1 Bioanalytical Support for Both *In Vitro* and *In Vivo* Assays Across Drug Discovery and Drug Development

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1.1 SUMMARY

Bioanalysis (BA) in a drug metabolism environment can be defined as the analytical techniques that are utilized to assay various samples that are the result of *in vitro* or *in vivo* studies of one or more new chemical entities (NCEs). BA has continued to evolve in response to the needs of the drug discovery and drug development teams as well as the continuing improvement of the analytical technology that is used for the various assays. This chapter provides an overview of the various assays that are commonly used in various stages of drug discovery and drug development. The chapter is divided into three main sections: bioanalytical strategies, *in vitro* assays, and *in vivo* assays.

For most of the assays discussed in this chapter, the analytical tool is high performance liquid chromatography (HPLC) combined with tandem mass spectrometry (MS/MS). HPLC-MS/MS is preferred because it provides excellent selectivity and is applicable to most small molecules. The strategy for how best to use HPLC-MS/MS for the different *in vitro* and *in vivo* assays is still a topic of debate within the bioanalytical community.

It is common to have different levels of analytical acceptance criteria that become stricter as one moves from the early lead optimization arena and moves into the development arena. Using this strategy, the higher throughput assays that are used in the

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screening stage would have minimal standards needed as part of the assay. This makes sense because these assays usually are used for many compounds with only a small number of samples per compound. As one goes to the higher levels, relatively smaller numbers of compounds are being assayed and the number of samples per compound is higher. So, for the higher level assays, it makes sense to increase the number of standards that are required to perform the assay.

There are multiple *in vitro* assays that are used for drug discovery screening of NCEs. The basic strategy for utilizing these various *in vitro* assays is to use them to screen NCEs in order to select those that have druglike properties. These tests are commonly used as part of the absorption, distribution, metabolism, and excretion (ADME) optimization phase of new drug discovery. The most commonly used assays are designed to measure one of the following parameters: permeability, metabolic stability, enzyme inhibition, or metabolism. Each of these assay types is described in this chapter as a subtopic.

In a drug discovery setting, *in vivo* assays are similar to *in vitro* assays in terms of the need for providing the results in a timely manner. The main difference between *in vitro* and *in vivo* assays is that *in vivo* assays are often more challenging because the sample matrix is more complex. Most in *vivo assays* rely on some form of HPLC-MS/MS for the analytical step. The main topics covered in this chapter are: pharmacokinetic (PK) screening, PK studies, and metabolite identification.

In conclusion, mass spectrometry is used throughout new drug discovery and drug development for multiple assays. The type of mass spectrometer that is used depends on the type of assay that is being performed. As mass spectrometers evolve and are improved, it can be safely predicted that they will continue to be the premier analytical tool for many of the assays that are performed at various steps in this process.

1.2 INTRODUCTION

BA in a drug metabolism environment can be defined as the analytical techniques that are utilized to assay various samples that are the result of *in vitro* or *in vivo* studies of one or more NCEs. BA has continued to evolve in response to the needs of the drug discovery and drug development teams as well as the continuing improvement of the analytical technology that is used for the various assays. As shown in Fig. 1.1, BA can be considered the cornerstone for the whole drug discovery process. Multiple review articles and book chapters have been published on the topic of BA used in a drug metabolism environment, and the reader may want to consult these in order to gain additional perspectives on this topic [1–14]. For a comprehensive review of all of these assays, one could also consult the book by Kerns and Di [15].

As shown in Fig. 1.1, lead optimization is a critical step in the drug discovery process. One of the goals of the lead optimization step is to improve the drug metabolism and pharmacokinetic (DMPK) properties of the initial lead compounds in order to find new compounds that are active in the pharmacological model and have acceptable DMPK properties. In order to improve DMPK properties, hundreds of NCEs are tested in various *in vitro* assays. The goal of these studies is to find compounds that can be predicted to have human DMPK properties that are acceptable for the proposed use of the compound [14–21].

