

PART I

OVERVIEW OF LC-MS BIOANALYSIS

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1

ROLES OF LC-MS BIOANALYSIS IN DRUG DISCOVERY, DEVELOPMENT, AND THERAPEUTIC DRUG MONITORING

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1.1 INTRODUCTION

Bioanalysis is a subdiscipline of analytical chemistry for the quantitative measurement of xenobiotics (chemically synthesized or naturally extracted drug candidates and genetically produced biological molecules and their metabolites or post-translationally modified products) and biotics (macromolecules, proteins, DNA, large molecule drugs, metabolites) in biological systems. Many scientific decisions regarding drug development are dependent upon the accurate quantification of drugs and endogenous components in biological samples. Unlike its sister subdisciplines of analytical chemistry such as drug substance and drug product analysis, one very unique feature of contemporary bioanalysis is that its measurement target is always at very low concentration levels, typically at low ng/ml concentration range and even at pg/ml for highly potent medicines. It is this very low concentration, compounded by coexisting endogenous or exogenous compounds with similar chemical structures to the target analytes at a much higher concentration (typically at $\mu\text{g/ml}$ to mg/ml range), that challenges bioanalytical scientists to accurately and definitively measure the analytes of interest.

Since its commercial introduction in the 1980s, liquid chromatography–mass spectrometry (LC-MS), or much more predominantly, tandem mass spectrometry (LC-MS/MS) has rapidly become standard instrumentation in any well-equipped bioanalytical laboratory. LC-MS is a

combination of the physicochemical separation capabilities of liquid chromatography (LC) and the mass (MS or MS/MS) separation/detection capabilities of mass spectrometry. In LC-MS bioanalysis, assay selectivity can be readily achieved by three stages of separation of the analyte(s) of interest from unwanted components in the biological matrix: (1) sample extraction (protein precipitation, liquid–liquid extraction, solid-phase extraction, etc.), (2) column chromatography, and (3) tandem mass spectrometric detection in selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) mode. Nevertheless, many factors, including matrix effect, ion suppression, and in-source breakdown of labile metabolites, can compromise the reliability of a LC-MS bioanalytical assay. These factors should be carefully evaluated during method development.

The focus of LC-MS bioanalysis in the pharmaceutical industry is to provide a quantitative measurement of the active drug and/or its metabolite(s) for the accurate assessment of pharmacokinetics, toxicokinetics, bioequivalence (BE), and exposure–response (pharmacokinetics/pharmacodynamics) relationships (Figure 1.1). The quality of these studies, which are often used to support regulatory filings and other evaluations, is directly related to the conduct of the underlying bioanalysis. Therefore, the application of best practices in bioanalytical method development, validation, and associated sample analysis is key to an effective discovery and development program leading to

