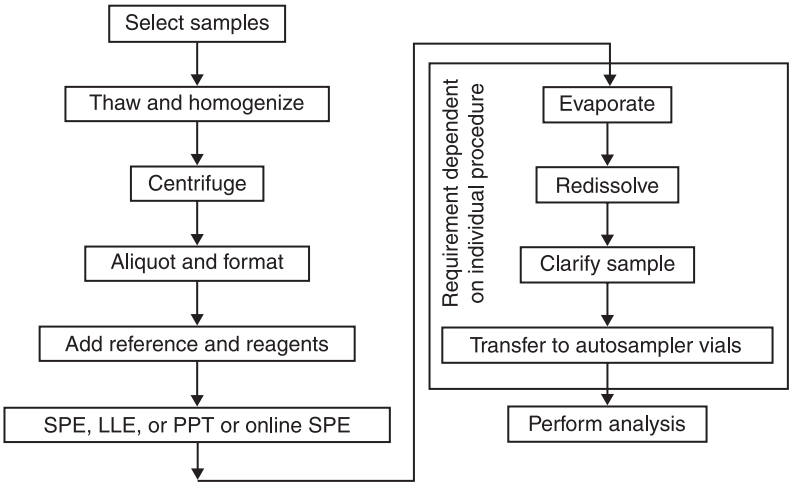


Figure 1.28. *Generic quantitative bioanalytical sample preparation scheme. (SPE = solid phase extraction; LLE = liquid-liquid extraction; PPT = protein precipitation). (Reprinted with permission from Chang et al., 2007a.)*

An alternative approach links the traditionally off-line sample preparation directly to the LC–MS system to utilize on-line SPE-type extraction. These systems have the advantages of unattended operation, and can use the LC-MS analysis time of sample one to prepare sample two, thereby saving time. Examples of such systems include the Symbiosis™ system from Spark Holland and the systems utilizing turbulent flow from Cohesive Systems/ThermoFisher (Ayrton et al., 1997; Alnouti et al., 2005; Kuhlenbeck et al., 2005; Koal et al., 2006; Smalley et al., 2007; Xu et al., 2007). The latter technique typically utilizes direct injection of the plasma sample, extraction of the analyte(s) onto a trapping column, and elution to an analytical column (Ayrton et al., 1997). The trapping column is engineered with relatively large diameter particles, and is operated at high flow rate. The theory and practice of this type of system are considered in greater detail in Chapter 10 of this book, both for quantitation and for metabolite identification.

Using a Symbiosis system, Alnouti et al. (2005) and Li et al. (2005) developed online SPE–LC–MS/MS methods for analysis of rat plasma without any prior sample processing. Direct plasma injection resulted in accuracy of 88–111% and 41–108% with and without on-line SPE, respectively. The precision was improved from 3–81% without SPE to 0.5–14% with SPE. Furthermore, Alnouti et al. (2005) demonstrated that the cost of quantitative bioanalysis can be reduced by reusing the on-line SPE cartridges up to 20 times without loss of accuracy, precision or analyte recovery.

Today, in the pharmaceutical industry, there exists a variety of technologies to which either partial or full automation of quantitative bioanalytical steps can make the process higher throughout. However, choosing the appropriate technology to automate requires an evaluation of several parameters, including number of samples, type of samples, and time required to automate in comparison to nonautomated work flow.

1.5.2 Improvements in Column Technology

Similar to MS, prior to the 1970s, only selected LC methods were available to the pharmaceutical scientists and most laboratories around the country packed their own columns, and HPLC was considered somewhat of a specialist tool. It was not until the 1980s that HPLC became a most practical analytical tool across the industry and the pharmaceutical scientist's tool of choice for separation, identification, purification, and quantification of drugs and metabolites. The past two decades have seen a vast undertaking in the development of column technology, especially in support of high-throughput bioanalysis. Today, more than 2 million analytical columns are sold per year (Unger, 2008).

To achieve the optimum reversed-phase LC separation, one needs to explore variables such as the analyte chemistry, mobile-phase composition (solvent type, solvent composition, pH, and additives), column composition, column particle size, and column temperature. For pharmaceutical analysis using mass spectrometry, the chemistry of an analyte is rarely changed beyond manipulation of the mobile phase pH, and even there options are limited. Volatile pH modifiers (buffers) are still preferred for LC–MS, and concentrations of these modifiers are kept low. Relatively simply mobile phases consisting of water, acetonitrile, and either formic acid (0.1% v/v), ammonium acetate (1–20 mM), or both have been common.

The column chemistry can be altered through changes to either or both the packing material and the bonded phase. The packing material used in LC–MS experiments is often based on 3–5-μm silica microspheres with a single pore-size (80–100 Å) distribution, with the smaller particle delivering higher efficiency separations. UPLC, as described above and in Chapter 4, extends this approach for both chromatographic and throughput efficiency gains. The common bonded phases used in LC–MS include the non-polar or "reversed phase" C18, C8, or phenyl, while less common options include the polar cyano and diol phases. Most of the separation issues that cannot be achieved by changing the mobile phase composition can be optimized by changing the bonded phase. In the example shown in Fig. 1.29, alternate selectivity for the test compounds was achieved by changing the bonded phase from either C18 or C8 to either cyano or phenyl. This type of alternate selectivity comes in handy when one is trying to separate a coeluting matrix or metabolite component from an analyte of interest.

