Mass Spectrometry in Drug Metabolism and Pharmacokinetics

Edited by

Ragu Ramanathan

A JOHN WILEY & SONS, INC., PUBLICATION

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Preface

Within the pharmaceutical industry, the mass spectrometer was long considered a useful and challenging analytical tool largely limited to the specialist user. The steady movement from specialist use to general use gained considerable speed in the 1990s, particularly due to the development of practical, sensitive liquid chromatography–mass spectrometry (LC–MS) interfaces and advances in the microelectronics. The rapid proliferation of quadrupole ion trap, linear ion trap, orbitrap, quadrupole mass filter, time-of-flight, and other types of mass spectrometers has impacted the industry from the earliest stages of disease determination through the final stages of clinical testing. This book, based on an American Society for Mass Spectrometry (ASMS) session, which I was fortunate enough to chair, will examine several of the ways in which mass spectrometry continues to have a profound influence on the direction and speed of drug discovery and development, especially in the area of drug metabolism (DM) and pharmacokinetics (PK).

To facilitate introduction to the topics contained in this book, the first chapter considers briefly the broader processes of drug discovery and development within the pharmaceutical industry. The specific roles of DM and PK, the applications considered throughout this book, are defined as well as major terms and concepts in mass spectrometry. Finally, the role of mass spectrometry in DM and PK is developed and the ensuing chapters introduced. For the experienced professional, this final section of the first chapter may represent the appropriate starting point in reading this book.

Chapter 2 systematically defines some of the important PK parameters and guides the reader through the types of quantitative LC–MS experiments performed to elucidate the PK parameters necessary to move a drug through discovery, preclinical development, and clinical stages. Chapters 3, 4, and 5 respectively introduce the readers to quadrupole mass filters and liner ion traps, time-of-flight mass

spectrometers, and Fourier transform (FTICR and Orbitrap) mass spectrometers and their applications in the area of DM and PK. The high-resolution LC–MS mass defect filter (MDF) approach is considered in Chapter 6. Today the MDF approach has been adapted by all the major mass spectrometer vendors to help accelerate drug discovery and development. Chapter 7 elegantly describes the utility of high-sensitivity radioactivity and mass spectrometry techniques for drug metabolism studies. While online electrochemical–LC–MS techniques available for generating metabolites are discussed in Chapter 8, Chapter 9 describes some of the LC–MS tools and techniques available for detecting and characterizing isomeric metabolites. Chapter 10 is dedicated to online sample processing and turbulent-flow LC–MS techniques. Finally, Chapters 11 and 12 present some of the laser desorption–based mass spectrometry applications in the DM and PK arena.

This book would have never been possible without the efforts and dedication of more than 35 co-authors and the editorial staff at Wiley. I am very grateful to Kevin B. Alton, Honggang Bi, Jimmy L. Boyd, Swapan K. Chowdhury, John R. Eyler, Michael L. Gross, W. Griffith Humphreys, Steven Michael, Richard Morrison, Noel Premkumar, Laszlo Prokai, Rasmy Talaat, Poonam Velagaleti, and Ronald E. White for their continued mentorship throughout my professional career. I am also very grateful to my parents, brothers, aunts, uncles, and grandmother for supporting my education and career. Finally, my deepest gratitude goes to my wife, Dil, and Vishan and Eshal for continuously supporting all my endeavors.

RAGU RAMANATHAN, PH.D.

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September, 2008

About the Editor

Ragu Ramanathan received a B.Sc. in Chemistry from the University of Southern Mississippi and a Ph.D. in Physical Chemistry/Mass Spectrometry from the University of Florida. His graduate research focused on coupling of electrospray ionization (ESI) to Fourier transform ion cyclotron resonance (FTICR) mass spectrometer. After spending three years as a postdoctoral research fellow with Professor Michael L. Gross at the Washington University, St. Louis, Missouri, Dr. Ramanathan managed the Center for Advanced Mass Spectrometry at the Analytical Bio-Chemistry Laboratories, Columbia, Missouri. In 1998, Dr. Ramanathan joined Schering-Plough Research Institute's (SPRI) Drug Metabolism and Pharmacokinetics (DMPK) Department and completed his tenure as a senior principal scientist in 2008. While at SPRI, Dr. Ramanathan was involved in the application of LC–MS for profiling and characterization of metabolites of drug candidates in the preclinical development and clinical stages. Dr. Ramanathan was with Pfizer Global Research and Development from 1999 to 2002 as a group leader of the Ann Arbor site biotransformation group. Dr. Ramanathan is currently an associate director at the Bristol-Myers Squibb, Co. and is responsible for elucidating biotransformation pathways of development drug candidates. Dr. Ramanathan's accomplishments include 35 peer-reviewed papers, 10 book chapters, and over 60 oral/poster presentations. He also served as a chairperson for the North Jersey ACS Mass Spectrometry Discussion Group and as a chairman for DMPK sessions of the American Society for Mass Spectrometry and Eastern Analytical Symposium meetings.

