PART I
Basic Concepts of Drug Metabolism and Disposition

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1 Progression of Drug Metabolism

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1.1 INTRODUCTION

In a certain sense, the field of drug metabolism (DM) is standing still. More specifically, the basic experiment of drug metabolism (i.e., administering a new drug to an animal or human and determining the structures, amounts, and disposition of the metabolites) has changed very little over a period of decades. Remarkably, the experimental design and resulting data set from a typical absorption, distribution, metabolism, and excretion (ADME) study conducted today would be instantly recognized and understood by DM scientists from 50 years ago. This is not the case with most other disciplines in the life sciences.

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For instance, 20 years ago protein sequencing was based on peptide chemistry, and the basic experiment was the assay of amino acids released from tryptic peptides, a process that required months or years to complete. Now, protein sequencing is based on nucleotide chemistry, and the basic experiment is the automated assay of oligonucleotides from partial hydrolysis of complementary deoxyribonucleic acid (cDNA) a process that takes about a day. The reason that ADME experiments have not evolved much is that we have never devised a surrogate for the whole human body for metabolism studies. All systems tried up to this point (e.g., animals, transgenic animals, perfused organs, in vitro incubations, three-dimensional (3D) microfluidic cell culture devices, in silico calculations) fail to reliably predict the actual metabolic fate of NCEs. To be sure, the technology we use for the ADME experiment has advanced greatly, but even with the newest methods, DM is still essentially a chemical exercise at its core, and the backbone technology remains mass spectrometry (MS).

However, if we consider the more complete picture, DM has expanded enormously in scope and level of understanding in those 50 years. While the chemistry-based core remains intact and actively growing, several other kinds of DM studies have layered over the core, all coexisting and relevant in contemporary DM science. Thus, in addition to the purely chemical description of the structures of metabolites and probable chemical mechanisms of their formation, we now have a very good biochemical understanding of the various enzymes that catalyze these biotransformation processes, as well as a cellular and genetic understanding of the expression and regulation of those enzymes. We are even making progress toward reliable prediction of the fates of xenobiotic substances in human beings (Anderson et al., 2009), although this goal remains out of reach for the present. The current level of understanding of DM is presented by several experts in Part I of this book.

1.2 HISTORICAL PHASES OF DRUG METABOLISM

Near the end of the twentieth century, I suggested that there had been four overlapping phases of DM in industrial drug discovery and development (White, 1998). These can be summarized as follows.

1.2.1 The "Chemistry" Phase (1950–1980)

During this period, only a descriptive account of the disposition of a new chemical entity (NCE) was provided, largely consisting of chemical information. Major urinary and fecal metabolites were isolated and identified by classic chemical techniques including column and thin-layer chromatography, crystallization, and derivatization. Eventually, spectroscopy was used for the structural elucidation, including mass spectroscopy, infrared, and nuclear magnetic resonance (NMR). These techniques required much smaller quantities to be isolated and allowed high-performance liquid chromatography (HPLC) to replace

column chromatography. Interestingly, early in this period R.T. Williams published his monograph *Detoxication Mechanisms*, which can be considered the first identification of DM as a discrete field of study (Murphy, 2008). The publication of that book also showed that academic researchers were beginning to think about the biological basis and implications of DM, although this way of thinking took some time to make an impact in the industrial world.

1.2.2 The "Biochemistry" Phase (1975–Present)

Starting in the mid-1970s, we began to determine the underlying biochemical processes responsible for the disposition of xenobiotics (e.g., which enzymes were involved). Illustrating the indistinct separation of these phases, the pioneers in this phase often came from a chemical background, and they sought to describe the enzymes in chemical terms. The proteins were isolated so that they could be treated as discreet chemical reagents, describable in classical chemical terms of composition, reaction stoichiometry, thermodynamics, and reaction mechanism. However, after about a decade or so, the chemical approach to studying the enzymes transitioned to a biochemical and cell biology approach in which enzyme kinetics and protein–protein and membrane–protein interactions became the "hottest" topics. All of the important DM enzymes were characterized, named, and even made commercially available to industrial researchers. The latest advance in the biochemistry phase was the realization that even the *exposure* of drugs to the drug-metabolizing enzymes was a biochemical event, mediated by physical enzymes called *transporters* (Wu and Benet, 2005). Advances in our understanding of the biochemistry of DM in academic laboratories were reflected in a greater expectation by regulatory agencies that a biochemical description be provided in addition to the purely chemical description of DM of an NCE.

