USING MASS SPECTROMETRY FOR DRUG METABOLISM STUDIES

Second Edition

Absorption
Distribution
Metabolism
Excretion

Pharmacokinetics (PK)

Dose NCE
(Drug) PO/IV

Plasma — A

Drug Levels —
& **PK** Parameters
LC-MS/MS

Bile/Urine — ME

Metabolite ID &
Drug Levels —
LC-MS/MS

Brain — D

Drug Levels —
LC-MS/MS or
MS IMAGE —
MALDI-MS/MS

EDITED BY
Walter A. Korfmacher

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This book is dedicated to the most important people in my life:

Madeleine Korfmacher
Joseph Korfmacher
Mary McCabe
Michael McCabe
Brian McCabe

Preface

The reason for writing this second edition is simple—science marches on and there are new topics and areas of interest that did not exist in 2003, when the first edition of this book was written. We now have UPLC and Orbitraps as well as DESI and DART to name a few of the new instruments and techniques that are now part of our toolset for using mass spectrometry for drug metabolism studies. This second edition is a completely new book with 14 new chapters that were written solely for this edition. While some of the topics are the same as in the first edition, the newly written chapters provide the latest thinking on how best to use mass spectrometry in a drug metabolism setting.

This book is designed to be a reference book for professionals in both mass spectrometry and various drug metabolism areas. It will also be a useful reference book for medicinal chemists working in the area of new drug discovery who want to learn more about drug metabolism and how it is used for participation in lead optimization efforts. The chapters are written by scientists who are experts in the topic and provide not only a summary of the current best practices but also an extensive review of recent scientific literature, including many references for further reading. Each chapter has been written so that it can be read separately from the other chapters, but, together, the 14 chapters cover a wealth of information on various topics that relate to mass spectrometry and drug metabolism studies.

Some of the chapters are written to cover general topics, while other chapters cover a specific area of interest. For example, there is a chapter on metabolite identification as well as a chapter on UPLC. There was also an effort on my part to include newer areas of interest to drug metabolism scientists. As one example of a new topical area, a chapter on biomarkers has been added in this second edition.

I would like to thank all the contributing authors for their efforts to make this second edition complete. I also thank CRC Press for supporting this effort. In addition, I would like to thank the management of Schering-Plough Research Institute for supporting my efforts to make this book a reality. Finally, I would like to thank my family for all their support, with special thanks to my wife, Madeleine.

Walter A. Korfmacher

Editor

Dr. Walter A. Korfmacher is a distinguished fellow of exploratory drug metabolism at Schering-Plough Research Institute in Kenilworth, New Jersey. He received his BS in chemistry from St. Louis University in 1973. He then went on to obtain his MS in chemistry in 1975 and his PhD in chemistry in 1978, both from the University of Illinois in Urbana. In 1978, he joined the Food and Drug Administration (FDA) and was employed at the National Center for Toxicological Research (NCTR) in Jefferson, Arkansas. While at the NCTR, he also held adjunct associate professor positions in the College of Pharmacy at the University of Tennessee (Memphis) and in the Department of Toxicology at the University of Arkansas for Medical Sciences (Little Rock). After 13 years at the NCTR, he joined Schering-Plough Research Institute as a principal scientist in October 1991.

Dr. Korfmacher is currently a distinguished fellow and the leader for a group of 20 scientists. His research interests include the application of mass spectrometry to the analysis of various sample types, particularly metabolite identification and trace organic quantitative methodology. His most recent applications are in the use of high-performance liquid chromatography (HPLC) combined with atmospheric pressure ionization mass spectrometry and tandem mass spectrometry for both metabolite identification as well as nanogram per milliliter quantitative assay development for various pharmaceutical molecules in plasma. He is also a leader in the field of developing strategies for the application of new mass spectrometry (MS) techniques for drug metabolism participation in new drug discovery and is frequently invited to speak at scientific conferences.