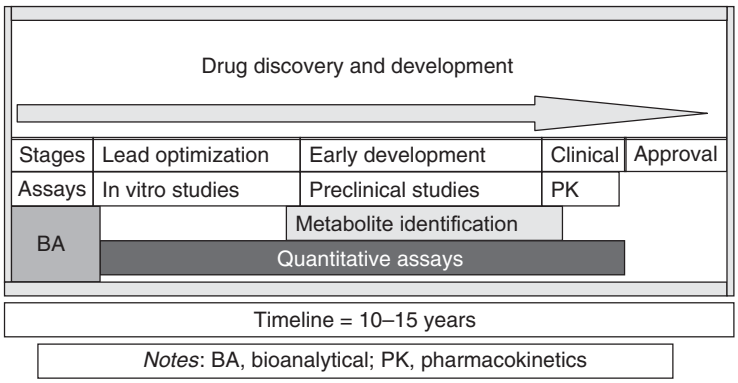

Figure 1.1 Schematic showing the various stages of new drug discovery and development as well as how bioanalysis (BA) is the cornerstone to the whole process.

This chapter provides an overview of the various assays that are commonly used in various stages of drug discovery and drug development. The chapter is divided into three main sections: bioanalytical strategies, *in vitro* assays, and *in vivo* assays. The chapter concludes with a summary that serves as a condensed version of the topic.

1.3 BIOANALYTICAL STRATEGIES

For most of the assays discussed in this chapter, the analytical tool is HPLC combined with MS/MS. HPLC-MS/MS is preferred because it provides excellent selectivity and is applicable to most small molecules [5,6,13,14,22–25]. The strategy for how best to use HPLC-MS/MS for the different *in vitro* and *in vivo* assays is still a topic of debate within the bioanalytical community.

It is common to have different levels of analytical acceptance criteria that become stricter as one moves from the early lead optimization arena and moves into the development arena. Table 1.1 shows how this type of strategy might be utilized.

Using this strategy, the higher throughput assays that are used in the screening stage would have minimal standards needed as part of the assay. This makes sense because these assays usually are used for many compounds with only a small number of samples per compound. As one goes to the higher levels, relatively smaller numbers of compounds are being assayed and the number of samples per compound is higher. So, for the higher level assays, it makes sense to increase the number of standards that are required to perform the assay.

1.4 *IN VITRO* ASSAYS

There are multiple *in vitro* assays that are used for drug discovery screening of NCEs [7,21,26–29]. The basic strategy for utilizing these various *in vitro* assays is to use them to screen NCEs in order to select those that have druglike properties [21,30–34]. These tests are commonly used as part of the ADME optimization phase of new drug discovery [14,35–37]. The most commonly used assays are designed to measure one

TABLE 1.1 Bioanalytical Strategies for Various Stages of Drug Discovery

Level	Drug Stage	Major Analytical Criteria	Example Assay	GLP[a]?
0	Early screening	No standard curve	Caco-2 cell	No
1	PK[b] screening	Two-point standard curve	CARRS	No
2	Lead optimization	Multipoint standard curve (no QCs[c])	Rat PK study	No
3	Lead qualification	Multipoint standard curve plus QCs	Multidose rat PK study	No
4	Development	Follow GLP rules	TK[d] studies	Yes

[a]Good laboratory practices.
[b]Pharmacokinetic.
[c]Quality control.
[d]Toxicokinetic.

of the following parameters: permeability, metabolic stability, enzyme inhibition, or metabolism. Each of these assay types is described in this section as a subtopic.

1.4.1 Permeability

The goal of the various permeability assays is to measure the ability of a compound to cross a cell membrane. This is an important characteristic of a druglike molecule that could be developed into an orally dosed drug. In order for an orally dosed drug to be effective, it must be absorbed across the intestinal gut membrane. The various permeability assays strive to measure the potential for a compound to be absorbed.

The first cell-based assay for permeability measurement is the Caco-2 assay [38]. The Caco-2 cell line is an immortal human colon carcinoma cell line that was commercialized for this assay. It is available in multiple formats, but each of these shares the same functional design. The experimental setup includes a chamber with two sides (apical and basolateral) that contain liquid and are separated by the cell membrane. The test compound can be added to either side. Once the experiment is completed, a sample of the liquid from each side is taken and assayed for the test compound.