Monolith-based column packing material emerged in the 1990s as potentially important in high throughput quantitative bioanalysis. High permeability, low pressure drop, and good separation efficiency are some of the attributes of monolithic columns. These attributes result from monolithic columns being designed to have a single piece of biporous solid material with interconnected skeletons and interconnected flow paths through the skeletons (Tanaka et al., 2002). While the larger through-pores (typically 2 μm) lead to reduced flow resistance, the smaller mesopores (12 nm) located on the silica skeleton lead to increased surface area needed to achieve separation efficiency. Data suggest that silica- and polymer-based monoliths are ideal for small- and large-molecules, respectively (Tennikova et al., 1990).

In a recent study Alnouti et al. (2005) evaluated conventional C18 and monolithic columns for online SPE–LC–MS/MS quantification of propranolol (ketoconazole

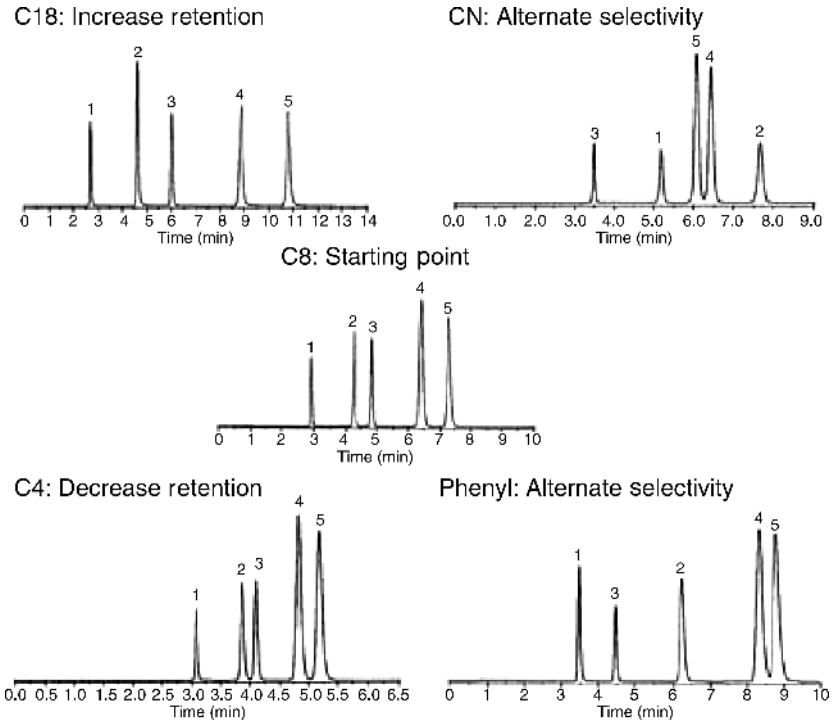


Figure 1.29. *LC separation of (1) norephedrine, (2) nortriptyline, (3) toluene, (4) imipramine, and (5) amitriptyline using columns with various bonded phases [all-ACE column: 250 mm × 4.6 mm, 5 μm; mobile phase 80/20 (v/v): methanol–25 m*M *phosphate buffer; usually, phosphate buffers are not preferred for MS applications]. (Reprinted with permission from Dolan, 2007.)*

was used as the IS) and diclofenac (ibuprofen was used as the IS) directly in rat plasma. As shown in Fig. 1.30, the LC–MS/MS run time was reduced from 4 to 2 min in going from a conventional Luna C18 to a monolithic column, while accuracy and precision of the method were maintained. The HPLC flow rates were 0.8 mL/min and 3.5 mL/min (split to deliver 1.5 mL/min to the MS) respectively for the C18 and the monolithic column approaches. The monolithic column approach demonstrates that high separation efficiencies can be achieved at a significantly increased HPLC flow rate. Several other groups also have evaluated the advantages and limitations of using monolithic columns for high throughput quantitative bioanalysis (Wu et al., 2001; Hsieh et al., 2002; Hsieh et al., 2003; Huang et al., 2006).

One weakness of the dominant reverse phase separations mechanism has been the poor retention of highly polar analytes, and hydrophilic interaction liquid chromatography (HILIC) has emerged as an alternative. In HILIC, a polar stationary phase such as silica gel is used to retain highly polar analytes. Mobile phases components similar to those described above for reverse phase separations are used, but the proportions of aqueous vs. organic are changed. Analytes are retained under conditions of relatively low water content, and eluted using increased water content.

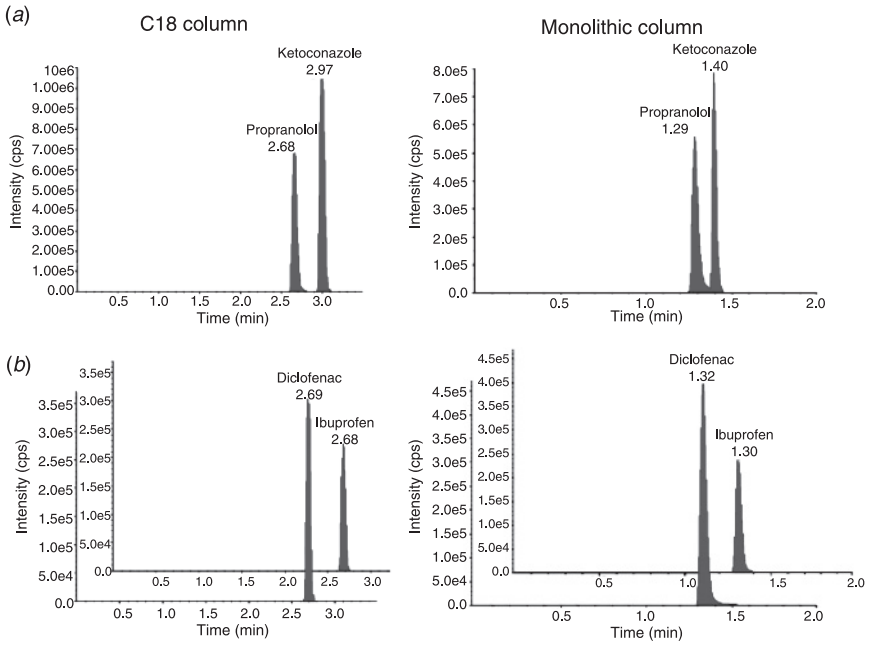


Figure 1.30. *LC–MS/MS (MRM) chromatograms of (a) propranolol (260→116)/ketoconazole (531→489) and (b) diclofenac (294→250)/ibuprofen (205→161) in rat plasma obtained with online SPE and either a Luna C18 column (left panels) or a Chromolith monolithic column (right panels) combination. (Reprinted with permission from Alnouti et al., 2005.)*