In 1999–2000, Dr. Korfmacher was the chairperson of the North Jersey Mass Spectrometry Discussion Group and in 2002 he received the New Jersey Regional Award for Achievements in Mass Spectrometry. Dr. Korfmacher is a member of the editorial boards of three journals: *Rapid Communications in Mass Spectrometry*, *Current Drug Metabolism*, and *Drug Metabolism Letters*. He is also an associate editor of pharmaceutical sciences for the *Journal of Mass Spectrometry*. Dr. Korfmacher has edited a book entitled *Using Mass Spectrometry for Drug Metabolism Studies* (CRC Press, 2005) and has written chapters in four additional books. He has more than 125 publications in the scientific literature and has made more than 75 presentations at various scientific forums.

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1 Strategies and Techniques for Bioanalytical Assays as Part of New Drug Discovery

Walter A. Korfmacher

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1.1 INTRODUCTION

The task of new drug discovery remains a formidable undertaking. Current estimates of the cost of bringing a new drug to the market are in the range of $1.2–$1.5 billion. There is also a significant time commitment—typically it takes 10–14 years to bring a compound from initial discovery to being an approved drug in the market. One of the reasons it takes so long and costs so much is that there is a lot of attrition along the way (most compounds fail). The challenge of new drug discovery is to sort through millions of compounds in a compound library to find a few initial lead compounds and then sift through thousands of new compounds as part of the lead optimization phase with the goal of getting 10–20 compounds that are suitable for development. As shown in Figure 1.1, it takes a total of 14 compounds selected for development in order to reach the goal of 1 compound that becomes a new drug on the market (for more on this topic see Chapter 2). It is this critical lead optimization phase during which there is a continuous need for getting thousands of compounds assayed that the capabilities of a higher throughput bioanalytical scientist are required.

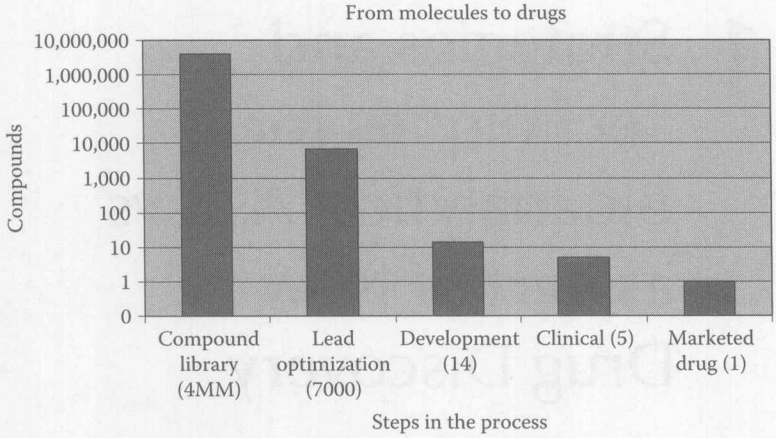

FIGURE 1.1 Schematic chart showing the effect of compound attrition in the drug discovery process from compound libraries to drug approval. The *x*-axis is the stage or point in the process. The *y*-axis is the number of compounds at that point.

The last 20 years have produced enormous changes in how new drug discovery is performed. Before 1990, most major pharmaceutical companies had little need for drug metabolism expertise before a compound was recommended for development. As long as a new compound showed efficacy in some *in vivo* model, it could be a candidate for nomination [1,2]. What changed the landscape was a study that showed that 40% of clinical compounds failed due to human pharmacokinetics [3]. This finding led major pharmaceutical companies to set up exploratory drug metabolism (EDM) departments. The goal of the EDM departments has been to reduce the attrition rate due to pharmacokinetic (PK) issues for new compounds in the clinic [4–6]. As documented by Kola and Landis [7], the desired results have been achieved; currently, less than 10% of new compounds in the clinic fail due to PK reasons. This change in strategy led to the need to develop higher throughput bioanalytical assays in a new drug discovery environment. During the last two decades, another significant change occurred—the high-performance liquid chromatography–tandem mass spectrometer (HPLC–MS/MS) became the instrument of choice for almost all *in vitro* and *in vivo* assays that are routinely performed by bioanalytical scientists in these EDM departments [6,8–22]. This new technology has continued to evolve. As shown in Figure 1.2, in addition to the triple quadrupole mass spectrometer, we now have access to new analytical tools such as the Exactive MS, the LTQ-Orbitrap MS, and the LTQ-FTMS system. The new analytical tools as well as other MS systems have become essential for both quantitative and qualitative assays that are now routinely performed in EDM departments as part of new drug discovery. The reader can find discussions on the utility of these MS systems as well as other types of mass spectrometers in various chapters of this book. This chapter will focus on strategies for quantitative bioanalytical assays to support various *in vivo* PK studies that are now routinely used in new drug discovery.