The assay is typically performed using HPLC-MS/MS. Various HPLC-MS/MS procedures have been described in the literature [39–48]. Because the goal of the assay is to measure the ratio of the test compound concentrations of the two compartments, in some cases the HPLC-MS/MS analysis merely provides a response level for each compartment; the ratio of the response levels is equal to the ratio of the compound concentrations of the two compartments [49].

In addition to being used as a drug discovery screen, the Caco-2 assay has also been validated as an *in vitro* assay for development compounds to assess their potential to act as either substrates or inhibitors of P-glycoprotein (P-gp) [50]. When used in this manner, compounds are subjected to "bidirectional" Caco-2 assays, in which the test compound is added to the apical side and basolateral side in separate experiments. This test measures the efflux ratio for the compound to determine if it is a substrate for

P-gp. Additional measurements can then be made with known model compounds to determine if the test compound is an inhibitor of P-gp. These results are then suitable to be included in regulatory submission documents.

Another permeability assay is the parallel artificial membrane permeability assay (PAMPA). The advantage of PAMPA is that the artificial membrane is simple to maintain and the assay can be performed using either HPLC-ultraviolet (UV) detection or even a UV plate reader [15]. The main disadvantage of PAMPA is that it measures only passive diffusion (there is no P-gp component as there is with Caco-2). Some drug discovery practitioners have suggested using PAMPA as an initial screen for permeability and waiting until the end of the lead optimization stage to use the Caco-2 cell assay [51].

1.4.2 Metabolic Stability

The goal for the metabolic stability assay is to measure the metabolic liabilities of a test compound. The two most common metabolic stability assays are based on either liver microsomes or hepatocytes. The advantage of using liver microsomes is that they are relatively inexpensive. The disadvantage of liver microsomes is that they only demonstrate phase I (CYP-mediated) metabolites unless additional cofactors are added to allow for phase II (conjugated) metabolism. The disadvantage of hepatocytes is that they are relatively expensive. The advantage of hepatocytes is that they are able to produce both phases I and II metabolites. In some cases, liver microsomes are used as an early screen for metabolic stability and hepatocytes are reserved for those compounds that are potential candidates for development.

Regardless of the microsomal stability process that is chosen, the bioanalytical challenge is the same. For a higher throughput metabolic stability assay, one might need to develop HPLC-MS/MS assays for 100–200 NCEs per week. Typically, this requires automated MS/MS method development software tools in order to meet this analytical challenge. Currently, most vendors for triple quadrupole MS/MS systems will include this type of software in their system. Kieltyka *et al*. [52] have described the utility of QuickQuan™ (Thermo) for the automated MS/MS method development of 500 compounds per month in order to use HPLC-MS/MS to assay these NCEs for metabolic stability. In their application, the compounds were tested in human, rat, and mouse microsomes for metabolic stability. They reported a success rate of >95% when using the automated method development procedure on more than 25,000 compounds.

1.4.3 Enzyme Inhibition

The goal of enzyme inhibition assays is to estimate the potential for a compound to produce drug–drug interactions due to enzyme inhibition in a clinical setting. The assays used for this screen are based on testing the effect of the NCE on cytochrome P450 (CYP) activity by monitoring various well-known CYP substrates. There are a small number of CYP isozymes that account for most of the CYP activity. For example, 50% of all drugs are metabolized by CYP3A4 and 30% of all drugs are metabolized by CYP2D6 [15]. If an NCE was found to significantly inhibit CYP3A4 or CYP2D6 at concentrations in the 1–10 μM range that could be a potential development issue for that NCE.

There are two common strategies for screening NCEs for their enzyme inhibition potential. The first strategy uses recombinant CYP isozymes and fluorogenic probe substrates. The assay readout is based on a fluorescence plate reader. The advantage of this assay is that it is high throughput and relatively low cost. The second strategy (considered the "gold standard" assay) is based on human liver microsomes (HLMs) and various probe substrates. The assay for this method is normally based on HPLC-MS/MS. Bell *et al*. [53] compared these two strategies and found that for NCEs, the fluorescence-based assay often produced results that did not correlate well with the HPLC-MS/MS-based assay.