Conversely, in conventional reverse-phase HPLC, very high water content is required to retain polar analytes. The high water content in turn hinders the ionization and desolvation process during LC–MS (Hsieh and Chen, 2005; Xue et al., 2006). Therefore, HILIC allows one to elute highly polar analytes with small amounts water and maintain good LC–MS sensitivity (Hsieh and Chen, 2005). In a recent

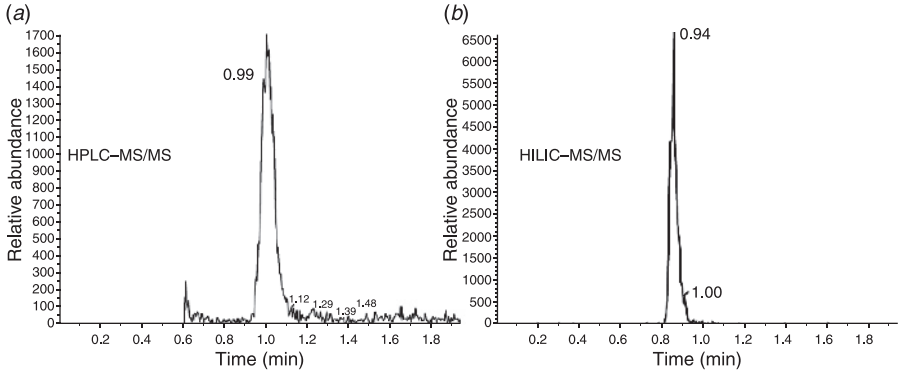


Figure 1.31. *LC–MS/MS (SRM) chromatograms for muraglitazar (517→186) in human plasma obtained using (a) HPLC and (b) HILIC. (Reprinted with permission from Xue et al., 2006)*

study, Xue et al. (2006) compared HPLC–MS/MS and HILIC–MS/MS for quantification of muraglitazar in human plasma and showed that the sensitivity of the LC–MS/MS assay can be improved by using HILIC (Fig. 1.31).

Under HILIC conditions, similar to polar analytes, polar endogenous matrix components such as phospholipids, peptides, and sugars also get retained longer. These polar matrix components can only be disrupted by using high buffer concentrations in the mobile phase. If high buffer concentrations are not an option, then the samples have to be appropriately processed to remove most of the endogenous matrix components before LC–MS/MS. Incomplete removal of these endogenous components is known to cause ion suppression during LC–MS/MS.

Table 1.5 compares various fast chromatographic approaches available for LC–MS/MS based quantitative bioanalysis.

TABLE 1.5. Comparison of Selected Fast Chromatographic Approaches

Approaches	Advantages	Limitations
Monoliths	Low back pressure, possibility to obtain high efficiency with $L_{col} > 1$ m; Compatible with conventional instruments Several monoliths available: organic and inorganic (e.g., carbon, zirconia, silica) Green chemistry (low organic modifier proportion at high temperature)	No straightforward method transfer Low resistance at high pH (pH > 7) and high pressure (>200 bars) Narrow-bore column not yet available (high solvent consumption, split with MS) Heating and cooling requirements (dedicated system) Stability of stationary phases
High-temperature liquid chromatography (HTLC)	Peak shape improvement of basic compunds (pK_a modification) Temperature an additional parameter for method development Possibility to couple HTLC with other fast-LC approaches (sub-2 μm, UPLC) Significant decrease in analysis time (up to 10 times)	Compound stability needs to be evaluated prior to analysis Not straightforward method transfer (selectivity changes with T) High back pressure with small particle size, limited efficiency
Sub-2-μm particles	Easy methiod transfer Many commercially available sub-2-μm particles (e.g., C_4, C_8, C_{18}, HILIC)	Limited compatibility with conventional instrumentation
Sub-2-μm particles at 1000 bars (UPLC)	Large decrease in analysis time (up to 20 times) Possibility to obtain high efficiency with $L_{col} \geq 15$ cm Easy method transfer	Few available stable stationary phases Dedicated instrumentation needed Solvent compressibility and frictional heating could exist at $\Delta P = 1000$ bars

Abbreviations: Lcol, length of column; T, temperature.
Source: From Guillarme et al., 2007.

1.6 SERIAL AND PARALLEL LC–MS APPROACHES FOR INCREASING QUANTITATIVE BIOANALYTICAL THROUGHPUT

The traditional, serial-mode, quantitative bioanalytical operation involves a single autosampler/HPLC column and a MS system. In this mode of operation, samples are injected one at a time. The serial-mode strategies used to increase the throughput include fast chromatography (Romanyshyn et al., 2000, 2001; Hop et al., 2002), automated data processing (Whalen et al., 2000; Fung et al., 2004; Briem et al., 2007), and pooling strategies (cassette dosing, pooling after individual dosing, simple sample screens, etc.) (Korfmacher et al., 2001; Kassel, 2004, 2005). Some of these strategies have resulted in 60 samples per hour. Today, fast chromatography is routinely achieved by utilizing UPLC instead of HPLC (Yu et al., 2006, 2007).