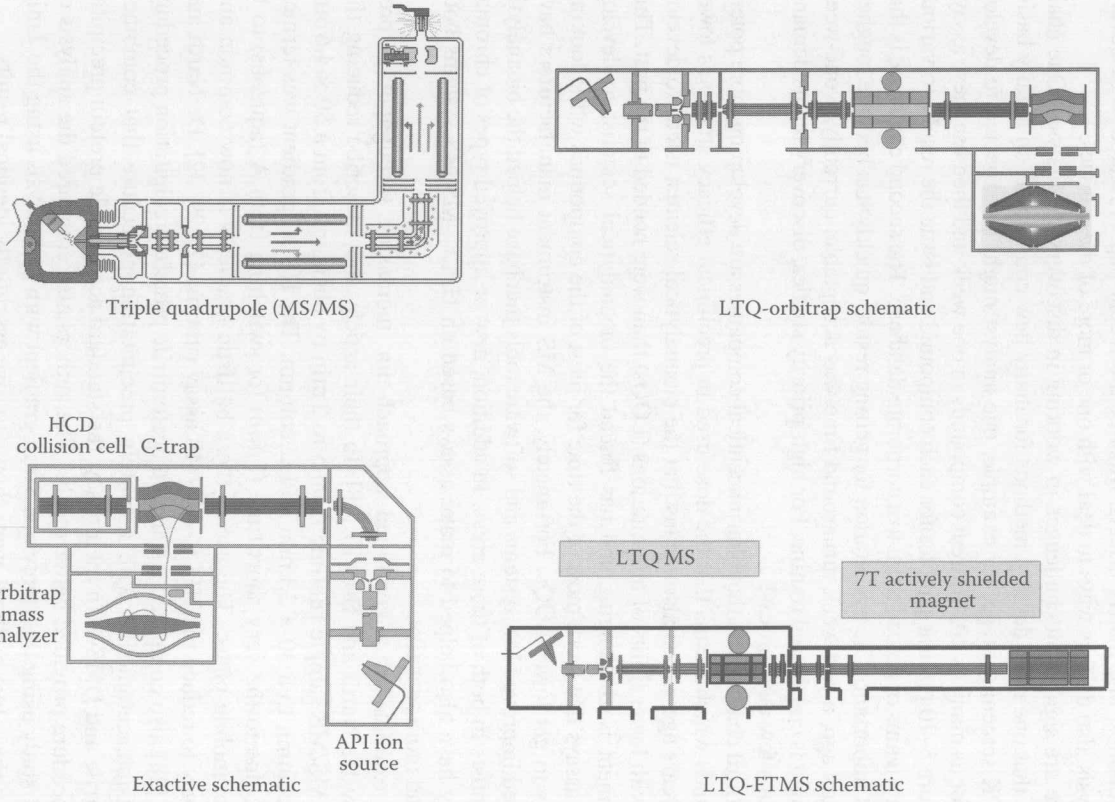

FIGURE 1.2 Four types of mass spectrometers that are used for various drug metabolism assays. (From Pappas, J., Thermo Fisher Scientific Inc., Waltham, MA. With permission.)

1.2 REVIEW OF RECENT LITERATURE

In the past several years, multiple reviews and book chapters have been published on topics related to discovery bioanalysis [10,15,19,20,22–27]. Most of the issues that are cited deal with one of four topic areas: sample preparation, faster assay run times, use of robotics, and matrix effects. Most of the recent papers related to discovery bioanalysis also discuss ways to deal with one or more of these issues.

There are significant challenges in working in discovery bioanalysis. One challenge is that one must develop methods for many new compounds on a daily basis. In the PK screening stage, for example, one analyst might be expected to develop assays for as many as 20 different compounds in one week and then use these assays to measure 5–10 plasma samples for each compound and issue the results to various discovery teams or upload them to a corporate database. The second challenge is that speed is important. The expectation for getting results quickly has become tougher. Five years ago, a two-week turnaround time was acceptable; currently, a one-week turnaround is considered routine. For high-priority studies, discovery teams demand results in a few days or less!