Multiple authors have described procedures for assaying enzyme inhibition potential using HLMs with various probe substrates and an HPLC-MS/MS assay [54–62]. The main differences in these assays are: (i) how many probe substrates are measured and (ii) what methodology was used for the HPLC-MS/MS assay. Chu *et al*. [59] described a 0.5 min HPLC-MS/MS assay that was able to provide enzyme inhibition potential results for CYP3A4 and CYP2D6. Plumb *et al*. [60] made use of ultra-performance liquid chromatography (UPLC) combined with MS/MS to assay six probe substrates in 0.5 min.

1.4.4 Protein Binding

Protein binding is another *in vitro* assay that relies on HPLC-MS/MS for the analytical step. The goal of the assay is to measure the free (unbound) concentration of an NCE when it is in human or laboratory animal plasma. Various methods for performing the protein binding experiment have been described [15,63,64]. The typical current strategy for screening NCEs for protein binding is based on 96-well equilibrium dialysis [63,65]. Owing to the fact that many compounds are >99.5% bound, it is important to be able to measure the low concentration of the test compound in the free fraction in these studies. Owing to the low concentrations of some of these samples, it is important to use HPLC-MS/MS for the analytical step. Wan and Rehngren [66] reported the use of a sample pooling approach for higher throughput screening of protein binding in a discovery setting: their assay was based on HPLC-MS/MS.

1.4.5 *In Vitro* Metabolite Identification

In vitro metabolism studies are a common part of the lead optimization strategy. In a drug discovery setting, the ability to provide metabolite information to the discovery teams is very helpful to the medicinal chemists. For example, knowing the site of oxidation of a compound that has a short *in vivo* half-life can lead to the synthesis of a new compound where that site is modified in a way that blocks the metabolic site; in some cases, this new compound may have a longer *in vivo* half-life. In another example, the metabolite may be active and have a better *in vivo* PK profile—in this example, the metabolite could become the new lead compound in the discovery program.

Typically, in order to perform an *in vitro* metabolism study, one will incubate the NCE in either liver microsomes or hepatocytes which will produce a sample that contains metabolites of the NCE. Microsomes provide a good picture of phase I metabolites (CYP-mediated reactions) while hepatocytes produce both phases I and II (conjugated) metabolites [25]. Multiple literature reviews and book chapters have been written on methods for performing metabolism studies in various matrices [9,25,27,67–75]. These

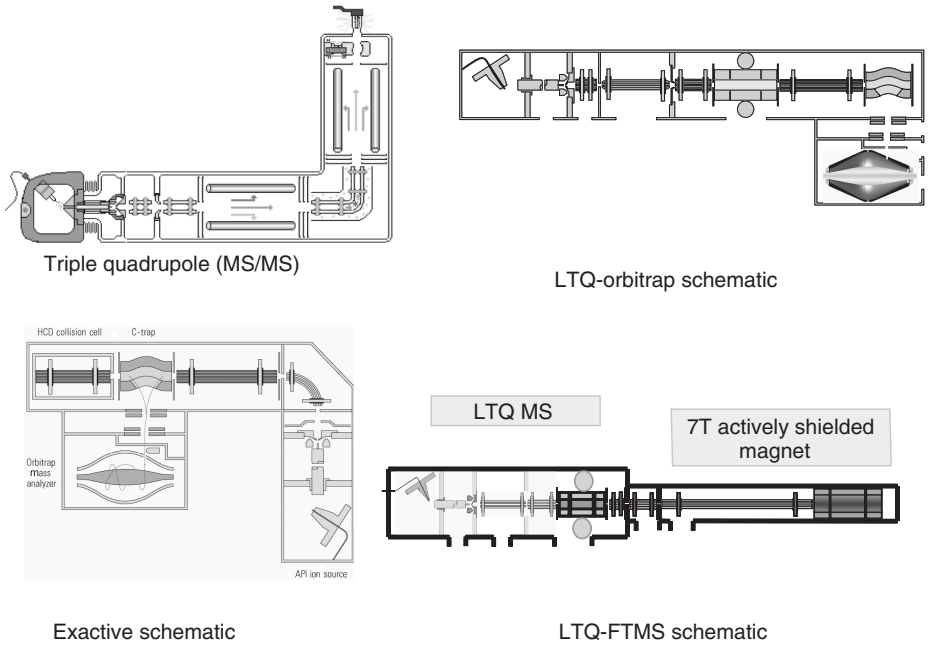

Figure 1.2 Schematic showing the multiple types of mass spectrometers that can be used for various BA assays in new drug discovery and development. *Source*: Figure provided by Jerry Pappas from Thermo-Fisher and used with permission. (See color insert.)

methods all rely on some form of HPLC-MS/MS analysis to find the metabolites and then to further characterize them in order to provide at least a partial structural identification.