In most traditional LC–MS quantitation, the mass spectrometer is utilized for analyte and IS detection for only a brief period in the overall analysis. Significant time is lost to the autosampler, gradient delay and re-equilibration, or isocratic flushing of late-eluting materials. Throughput can be improved by establishing additional chromatographic systems in parallel. Some systems utilized simultaneous, parallel LC separations followed by detection using a multiplexed "MUX" MS interface (Fig. 1.32) (Deng et al., 2001, 2002; Jemal et al., 2001; Rudewicz and Yang, 2001; Yang et al.,

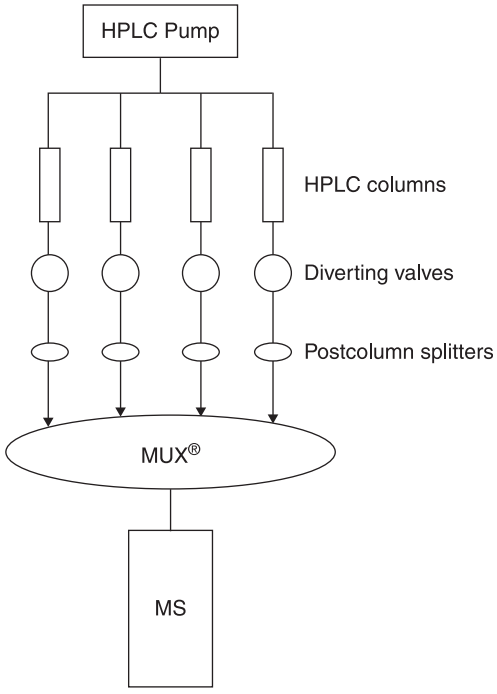


Figure 1.32. *Schematic of parallel LC–MS system consisting of binary pump, autosampler, and mass spectrometer used in combination with four LC columns connected to four-sprayer MUX interface. (Reprinted with permission from Fung et al., 2003.)*

2001a,b; Fang et al., 2002, 2003; Morrison et al., 2002; Fung et al., 2003). Other systems used parallel LC separations with a time offset such that elution from each column occurred in a discrete time period (Zweigenbaum and Henion, 2000; van Pelt et al., 2001; Xia et al., 2001). A single LC could be coupled to multiple columns, and varying lengths of "delay" tubing used to create offset elution from the columns. Alternatively, multiple independent autosamplers, pumps, and columns can be connected to a common mass spectrometer through a switching valve. Injection times are offset across the LC systems (typically two or four parallel systems), and the injection/elution/detection process is tracked through the software. This latter technique has been successfully commercialized through the Cohesive/ThermoFisher system mentioned above with or without the turbulent flow component (Chapter 10).

1.7 HIGHER THROUGHPUT QUANTITATIVE ANALYSIS WITHOUT LIQUID CHROMATOGRAPHY

A faster option is to eliminate the chromatography step altogether. In the early years of API–LC–MS, many users hoped that the techniques of ESI and APCI would facilitate just that. However, it was quickly recognized that ion suppression or metabolite coelution caused irreproducible or inaccurate results for many analytes (Cohen and Gusev, 2002). More recently, there has been considerable renewed interest in the use of desorption ionization techniques for quantitation (Notari et al., 2006). By eliminating the necessity for chromatographic separation, desorption-based techniques can achieve order-of-magnitude increases in throughput compared with high-speed LC–MS techniques. Recent examples include the commercial LDTD (laser diode thermal desorption, Phytronix Technologies) (Edge et al., 2008; Koers, 2008; Tremblay et al., 2008) and DESI (Prosolia, Inc.) systems. Use of desorption techniques for quantitative analysis is considered in Chapter 11 of this book.

1.8 MASS SPECTROMETRY IN QUALITATIVE ANALYSIS

The qualitative applications of MS in drug metabolism and pharmacokinetic studies are generally focused on addressing at least one of the two fundamental questions in DMPK. As summarized by Ma et al. (2006) and Prakash et al. (2007), we are looking to determine what drug-derived analytes are in a sample and how much there is. The first part of the question centers on identification of an unknown, a challenge for which MS is eminently suited. The combination of chromatographic separation, identification of molecular weight (and possibly elemental composition), and structural information via MS^n fragmentation is currently the strongest technique available to the drug metabolism group for the analysis of biological samples.

From the perspective of the pharmaceutical industry, the attention given to metabolites started to increase when the Pharmaceutical Research and Manufacturers of America (PhRMA) commissioned a review of the role of metabolites in drug induced toxicity. This group, called the metabolites in safety testing (MIST) committee, partnered with the U.S. Food and Drug Administration, convened several

workshops, and in 2002 published a proposal outlining the types of drug metabolism studies relevant to safety assessment at all major phases of drug development. This publication, commonly referred to as the MIST document, very carefully addressed some of the contemporary issues in the safety evaluation of drug candidates and discussed how best to use the metabolite data. A brief timeline of recent publications relevant to metabolite issues is presented in Table 1.6.