The third challenge is that pharmaceutical companies are developing more potent compounds which means that the dose used in preclinical efficacy studies is lower than 5 years ago which also means that the bioanalytical scientist needs to develop assays with lower limits of quantitations (LOQs) than were needed in the past. This environment is challenging and has forced the bioanalytical scientist to develop generic assays that work most of the time for most of the compounds while looking for ways to get lower LOQs. Fortunately, the MS instrument manufacturers have developed improved MS systems and software tools that have helped the bioanalytical scientists in both of these areas. In addition, new or improved types of chromatography have also helped to make assays based on HPLC–MS/MS systems both faster and more sensitive.

One example of a combined approach for increasing throughput is provided by De Nardi and Bonelli [28]. In their article, they describe reducing the HPLC–MS/MS sample runtime from 5 to 2 min by changing from a 50 × 4.6 mm HPLC column to a 30 × 2.1 mm HPLC column. The HPLC gradient was termed ballistic due to the very short time (1 min) for switching from A (aqueous) to B (organic) mobile phase. The use of these ballistic gradients is now common and is one way to reduce the HPLC–MS/MS assay runtime [22,29–32]. De Nardi and Bonelli [28] also compared a standard acetonitrile protein precipitation procedure (1:3::plasma:acetonitrile) with a protein precipitation procedure that combined acetonitrile and DMSO. In their assay, the standard acetonitrile protein precipitation procedure provided better results. The authors also compared the analysis of a rat PK study using their previous 5 min gradient with an analysis using the 2 min gradient; these two methods were shown to give essentially identical results.

Briem et al. [33] described a combined approach for the higher throughput sample preparation and analysis of plasma samples from discovery preclinical PK studies. Their process combines robotic sample preparation with fast HPLC–MS/MS analysis to speed up the assay plus they add additional steps based on understanding the PK properties of the compound and checking for matrix effects to ensure that the

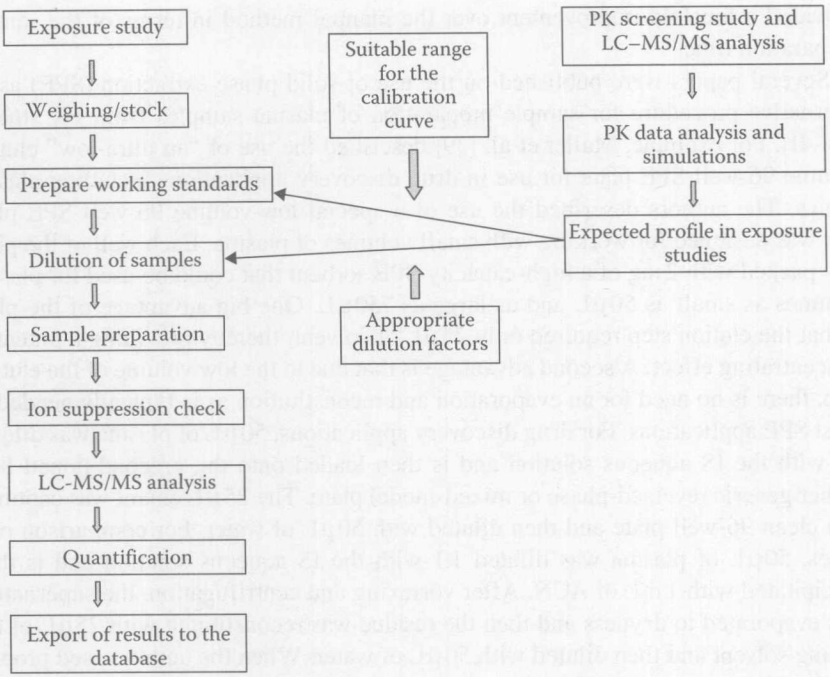

FIGURE 1.3 Workflow for discovery PK studies using the expected PK profile to guide the assay setup. (Adapted from Briem, S. et al., *Rapid Commun. Mass. Spectrom.*, 21, 1965, 2007. With permission.)

data is accurate. Figure 1.3 shows their proposed workflow for the analysis of plasma samples from discovery PK studies. In their scheme, they make use of initial rat PK screening data to calculate various PK parameters for a new chemical entity (NCE) and then these PK parameters are used to predict the likely plasma concentrations of the NCE in subsequent PK studies. The primary value of this procedure was to guide the analyst regarding which study samples should be diluted before the sample analysis was performed, thereby reducing the times that samples would have to be reassayed because they were above the limit of quantitation. This type of advanced planning for a discovery PK study is reasonable and is a good example of how a little extra planning can save time and effort in the long run.