There are multiple types of mass spectrometers that have been used for various BA assays. Figure 1.2 shows four example MS systems that have been used by various drug metabolism researchers. While various strategies have been used in the past to find the metabolites in the *in vitro* sample, currently, most researchers would use higher mass resolution mass spectrometers plus one or more software filtering tools to simplify the task of determining what metabolites are present in the sample. Most triple quadrupole mass spectrometers are unit mass resolution (not high mass resolution). Unit mass resolution is sufficient to separate a compound that has a mass of 520.4 from a second compound that has a mass of 521.4. A high mass resolution mass spectrometer can separate a compound that has a mass of 521.45 from a second compound that has a mass of 521.46. This capability (high mass resolution) is very helpful in finding metabolites in a complex matrix because most of the endogenous material will not have the same exact mass as the metabolites. Examples of mass spectrometers that have high mass resolution capabilities are quadrupole-time-of-flight (QTOF) MS systems as well as Orbitrap MS systems [76].

In addition, because many of the metabolites will have predictable masses (e.g., +16.0 for the addition of an oxygen atom to the NCE), one can use specialized software tools in conjunction with the high mass resolution mass spectrometers to look for metabolites. An example of this type of software is the "mass defect filter"

software described by Zhu *et al*. [75,77]. This filter takes advantage of the fact that most druglike chemicals have an exact mass that is different from most endogenous materials (even though the nominal mass may be the same).

1.5 *IN VIVO* ASSAYS

In a drug discovery setting, *in vivo* assays are similar to *in vitro* assays in terms of the need for providing the results in a timely manner. The main difference between *in vitro* and *in vivo* assays is that *in vivo* assays are often more challenging because the sample matrix is more complex. Most *in vivo* assays rely on some form of HPLC-MS/MS for the analytical step [5,6,22–24,78].

1.5.1 PK Screening

PK screening refers to higher throughput *in vivo* studies that are designed to differentiate multiple compounds in the lead optimization phase of new drug discovery. Typically, groups of compounds will be tested in a standardized manner in order to rank order them or screen out compounds quickly. Various researchers have proposed different types of PK screening assays; most of these assays are based on a rodent model.

One of the more popular assays for PK screening was based on the concept of using cassette dosing [79–84]. Cassette dosing makes use of the ability of HPLC-MS/MS to analyze multiple compounds in one assay. This ability allowed the researchers to dose multiple compounds into one laboratory animal and then obtain individual concentrations for each compound over a series of time points such that PK parameters such as area under the curve (AUC) or maximum concentration ($C_{\max}$) could be measured for each compound. Cassette dosing has the advantage that it requires fewer laboratory animals than would be needed for the standard individual dosing model. Several pharmaceutical companies found cassette dosing to be useful for screening NCEs. Cassette dosing also has disadvantages. For example, cassette dosing can produce incorrect PK parameters in many cases [85]. Another disadvantage is that the assay takes more effort to develop due to the mixture analysis and the planning has to take into account the possibility that compounds (or their metabolites) could interfere with each other if they are not separated chromatographically and are isobaric.

Korfmacher *et al*. [86] described a methodical PK screening system that was not based on cassette dosing. The method was called *cassette-accelerated rapid rat screen* (CARRS). The described system was based on cassette analysis where groups of six compounds are orally dosed individually into two rats and six time points (0.5, 1, 2, 3, 4, and 6 h) are taken from each rat. The samples are pooled across the two rats, yielding a total of six plasma samples per test compound. The assay used a simple two-point standard curve. The result was that all the samples for one cassette could be placed on one 96-well plate—this allowed for efficiencies in sample preparation and HPLC-MS/MS analysis. Mei *et al*. [87] reported on a retrospective study of the CARRS data and found that it correlated well with standard ("full") PK studies. In addition, Mei *et al*. reported that CARRS provided a screening efficiency of about 50% (i.e., about one-half of the NCEs tested in CARRS were found to provide such low oral AUC levels that they were eliminated from further consideration as a potential-lead

compound). CARRS has been used successfully as a PK screening tool on over 20,000 compounds.