TABLE 1.6. Recent Publications/Events on Metabolite in Safety Testing

Year	Organization/Reference	Outcome/Recommendations
1999	PhRMA—Commissioned a survey of current practices in dealing with metabolites in safety testing	• Several workshops held to discuss metabolites in safety testing approaches across pharmaceutical industry and the expectations from FDA.
2002	Baillie et al.—MIST Document Published	• Perform radiolabeled human ADME study as early as possible to define a major circulating metabolite. • A major circulating metabolite is defined to be a drug derived component(s) accounting for 25% or more of the AUC of total circulating drug derived components (relative abundance). • Any major human circulating metabolites should be considered for monitoring in toxicological and/or clinical studies. • If a metabolite is pharmacologically active or structural alert (i.e., contains a reactive functional group, glutathione conjugates) then it should be considered for monitoring in toxicological and/or clinical studies. • If a unique or human specific metabolite is observed during in vitro cross-species comparison studies, additional toxicological studies are warranted. • If a unique or human specific circulating metabolite is absent or present at relatively low concentration in toxicological species, separate studies to evaluate the toxicity of the human specific metabolite are warranted. • Carcinogenicity studies on a major metabolite are not recommended.
2003	Hastings et al.—Letter to the Editor	• Raised some concerns about a major metabolite being defined as a metabolite representing 25% of the systemic exposure compared to the parent drug. • Provided clear evidence that a minor metabolite could very easily produce toxicity. • The authors stated that the agency reserves the right to request carcinogenicity studies on major metabolites where required.

(*Continued*)

TABLE 1.6 *Continued*

Year	Organization/Reference	Outcome/Recommendations
		• Mentions that a formal guidance on the issue of drug metabolites in safety testing is warranted.
2003	Baillie et al.— Response to Hastings et al.	• Welcomed a formal guidance from the U.S. FDA • Additional clarification provided to distinguish between *major* and *unique* metabolites. • Emphasized a case-by-case approach to metabolite safety studies rather than formally outlining a set of studies for all circumstances.
2005	Smith and Obach—Commentary on MIST	• Proposed using the absolute abundance of a metabolite rather than relative abundance value as suggested in the MIST document.
2005	FDA—Draft Guidance on Safety Testing on Drug Metabolites is Published	• As early as possible, assess species differences in metabolism of a drug (in vitro studies, nonclinical animal studies). • Perform metabolic evaluations in humans as early as possible (radiolabeled or non-radiolabeled). • Early identification of unique or major human metabolites can provide clear directions for testing in animals, assist in interpreting and planning of clinical studies, and prevent delays in drug development/approval. • All human circulating metabolites that account for $>10\%$ of the administered dose or systemic exposure (whichever is less) and that were not present at sufficient levels to permit adequate evaluation during nonclinical animal studies should be considered for additional safety/toxicological testings. • If the systemic exposure for a major human circulating metabolite is equivalent to that observed in nonclinical toxicological species, then the metabolite levels may be sufficient to limit additional toxicity testing using the major human metabolite. • As needed, perform carcinogenicity studies on major human metabolites.
2006	Prueksaritanont, Lin and Baillie—Publication	• Highlighted the kinetic, metabolic, exposure, and toxicity differences of a preformed or synthetic metabolite(s) compared to that of a metabolite(s) generated endogenously from a parent drug. • Safety evaluations involving preformed or synthetic metabolite(s) of a drug should take metabolite kinetics into considerations.

(*Continued*)

TABLE 1.6 *Continued*

Year	Organization/Reference	Outcome/Recommendations
2008	FDA—Guidance on Safety Testing on Drug Metabolites is Published	• No major changes to recommendations provided in the Draft Guidance. • Focus changed from metabolites accounting for >10% of the administered dose (which could potentially apply to circulating as well as metabolites in excreta) to circulating metabolites accounting for >10% of parent drug's systemic exposure. • A new term, disproportionate drug metabolite, was introduced. Disproportionate drug metabolite was defined as "a metabolite present only in humans or present at higher plasma concentrations in humans than in the animals used in nonclinical studies. In general, these metabolites are of interest if they account for plasma levels greater than 10 percent of parent systemic exposure, measured as area under the curve (AUC) at **steady state**." • An updated decision tree flow diagram summarizing some of the steps required to assess safety of a major human drug metabolite is provided (Fig. 1.33).

Abbreviations: PhRMA, The **Ph**armaceutical **R**esearch and **M**anufacturers of **A**merica; MIST, **M**etabolites in **S**afety **T**esting; FDA, United States **F**ood and **D**rug **A**dministration.

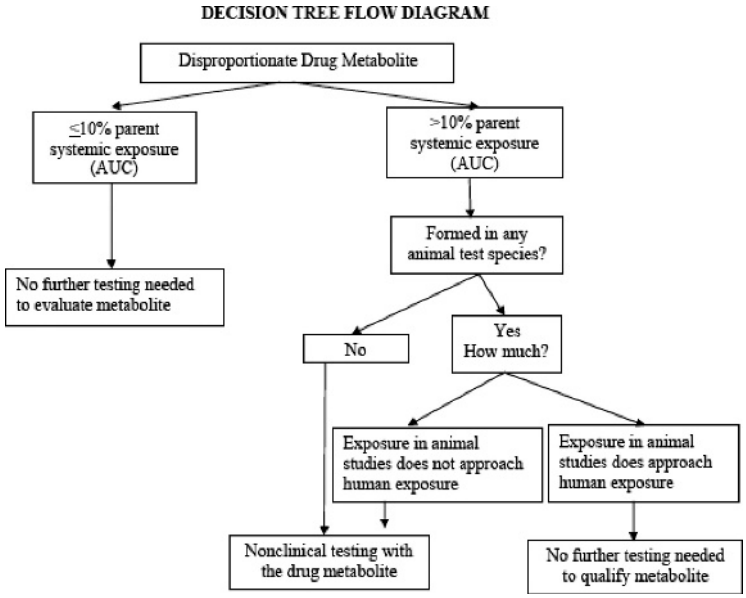


Figure 1.33. *A decision tree flow diagram describing some of the studies needed to determine safety of a human drug metabolite. (Food and Drug Administration (2008). Guidance for Industry: Safety Testing of Drug Metabolites.)*