Briem et al. [33] also describe the use of a liquid handler to perform the sample preparation steps in an automated manner. The sample preparation consisted in taking a 25 µL aliquot of the plasma sample and adding 150 µL of acetonitrile (ACN) containing the internal standard (IS) and then following typical protein precipitation (PPT) procedure steps. The authors noted that the 1:6 ratio of plasma to ACN worked well for the liquid handler and exceeded the 1:3 criterion reported by Polson et al. [34] as the minimum ratio of plasma to ACN needed to remove at least 96% of the proteins from the plasma sample. The authors also noted that use of EDTA (instead of heparin) as the anticoagulant helped to avoid clots in plasma as has been reported previously by other authors [35–37]. The authors also stated that the robotic method

provided a fourfold improvement over the manual method in terms of the sample preparation time.

Several papers were published on the use of solid phase extraction (SPE) as an alternative procedure for sample preparation of plasma samples from PK studies [38–41]. For example, Mallet et al. [39] described the use of "an ultra-low" elution volume 96-well SPE plate for use in drug discovery applications including plasma assays. The authors described the use of a special low-volume 96-well SPE plate that was designed for working with small volumes of plasma. Each well of the plate was packed with 2 mg of a high-capacity SPE sorbent that could be used for plasma volumes as small as 50 µL and as large as 750 µL. One big advantage of the plate is that the elution step required only 25 µL of solvent, thereby providing a potential concentrating effect. A second advantage is that due to the low volume of the elution step, there is no need for an evaporation and reconstitution as is typically needed in most SPE applications. For drug discovery applications, 50 µL of plasma was diluted 1:1 with the IS aqueous solution and is then loaded onto the preconditioned SPE (either generic reversed-phase or mixed-mode) plate. The 25 µL eluant was captured in a clean 96-well plate and then diluted with 50 µL of water. For comparison purposes, 50 µL of plasma was diluted 1:1 with the IS aqueous solution and is then precipitated with 1 mL of ACN. After vortexing and centrifugation, the supernatant was evaporated to dryness and then the residue was reconstituted with 25 µL of the eluting solvent and then diluted with 50 µL of water. When the authors used propranolol as a test compound for this sample preparation comparison, the results were dramatic. As can be seen in Figure 1.4, while the signal for the sample from the PPT