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Evolving Role of Mass Spectrometry in Drug Discovery and Development

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Mass Spectrometry in Drug Metabolism and Pharmacokinetics. Edited by Ragu Ramanathan

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).

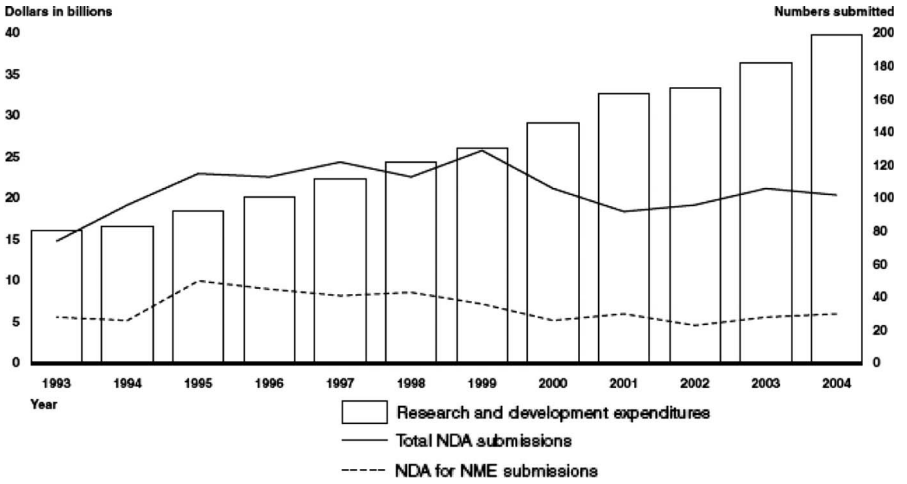

Figure 1.1. *1993–2004 Pharmaceutical R&D expenses, total new drug applications (NDAs), and NDAs for new molecular entity (NME) submission trends. [Reprinted with permission from the U.S. Government Accountability Office (GAO) 2006.]*

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

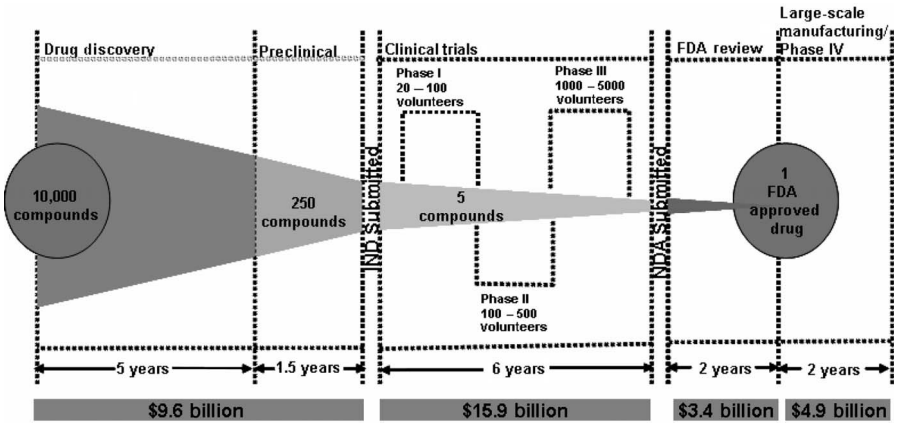

Figure 1.2. *Complex pathway of pharmaceutical R&D involved in bringing a new drug to the market. (Adapted from PhRMA, 2006.)*