1.8.1 Common Phase I and Phase II Biotransformation Pathways

Upon exposure to living systems, drugs undergo metabolism or biotransformation as a detoxification process to form more polar forms (metabolites) of the drug that can be readily eliminated. Undesired consequences of biotransformation include rapid clearance, formation of active metabolites, formation of reactive metabolites, and drug–drug interactions due to enzyme induction or inhibition. As described previously, metabolism can occur in different parts of the body, but the gut and liver are the major sites of metabolism. Several enzymes are involved in detoxification of drugs. These enzymes and their role in metabolism have been discussed in detail by Ghosal et al. (2005) and Johnson (2008a). Briefly, the drug detoxification pathways involve phase I and phase II metabolic reactions. As shown in Table 1.7, the phase I processes involve reactions such as oxidation, reduction, and hydrolysis. Among the phase I reactions, oxidative reactions can be catalyzed by either cytochrome P450s (CYP450) or nonmicrosomal enzymes such as monoamine oxidases (MAOs), peroxidases, and flavin-containing monooxygenases (FMOs). Among the drug-metabolizing enzymes, the CYP superfamily has

TABLE 1.7. Examples of Common Drug Biotransformations

Type of Biotransformation	Net Transformation	Nominal m/z Shift	Exact m/z Shift
	Phase I		
Hydroxylation/N-oxidation/S-oxidation	$+O$	+16	+15.9949
Dihydroxylation	$+2O$	+32	+31.9898
Dehydrogenation or reduction	$-H_2$	−2	−2.0156
Demethylation	$-CH_2$	−14	−14.0156
Deethylation	$-C_2H_4$	−28	−28.0312
Depropylation	$-C_3H_6$	−42	−42.0468
Oxidative deamination	$-NH_3,+O$	−1	−1.0316
Oxidative dechlorination	$-Cl,+OH$	−18	−17.9662
Oxidative defluorination	$-F,+OH$	−2	−1.9957
Hydration	$+H_2O$	+18	+18.0105
Methyl to an acid	$-H_2,+O_2$	+30	+29.9742
	Phase II		
Glucuronidation	$+C_6H_8O_6$	+176	+176.0321
Sulfation	$+SO_3$	+80	+79.9568
Glutathione conjugation	$+C_{10}H_{15}N_3O_6S$	+305	+305.0681
	$+C_{10}H_{17}N_3O_6S$	+307	+307.0837
Cysteine–glycine conjugation	$+C_5H_{10}N_2O_3S$	+178	+178.0410
Cysteine conjugation	$+C_3H_7NO_2S$	+121	+121.0196
N-acetyl-cysteine conjugation	$+C_5H_9NO_3S$	+163	+163.0301

Note: Exact masses of elements: C = 12.000000; N = 14.003074; O = 15.994915; H = 1.007825; F = 18.998403; Cl = 34.968853; S = 31.972072.

been extensively studied because they account for more than 90% of oxidative biotransformation of drugs and xenobiotics (Ramanathan et al., 2005). To date, 750 CYP450s or polymorphs have been sequenced and 55 of them have been characterized as human isoforms (Ramanathan et al., 2005). As listed in Table 1.7, microsomal oxidations include aromatic and side-chain hydroxylation, N-oxidation, S-oxidation (sulfoxidation and sulfonation), N-hydroxylation, N-, O-, S-dealkylation, deamination, dehalogenation, and desulfation. Although MAOs, FMOs, and peroxidases have been associated with several of the above-mentioned biotransformation processes, their involvement is of lesser importance. According to the FDA's guidance on safety testing on drug metabolites (Food and Drug Administration, 2008), "metabolites formed from Phase I reactions are more likely to be chemically reactive or pharmacologically active and, therefore, more likely to need safety evaluation."

Phase II processes lead to ultimate detoxification reactions involving modification of a functional group ($-OH$, $-NH_2$, $-SH$, or $-COOH$) by a bulky and polar groups such as glucuronides, sulfates, amino acids, and/or glutathiones. Among these reactions, glucuronidation is the most common phase II reaction, and it is catalyzed by the enzymes called uridine diphosphate glucuronosyltransferases (UGTs). Most often, phase II reactions terminate the pharmacological activity of a drug. According to the FDA's guidance on safety testing on drug metabolites (Food and Drug Administration, 2008), "Phase II conjugation reactions generally render a compound more water soluble and pharmacologically inactive, thereby eliminating the need for further evaluation. However, if the conjugate forms a toxic compound such as acylglucuronide (Faed, 2003), additional safety assessment may be needed." Only in a few cases Phase II metabolites have been found to be pharmacologically active, for example, the phenolic glucuronide conjugate of Ezetimibe (Patrick et al., 2002) and morphine-6-glucuronide (Ishii et al., 1997).

The reasons for identification of metabolites are manyfold but all boil down to human safety of drugs under clinical investigation. Initially metabolites of a drug are characterized with in vitro systems (microsomes, hepatocytes, S9 fractions, etc.) and later lead compounds are assessed using mouse, rat, rabbit, dog, and/or monkey. Subsequently, metabolites in humans are identified following drug administration to assure that the nonclinical species undergoing safety assessment are adequately exposed to human metabolites of the drug (Smith and Obach, 2006).

1.8.2 Metabolite Profiling, Detection, and Characterization Process Flow

Metabolite identification can be conducted on several levels, ranging from straightforward analyses for targeted species to more complex analyses utilizing radiometric detection, MS, and possibly other detectors. A process flow for profiling and characterization of metabolites by LC–MS is presented in Fig. 1.34. There is an increasing emphasis on quality metabolite data from relatively early in discovery (Fernandez-Metzler et al., 1999), and some of the fundamental approaches that can be used have been summarized by Anari and Baillie (2005).