Liu *et al*. [88] described an oral PK screening model based on mice that they called *snapshot PK*. Their model used two mice per compound and provided an oral AUC value in a systematic manner similar to the CARRS model. The snapshot PK was reported to have a screening efficiency of >80%. In addition, because the snapshot PK model was based on mice, less compound was required for dosing the two animals than what would be needed for a similar screen in rats (e.g., CARRS).

1.5.2 PK Studies

PK studies whether in a discovery setting or in a "bridging to development" study are normally carried out in one of two ways [oral (PO) only or intravenous (IV)/PO]. If one is trying to get a full PK picture of a compound in a laboratory animal, then it would be common to dose one set of animals via PO administration and another set (same species) via intravenous IV administration at a similar dose. By comparing the AUC values of the IV and PO dose, one can obtain the important parameter, percentage bioavailability (F), using the following equation:

$$\%F = \frac{(\mathrm{AUC_{PO}} \times \mathrm{Dose_{IV}})}{(\mathrm{AUC_{IV}} \times \mathrm{Dose_{PO}})}$$

where

F = bioavailability
$\mathrm{AUC_{PO}}$ = area under the concentration versus time curve after PO dosing
$\mathrm{Dose_{IV}}$ = dose for the IV administered compound
$\mathrm{AUC_{IV}}$ = area under the concentration versus time curve after IV dosing
$\mathrm{Dose_{PO}}$ = dose for the PO administered compound.

In addition, multiple additional PK parameters can be derived from a single PO/IV PK study [7,15,89]. The second common PK study is a single rising dose (SRD) study. In a PO SRD study, several sets of laboratory animals are dosed with the test compound PO, but the dose is increased with each set. This study can be used to determine if the AUC increases with the increasing dose. A well-behaved (from a PK perspective) compound may show an almost linear relationship between dose and AUC, while many compound will show a nonlinear increase in the AUC versus the dose of the test compound.

There are multiple steps involved in the BA of a PK study. As shown in Fig. 1.3, these steps involve alignment and coordination across various specialties in order to obtain the test compound, dose the compound, collect the samples, and assay the samples. All of this needs to be done in a timely manner when working in a lead optimization environment.

Once a compound is in development, various safety studies are performed using laboratory animals (typically rat and dog or monkey). In these studies, it is common to take blood samples to measure the exposure of the test animals to the test compound—this type of study is referred to as *toxicokinetic (TK) study*. For TK studies, one must always use good laboratory practices (GLPs) when assaying the plasma samples from the study [90–94].