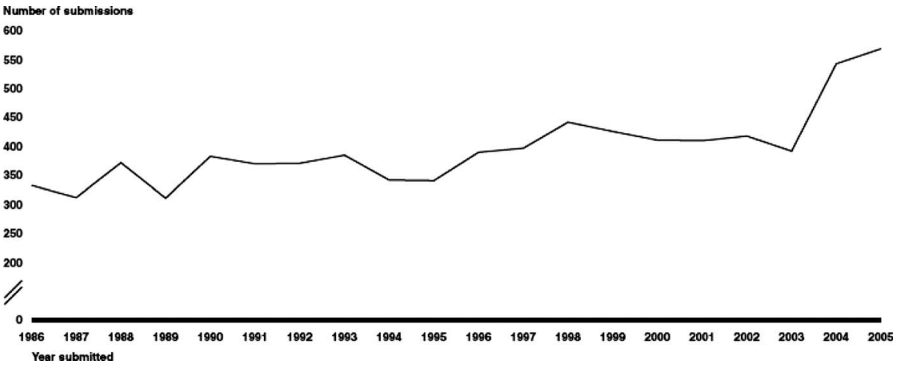

Figure 1.3. *Increase in INDs in recent years. Data are for commercial INDs. (Reprinted with permission from GAO, 2006.)*

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

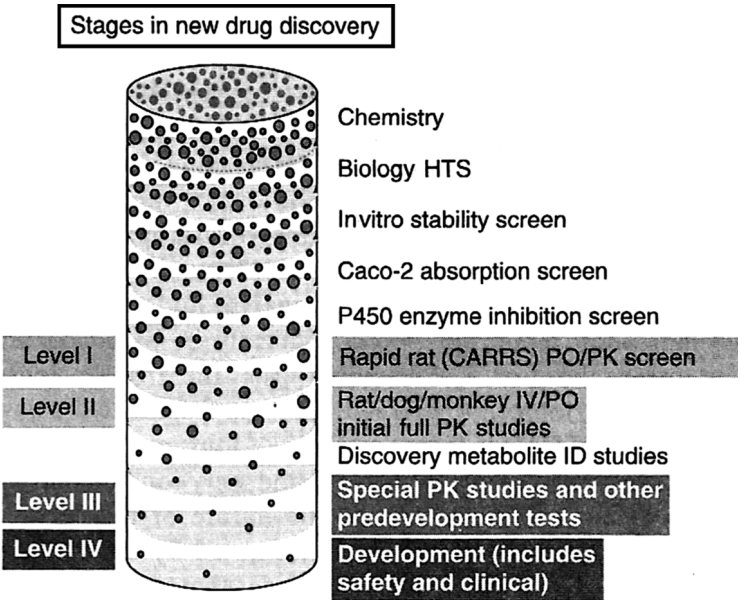

Figure 1.4. *NCE/NME progression scheme showing the various discovery stage liquid chromatography–mass spectrometry (LC–MS) and LC–tandern MS (LC–MS/MS) assays used for selecting NME/NCE to advance into development. (Reprinted with permission from Korfmacher, 2005.) (CARRS, Cassette accelerated rapid 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 × 12.011) + (25 × 1.0079) + (1 × 14.0067) + (1 × 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 × 12) + (26 × 1) + (1 × 14) + (1 × 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 × 12.0000) + (25 × 1.0078) +(1 × 14.0031) + (1 × 15.9949) + (1 × 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

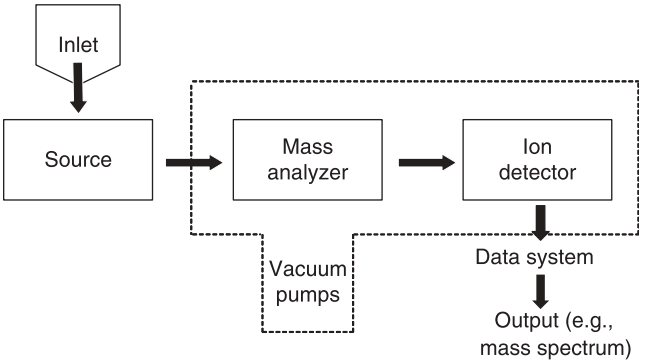

Figure 1.5. *Components of a mass spectrometer.*

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