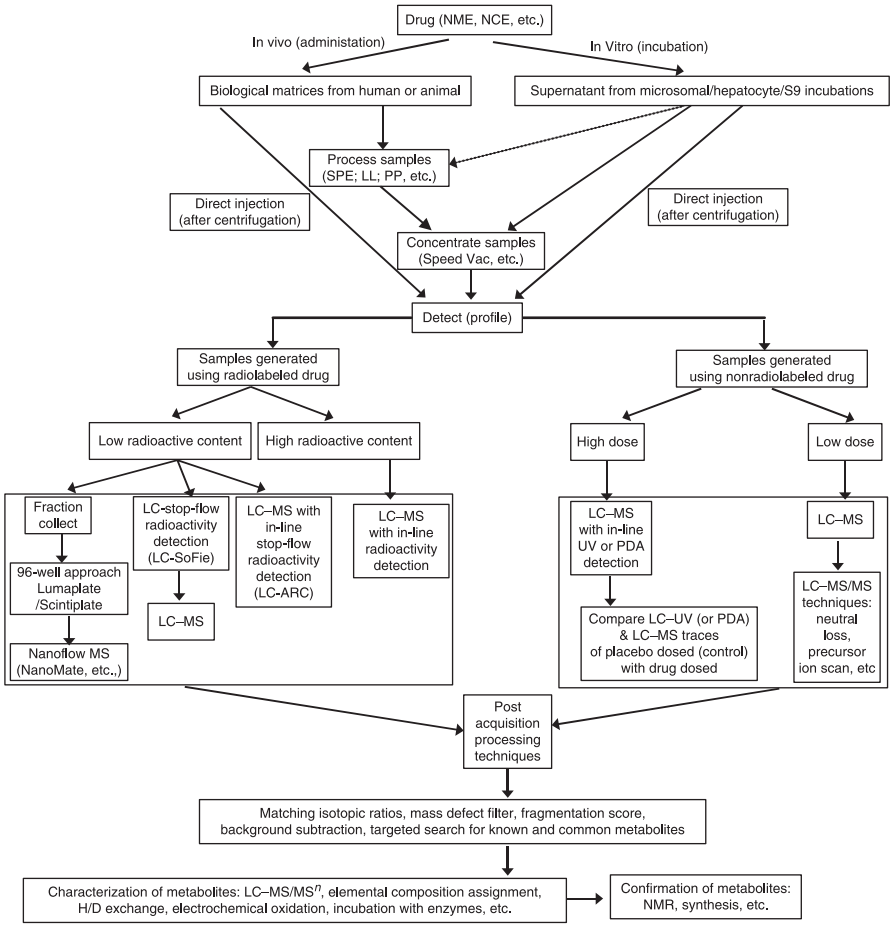


Figure 1.34. *Generic process flow for metabolite detection and characterization using LC–MS and other auxiliary techniques.*

At early stages of drug development, in the absence of radiolabeled drug, the major challenge in identification of metabolites is the presence of large amounts of mass spectral background from matrix components. To facilitate detection, initial screening for metabolites may utilize a list of targeted transformations based on the starting drug mass. Common transformations are converted to a list of target precursor ion m/z using the known mass shift of the transformation and the starting mass of the drug. The targeted list may be generic or can be modified based on known or predicted transformations expected for the compound/compound class being analyzed (Anari et al., 2004; Tiller et al., 2008). A few common example transformations are shown in Table 1.7. Reactive metabolites, discussed in detail by Johnson (2008b) and Obach et al. (2008), may be targets of especially high interest if such a structure is anticipated (Ma and Subramanian, 2006; see also Chapter 4). According to the

FDA's guidance on safety testing on drug metabolites (Food and Drug Administration, 2008), "Metabolites that form chemically reactive intermediates can be difficult to detect and measure because of their short half-lives. However, they can form stable products (e.g., glutathione conjugates) that can be measured and, therefore, may eliminate the need for further evaluation."

Once a targeted list is assembled, the appropriate LC–MS instrument can be set up to acquire both MS and MS/MS data (or MS^n data for traps) in an automated fashion. The MS/MS acquisitions would only be triggered by detection of a targeted precursor ion (from the list) at a minimum specified intensity. Linear ion trap quadrupole instruments are increasingly popular for this type of work (Hopfgartner and Zell, 2005) and are discussed in Chapter 3 of this book.

1.8.3 New Opportunities with Hybrid Mass Spectrometers

While much of the MS and MS/MS data acquisition has been performed using triple-quadrupole instruments, hybrid instruments capable of high resolution/high mass accuracy are increasingly being used for routine metabolite screening. Such systems include the Q-TOF, IT–TOF, LTQ–FTICR, and LTQ–Orbitrap configurations (Erve et al., 2008; Tiller et al., 2008). While foregoing none of the traditional MS and MS/MS data typically available, these instruments offer the added advantage of m/z measurements with high accuracy. For targeted structures with known elemental composition, the observed m/z would either be consistent with or refute the proposed structure.

When a targeted list is not used to drive the process, analysis may be conducted to assess all metabolites present. The observed m/z is compared with all possible elemental compositions that could produce the observed value, within constraints imposed by the user as described below. Accuracy and precision in the m/z measurement become critical in limiting the possible compositions to the greatest extent possible, greatly simplifying data reduction (Grange and Sovocool, 2008; Ruan et al., 2008). These techniques are discussed in Chapters 4 and 5.