1.2.3 The "Genetics" Phase (1990–Present)

In this phase, we began to account for individual variations in the pharmacokinetic rates and molecular sites of metabolism by genotyping human test subjects with respect to an ever-growing list of genetically polymorphic drug-metabolizing enzymes. Equally important, regulation of DM enzymes was recognized to occur mainly at the gene expression level, whether resulting from heredity, disease processes, or environment. This pharmacogenetic characterization has become a routine expectation for the registration packages of NCEs and continues to expand. As before, the new genetic information did not replace any previous requirements for DM information but instead added an additional dimension to that information package.

1.2.4 The "Biology" Phase (2010 and Beyond)

We are beginning to view drug metabolism in terms of systems biology. This involves taking a holistic view of the simultaneous interaction of a xenobiotic

molecule with all the enzymes and receptors in the human body. Some of these receptors are the pharmacological targets that lead to therapeutic benefits, some are unintended targets that generate adverse events and toxicities, and some are the enzymes and nuclear receptors of DM. In our overall description of the disposition of the compound, interactions of the compound with these DM targets are especially complex to relate to safety and efficacy. When designing a practical clinical medicine, we need to establish a balance between too rapid metabolism, leading to reduced efficacy, and too sluggish metabolism, leading to accumulation and possible toxicity. In the clinic, we need to determine whether metabolism decreases or increases the desired pharmacological effect (i.e., active metabolites). And, finally, in this holistic biological view, we need to assess how the metabolites interact with all the off-target human enzymes and receptors, especially the phenomenon of reactive metabolites covalently binding to proteins and nucleic acids, leading to toxic sequelae (Baillie, 2009).

These four phases are graphically depicted in Figure 1.1. They are layered in the figure because we continue to do all the activities of each preceding phase as we proceed through the evolution of industrial DM. Thus, the total amount of DM characterization work for a new drug has increased dramatically over the

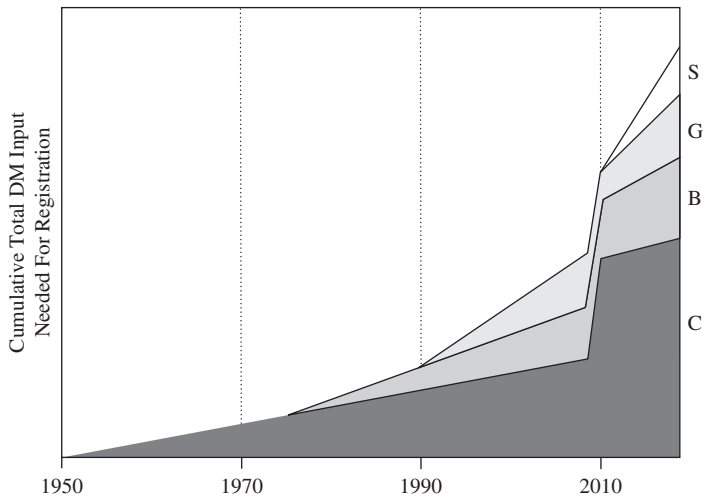

FIGURE 1.1 Progression of amounts of DM information required for regulatory filing of a new chemical entity. The vertical axis is the total amount of DM information in the registration application on a relative scale. The information classes are segregated as discussed in the text. The chemistry component (C, black) continually increases with time but abruptly increases about 2010 due to enhanced regulatory surveillance of metabolites. Starting around 1975, biochemistry (B, dark gray) begins to be included in the DM characterization and slowly increases with time. Genetics information (G, light gray) continues to increase, mainly in clinical trials. The apparent jump in B and G work around 2010 is due to the abrupt increase in C. Actual B and G work would not increase. Systems biology (S, white) is nearly zero in 2010 but is expected to increase subsequently.

years. The meaning of the step increase in work at around 2010 in the figure will be discussed in the next section.

1.3 NEXT STEP IN THE PROGRESSION OF DM

The first three of these historical phases of industrial DM serve to summarize and rationalize the scientific questions of yesteryear and today. The questions of tomorrow are described by the biology phase. However, now we can also discern the beginning of an additional new trend that could be called the "regulatory" phase. This phase is not primarily concerned with the physiological *process* of DM, as are the other phases. Instead, the regulatory phase is concerned with the human *safety* of the metabolites, once they are formed. But even though the focus of this phase is safety, it may well produce the greatest increment of additional DM work to be done in the future.