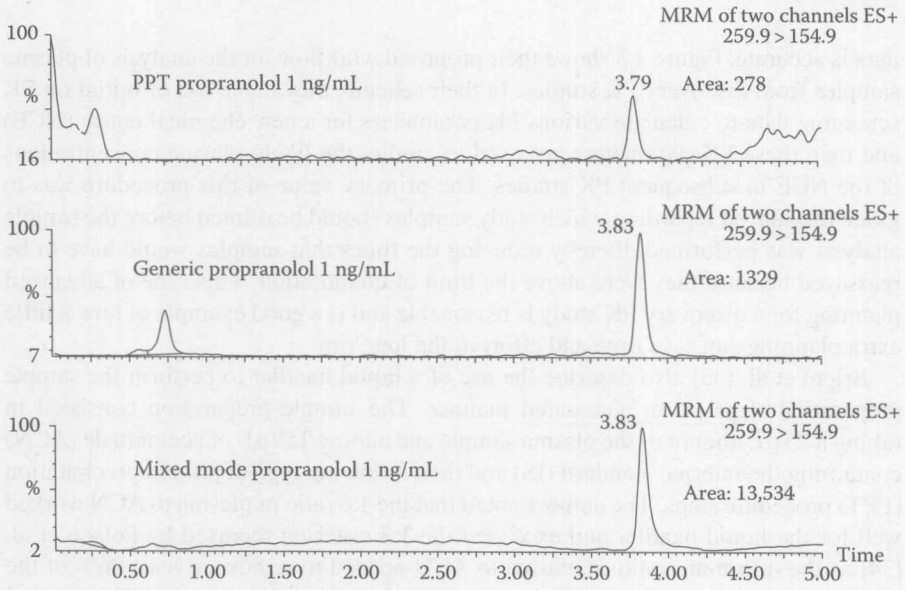

FIGURE 1.4 A comparison of three extraction protocols for the analysis of rat plasma samples for propranolol. (Reprinted from Mallet, C.R. et al., *Rapid Commun. Mass Spectrom.*, 17, 163, 2003. With permission.)

was acceptable, the signal from the generic SPE method was fourfold higher and the mixed-mode SPE method produced an impressive 40× higher signal from the same concentration. The authors also showed that their mixed mode SPE system was suitable for assaying a drug and at least two of its metabolites. Figure 1.5 shows the HPLC–MS/MS analysis of terfenadine and two of its metabolites at a concentration of 0.5 ng/mL in plasma; all three compounds were easy to detect at this concentration using this sample cleanup procedure.

Xu et al. [42] described the use of low-volume plasma sample precipitation procedure that was well suited for drug discovery PK assay applications. In this method, only 10 µL of plasma was used, and it was subjected to PPT using 60 µL of ACN (spiked with the IS) and then assayed using a generic HPLC–MS/MS procedure. In this report, the comparison was made between a "standard" PPT procedure and the proposed low-volume PPT procedure. In the standard-volume (1:3) PPT procedure, a 50 µL plasma sample was precipitated with 150 µL of ACN and the supernatant was transferred to another 96-well plate and a 5 µL aliquot was injected into the HPLC–MS/MS system. In the low-volume (1:6) PPT procedure, a 10 µL plasma sample was precipitated with 60 µL of ACN and the supernatant was transferred to another 96-well plate and a 5 µL aliquot was injected into the HPLC–MS/MS system. In each case the samples were assayed for a discovery test compound using a fast 1.5 min gradient assay. In the first comparison, blank plasma was assayed using either the low- or high-volume procedure. As shown in Figure 1.6, the low-volume procedure produced a significantly lower background signal than did the high-volume procedure. These data show the value of effectively injecting less matrix material into the HPLC–MS/MS system. The authors then showed the results of assaying

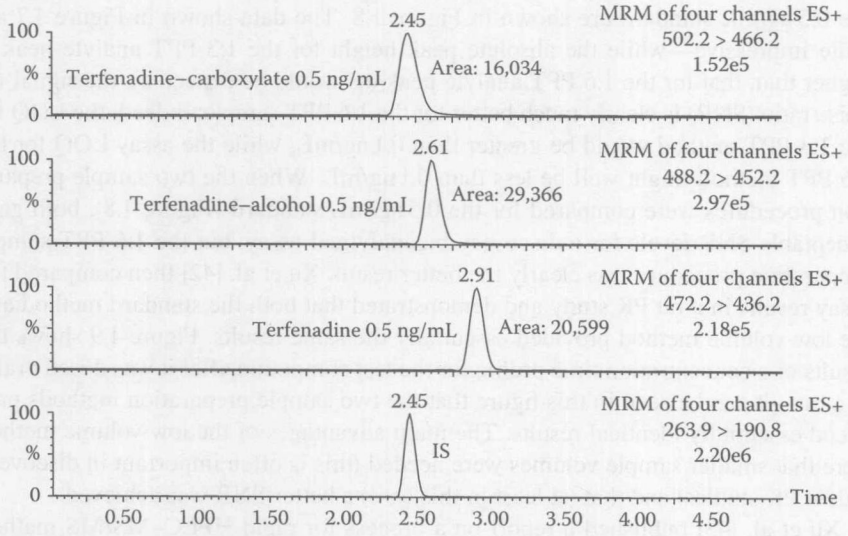

FIGURE 1.5 HPLC–MS/MS assay result for terfenadine and metabolites using a mixed-mode extraction protocol. (Reprinted from Mallet, C.R. et al., *Rapid Commun. Mass Spectrom.*, 17, 163, 2003. With permission.)