1.8.4 Auxiliary Techniques to Facilitate Metabolite Profiling and Identification

Studies utilizing dosage of drug labeled with radioisotopes such as ^{14}C or ^{3}H are frequently used to create a complete mass balance, including all metabolites (Ramanathan et al., 2007a,b). Analysis of both eliminated and circulating metabolites is accomplished by simultaneous use of radio flow detection and mass spectrometric analysis. While targeted analysis may facilitate identification of most major metabolites, radiotracer studies are still considered the benchmark for complete metabolite profiling. An update on this technique is provided in Chapter 7, in which methods for determining low levels of circulating metabolites are assessed.

Identification of a metabolite mass shift such as $+16$ clearly implies addition of oxygen, but does little else. The nature of the functional group and its location on the molecule may be determined through MS/MS analysis, but more stringent experimental

techniques may be needed. Derivatization techniques optimized for different functional groups may be useful (Hop and Prakash, 2005) but can be relatively cumbersome. A common probe for determining the nature of the functional group is hydrogen/deuterium (H/D) exchange. Depending on the nature of the functional group, different adjacent hydrogen atoms may be easily exchanged with deuterium. Analysis using D_2O instead of H_2O in the mobile phase can be sufficient to facilitate such exchange, and these techniques are explored in Chapter 9 of this book.

While highly successful, the API interfaces are not optimal for all small molecules. For cases where an analyte appears to ionize and vaporize with poor efficiency, a fundamental change in the analyte can be made prior to the API source. The use of coulometric assisted ionization is described in Chapter 8. In this method, electrochemistry techniques are used to alter the analyte. Improvements in specificity and sensitivity can result.

Mass spectrometry is also being used to help assess the ability of the drug to reach the target location in the body (McLean et al., 2007; Cornett et al., 2008; Kertesz and Van Berkel, 2008). The ability to determine the presence of the drug in the target organ, and even the drug/metabolite profile within the target organ, is explored in Chapter 12.

1.8.5 Tools and Techniques for Streamlining Metabolite Detection and Characterization

With the proliferation of mass spectrometers in drug metabolism came a vast amount of data to be processed and understood. In particular, techniques and tools for identification of unknown compounds such as metabolites, degradation products, or impurities have seen continual improvement in recent years, especially with respect to data obtained under high-resolution (and presumed high-mass-accuracy) conditions. A few possible approaches are summarized here.

One common target of data reduction is determination of the elemental composition for a particular observed m/z. Unfortunately, as m/z increases, so does the number of possible combinations of atoms that could produce the observed ion. The use of high-resolution instruments allows one to limit the possibilities of the observed m/z values through greater accuracy and specificity. High resolution should improve specificity but needs to be coupled with careful management of calibration to ensure high and known accuracy. Knowledge of the effective error bars on the m/z measurement permits limiting the search for matching formulas. Most instrument data systems now include algorithms for converting measured m/z to postulated elemental composition. The user is permitted to specify types of atoms to include (e.g., only C, H, N, and O), maximum numbers for each, and an estimate of the degree of bond saturation in the molecule. The result is a list of possible formulas that fit the criteria and the relative error of each from the observed m/z value.

These techniques still are not sufficient for true specificity. As mass increases from 200 to 500, the number of formulas mathematically possible for that mass increases rapidly, and a mass accuracy yielding a single, unambiguous formula quickly becomes impossible (Kind and Fiehn, 2006; Kind et al., 2007). To reduce the possibilities, the atom types and abundance required to produce the observed isotope

pattern can be included in screening for possible formulas. In addition to the isotope pattern itself, an accurate mass measurement of each isotope peak can be considered. Only a proposed formula that satisfies the constraints for the original m/z (accuracy, atom type and number limitations, etc.), the isotope pattern, and is consistent with the proposed formulas at each isotope m/z would now be acceptable (Thurman, 2006; Cuyckens et al., 2008; De Maio et al., 2008; Gallagher et al., 2008; McGibbon et al., 2008).

While highly accurate mass measurement has become more common, efforts are still made to maximize the utility of so-called low-resolution instruments. It is reported to be possible to produce highly accurate data from unit-resolution instruments using commercial products such as MassWorks™ (Gu et al., 2006), theoretically facilitating the use of a quadrupole instrument for high-mass-accuracy work. Given the proliferation of LITs, this is an attractive (and relatively inexpensive) option. However, the possibility will still exist for signal overlap at low resolution that would be resolved at high resolution.

In an effort to further simplify data, the concept of the mass defect filter was introduced (Chapter 6). The mass defect filter utilizes the well-characterized changes in mass introduced for different types of metabolite and, in conjunction with highly accurate m/z measurement, yields stringent requirements for m/z changes that must be met. For example, introduction of oxygen to a molecule produces a nominal mass increase of 16. In more exact terms the change is 15.9949. Therefore, introduction of an oxygen atom would reduce the mass defect of the resulting molecule by approximately 0.005 u.

In addition to software tools to help postacquisition processing, software tools to help mass spectral interpretation, particularly MS/MS, have taken new strides as well (Heinonen et al., 2008). One example of such a software tool is the MathSpec™ program. The details of the MathSpec approach have been explained (Sweeney, 2003). MathSpec software is used in conjunction with MS/MS spectra obtained under high-resolution conditions. The software systematically attempts to assemble possible parts (from the MS/MS fragment data) of the molecule into a rational molecule. Other examples of structure elucidation software include HighChem's Mass Frontier and ACD/Labs ACD/MS Manager (Bayliss et al., 2007). Other metabolite prediction software tools such as Meteor are also being incorporated into LC–MS software as tools to help accelerate metabolite detection and characterization (Testa et al., 2005; Ives et al., 2007).

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