1.3.1 New Regulatory Expectation

Regulatory interest in metabolites has developed into a formal Guidance for Industry (Safety Testing of Drug Metabolites) issued by the U.S. Food and Drug Administration (FDA), which instructs sponsors on the qualitative and quantitative characterization of metabolites in both clinical and preclinical toxicological settings (U.S. FDA, 2008). A similar concern about metabolite safety is expressed in a Guidance from the International Conference on Harmonisation (ICH), currently in draft stage (ICH, 2009). We may succinctly state the requirement as follows: Human circulating metabolites that exceed 10% of the total exposure of all drug-related materials in circulation at pharmacokinetic steady state require safety assessment before large-scale clinical trials can proceed. These Guidances have implications for bioanalysis and safety assessment, but here we wish to focus on the implications for biotransformation studies. Development of these Guidances, though initiated by an industry-sponsored group (Baillie et al., 2002), has resulted in an inevitable regulatory emphasis on metabolite characterization much earlier than traditionally carried out. Importantly, the relative levels of parent drug and metabolites at pharmacokinetic steady state have been little studied previously. Consequently, we can expect surprises, possibly even new phenomena, as we watch the time evolution of drug metabolism during the approach to steady state. The traditional approach to definitive metabolite characterization (i.e., single-dose ^{14}C-labeled clinical ADME studies performed during Phase II or III) is inadequate for the new regulatory demands and either a new approach to ^{14}C studies or new nonradiolabel-based methodology is required.

1.3.2 New Challenges for Technology

Distressingly, both the qualitative and quantitative requirements of the new paradigm potentially push the existing technology past present limits.

Qualitatively, technology is now needed for metabolite detection in early clinical development. Up to this time, MS was only required to elucidate the chemical structures of metabolites, augmented as necessary by NMR and synthesis of authentic standards. However, *detection* of the presence of metabolites in a sample relied on the observation of nonparent radioactive peaks in the chromatogram. Now we are asking the mass spectroscopist to also detect the metabolites, without the benefit of radiolabel or prior knowledge of the structures. This requirement for MS-based detection as well as characterization has necessitated the development and validation of new algorithms of data acquisition and processing. In fact, as will be seen in later chapters, reliable nonradiometric MS-based methods of metabolite detection are now a reality.

Quantitatively, technology limits are pushed in two ways. First, the mass spectroscopist is asked to estimate the concentration of each metabolite in a plasma profile and categorize it as more or less than 10% of the total. This is problematic because the structures may not be completely known and authentic standards may not be available. Second, for those metabolites at levels more than 10%, a validated assay will be required, pushing the sensitivity limits that may be required in some cases. As the typical drug candidate becomes ever more potent, with ever smaller administered doses, the accurate estimation of analyte exposures that are as much as an order of magnitude *less* than that of the parent could become a challenge. Experts will discuss technological advances in MS related to the problems of detection and estimation of amounts of metabolites in Part II of this book and the experimental application of this technology to these problems in Part III. However, we can put some perspective here on the magnitude of the challenge.

1.4 PERSPECTIVE ON THE MAGNITUDE OF THE CHALLENGE

1.4.1 Ultimate Limits on Metabolite Quantitation

Let us start by considering whether there is any amount of metabolite that is too little to be meaningful. Since the present regulatory criterion for the amount of metabolite requiring assay is expressed as a *percentage* of a variable quantity (dose), then as new, more potent drugs are introduced, there is no regulatory lower limit on the absolute quantity of a metabolite that might need to be detected and assayed. So a literal reading of the Guidances is that no amount of metabolite is too little to be of regulatory interest. This approach to metabolite safety assessment has been questioned on the basis of the likelihood of metabolite-driven toxicity from minute body burdens (Smith and Obach, 2009), and it seems unlikely that regulatory authorities would apply the 10% rule to very low dose drugs (ICH, 2009). However, for this examination of limits, let us assume the worst case, that the 10% rule was always applied. Is there any other limitation of how much metabolite might need to be detected and quantitated? Actually, a moment's practical consideration of the question

of lower limit reminds us that there is a fundamental limit imposed by nature (i.e., one molecule). Obviously, in this hypothetical limiting case, one molecule in a whole human body could only be assayed by exsanguinating the subject, so the stipulation that *the subject must survive the assay* clearly requires many more molecules than one in the body for detectability. To estimate *how* many more, in the next section we will use the "one-molecule" concept in the opposite direction (i.e., from the detector's point of view).

1.4.2 Practical Limits on Metabolite Quantitation

At very low concentrations, the discrete nature of molecules means that a detector signal no longer appears to be continuous. At the ultimate limit, either zero or one molecule will enter the inlet of the mass spectrometer; one cannot analyze half a molecule. Thus, one molecule entering the mass spectrometer inlet during a single duty cycle is the natural ultimate limit of sensitivity. Working backward from this limit and assuming that about 5% of the injected mass is actually sampled in a single duty cycle, we see that at least 20 molecules would have to be injected, on average, to get one into the inlet during a single duty cycle. Given a 10-μL injection from a 100-μL reconstituted extract of a 100-μL plasma aliquot of an original 1-mL plasma sample, we can estimate that plasma from a human subject would have to contain at least 2000 molecules per milliliter to be measurable in a practical sense by current liquid chromatography (LC)/MS laboratory methods. Although one could propose taking a larger plasma sample from a subject, reconstituting the extract in a smaller volume, and injecting a larger fraction onto the instrument, there are natural limitations for these volumes as well. For instance, a full pharmacokinetic profile for a subject that required 10 mL of plasma (i.e., about 20 mL of blood) for each of 10 time points would surely be close to the limit that physicians would ever accept under any circumstances, and even larger samples would clearly be out of the question. Thus, for this thought experiment, let us accept that a realistically and routinely measurable concentration could not be much less than 1000 molecules per milliliter of plasma based on the natural limit of one molecule interacting with a mass spectral detector. Application of Avogadro's number to this ensemble of 1000 molecules allows us to state that plasma concentrations less than about 1 attomolar (1 aM, 10^{-18} molar) will never be accessible in a practical sense.