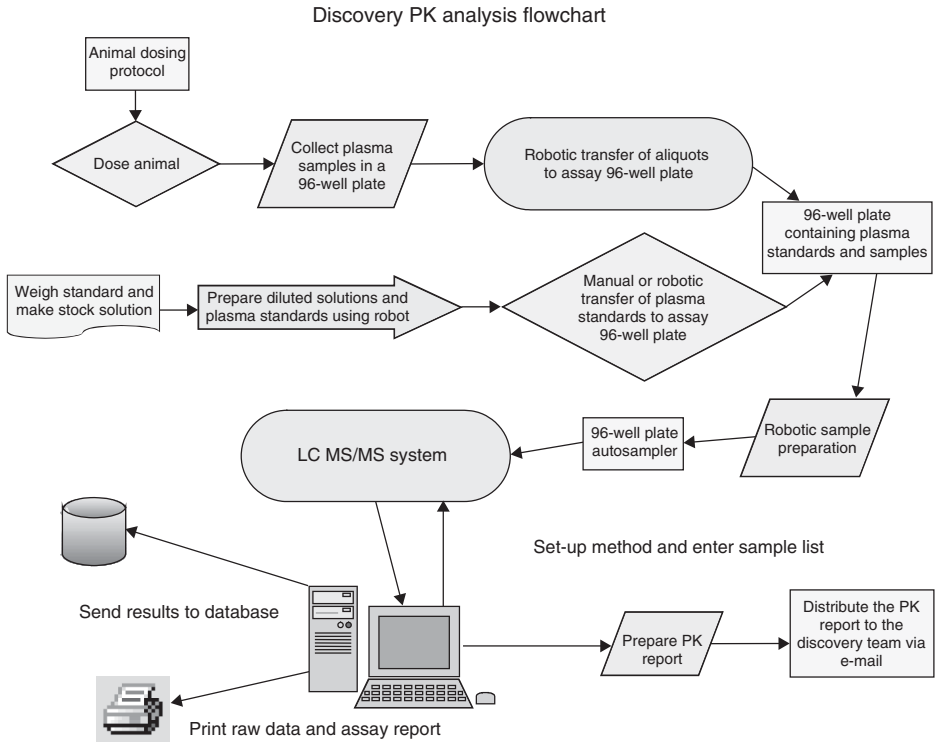

Figure 1.3 Schematic showing the various steps that are taken when assaying compounds in a discovery PK setting. *Source*: Figure was reprinted with permission from Korfmacher [5]. (See color insert.)

1.5.3 *In Vivo* Metabolite Identification

Metabolite identification is an important part of both new drug discovery and drug development [95]. During the new drug discovery stage, metabolite identification can be very helpful in guiding medicinal chemists in two ways: (i) by pointing toward the sites of metabolism on the NCEs, medicinal chemists can modify the lead compounds in ways that could block the metabolism; and (ii) in some cases, if the metabolite is active and has favorable PK properties, it may become the new lead compound in the series.

During the developmental stage, it is important to identify all major human metabolites of the test compound as well as its major metabolites in the rodent and large animal species used in the safety studies [96]. The Metabolites in Safety Testing (MIST) guidelines that were issued by the FDA (US Food and Drug Administration) have added a requirement that metabolite testing must be done using samples from studies where the test compound has reached steady state [97–100]. These requirements have mandated that pharmaceutical companies show that they have similar or higher exposure in animal safety studies than clinical studies for not only the test compound, but also for all major metabolites of the test compound [97–100].

Multiple authors have described various techniques for detecting or profiling metabolites during the new drug discovery stage as well as the development stage [1,3,27,68,69,72,74,101–109]. Most of these methods rely on using either a triple

stage quadrupole (TSQ) MS/MS system or a hybrid quadrupole-linear ion trap (QTrap) for detecting the metabolites and then getting product ion spectra that are needed to provide structural identification of the metabolites [72,110–114]. The QTrap MS/MS system has unique advantages that make it uniquely suitable for both quantitative assays as well as metabolite identification studies [111,115].

Higher mass resolution mass spectrometers have become an important tool for metabolite identification studies. The hybrid QTOF mass spectrometer has been used extensively for this purpose [101,116–118]. One major advantage of the QTOF mass spectrometer is that it can be used to obtain both high mass resolution mass spectra as well as high mass resolution product ion spectra of the metabolite.

If one obtains full scan data in a high mass resolution mode, then one can use special software tools to help differentiate the dosed compound and its metabolites from endogenous background materials. One of the software tools that can be used for this purpose is referred to as *a mass defect filter* [68,75,77,119–121]. The mass defect filter takes advantage of the fact that most pharmaceutical test compounds have an accurate mass that is shifted (less than) the exact nominal mass. This shift can be measured using a high mass resolution mass spectrometer and can be used to differentiate the compound and its metabolites from isobaric (same nominal mass) interferences. The mass defect filter is a software tool that makes this capability easy to use when looking for metabolites of a dosed compound. Zhu *et al*. [108,109] has recently described two additional high mass resolution software filters that can be used to help find metabolites when assaying complex biological matrices.

1.6 CONCLUSIONS

Mass spectrometry is used throughout new drug discovery and drug development for multiple assays. The type of mass spectrometer that is used depends on the type of assay that is being performed. As mass spectrometers evolve and are improved, it can be safely predicted that they will continue to be the premier analytical tool for many of the assays that are performed at various steps in this process.

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