1.4.3 Natural Limit Due to Dose Size

Now let us translate the natural-limit concept back to the real-world quantity of dose. If a midrange volume of distribution of 50 L is assumed for a typical drug, then a meaningful detection limit of 1 aM for a metabolite implies a concentration of 10 aM for the parent drug (i.e., metabolite is 10% of parent), which is equivalent to a total dose of 500 attomol. If the drug has a molecular weight (MW) of 400, then, the dose would be 0.2 pg. For comparison, what is

the lowest dose of drug for which we are ever likely to be expected to characterize circulating metabolites? The most potent substances administered therapeutically are hormones, and their doses are exceedingly low. For instance, the labor-inducing hormone oxytocin is effective at a vanishingly low dose of about 40 ng, while the calcitriol dose is only about 4 μg. These examples may represent the lowest human doses that will ever be used for any drug. Amazingly, some actual drugs already approach these dose levels. For instance, the inhaled β-agonist formoterol is given at 12 μg, and the inhaled glucocorticoid beclomethasone is delivered at 40 μg. Circulating levels of these two drugs and their metabolites are almost unmeasurable (C_{max} in each case is ca. 10 pg/mL). The most potent oral drugs are also in the same range, with the dose of clonidine being about 100 μg. If, as suggested above, future drug candidates will not be more potent than the most potent drugs in use today, then we can say that levels of metabolites requiring detection and characterization will likely never be less than about 1 pg/mL. This is good news because, although they are not routine, MS-based assays with picogram/milliliter sensitivity are already in use. Thus, we can see that there is a natural limit to the ultimate sensitivity required to detect and characterize circulating metabolites, and it is not far from what our current MS technology already allows us to do. Most new drugs in development are dosed in the 1 to 1000-mg range, and metabolite levels for these drugs are well within contemporary MS sensitivity.

1.5 ARE THERE MORE SENSITIVE ALTERNATIVES TO MS?

An interesting extension of the concept of natural limitation is to remove the assumption that one molecule gives one quantum of signal (i.e., destructive analysis). Spectroscopic methods such as NMR and fluorescence can time integrate numerous signal pulses from a single molecule, resulting in no theoretical limit to how much signal could be accumulated with unlimited acquisition time. In fact, quantitative NMR spectroscopy has been proposed as a readily available solution to the main problems of implementing the Guidances, namely recognition and quantitation of metabolites without radiolabel at steady state (Espina et al., 2009; Vishwanathan et al., 2009). However, both NMR and fluorescence still face the indivisibility of individual molecules when applied to ex vivo samples such as plasma, so that a practical lower limit of concentration would still exist. Moreover, given the insensitivity of NMR relative to MS, it is unlikely that NMR could ever access concentrations that MS could not, even with the advantage of signal accumulation. Conversely, fluorescence might conceivably achieve the requisite sensitivity, but unlike MS and NMR, fluorescence is not generally applicable to all drug candidates. Clearly, then, the only currently available technology for low-level metabolite characterization is MS, which combines sensitivity, structural information, and general applicability.

1.6 SUMMARY

In summary, DM is a traditional yet dynamic discipline, comprising a constant core overlaid with evolving successive layers of related activities. The core activity of detecting and determining the chemical structure of human metabolites has changed little in decades and, in fact, is the starting point for all the other activities in areas such as enzymology, regulation, and genetics. For example, it is meaningless to inquire which cytochrome P450 (CYP) enzyme is principally responsible for the metabolism of a new drug until the chemical structure of the major metabolite shows that it was likely formed by a CYP enzyme. Today, structural elucidation of a metabolite almost always begins with MS, followed by complementary methods such as NMR, as necessary. Conversely, *detection* of metabolites has traditionally been accomplished by radiochromatography. However, in response to evolving regulatory expectations, it is likely that detection will become the job of MS also. Thus, MS is the most important single technique in DM and is likely to remain so going forward into the future. This conclusion explains the need for continued advancement of DM applications of MS technology, as described in the remainder of this book.

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