4 ROLES OF LC-MS BIOANALYSIS IN DRUG DISCOVERY, DEVELOPMENT, AND THERAPEUTIC DRUG MONITORING

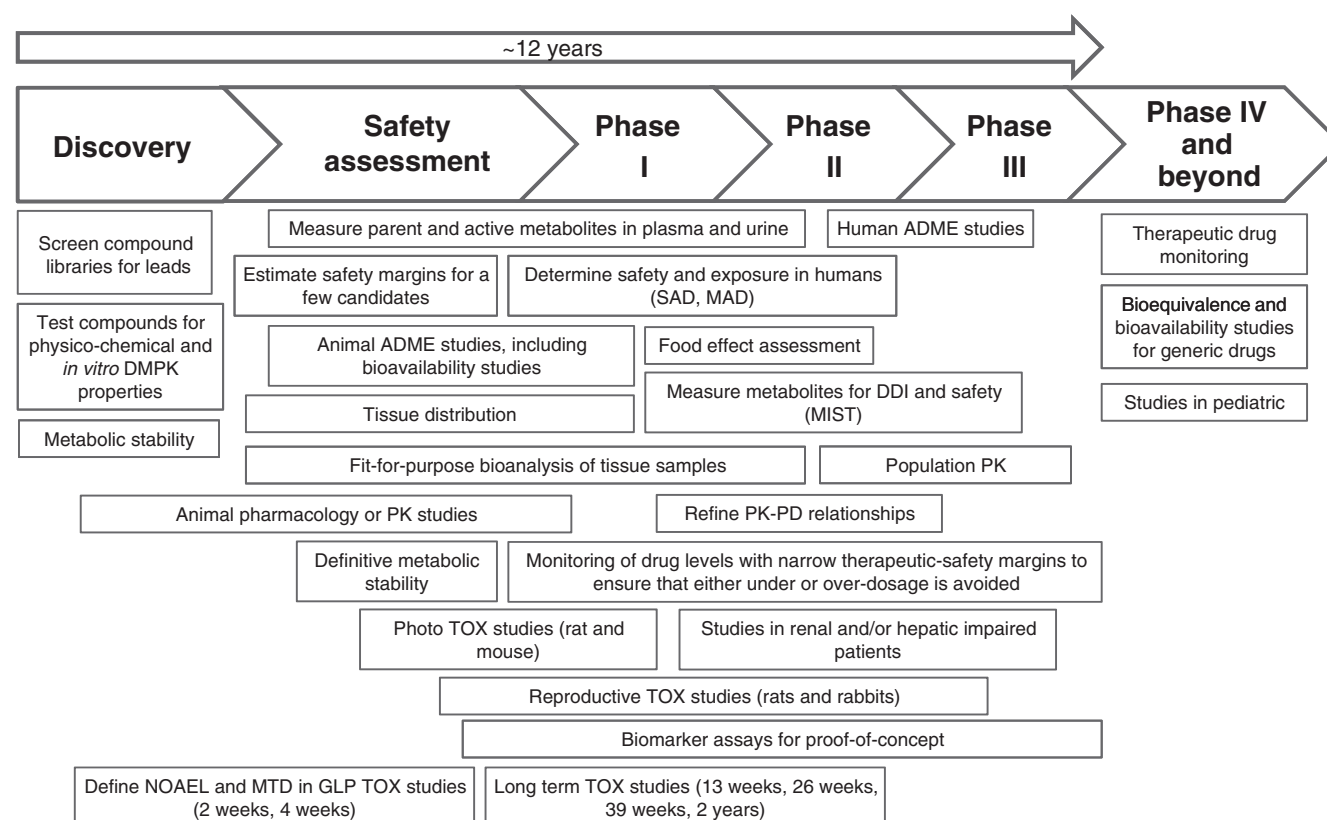


FIGURE 1.1 A flowchart of drug discovery and development, and postapproval studies of drugs where LC-MS bioanalysis plays important roles.

the successful registration and commercialization of a drug product.

1.2 LC-MS BIOANALYSIS IN DRUG DISCOVERY

Before the introduction of combinatorial chemistry, many drug candidates came from natural products where an active compound was isolated and its chemical structure was characterized using NMR, MS, IR, and derivatization or selective degradation chemistry. Screening entailed an assessment of bioactivity and physicochemical data compared to known databases. High-resolution mass spectrometry played a critical role allowing molecular formula searches from accurate mass data. Similarly, spectral databases allowed positive confirmation or class assessments. This process helped to ensure that novel compounds were selected. Since the introduction of combinatorial chemistry 20 years ago, the analyst's role in early drug discovery has shifted to the development of highly efficient LC-MS analytical methods to support quantitative analysis. The drug discovery process begins with compound library development and ends with the selection of preclinical drug candidates for preclinical safety assessment. LC-MS bioanalysis plays an important role throughout this process.

1.2.1 Structure-Activity Relationships from High-Throughput Screening

High-throughput LC-MS assays can be employed for the determination of solubility, membrane permeability or transport, protein binding, and chemical and metabolic stability for a large number of compounds that have been identified as "hits" (Janiszewski et al., 2008). Thousands of compounds per year go through some or all of these screening procedures. The *in vitro* studies validate *in silico* assessments performed prior to synthesis and select compounds for moving forward in development.

1.2.2 Structure—PK-PD Relationships

Selected compounds from high-throughput screening are subsequently evaluated in pharmacology models for efficacy. Provided the targeted biochemistry is applicable to LC-MS analysis, high-throughput screening of potential biomarkers can be performed in pharmacology studies via either a targeted pathway or a metabolomics approach. If successful, discovery biomarkers may be useful in preclinical and clinical studies. Simple examples include steroid biomarkers such as testosterone or dihydrotestosterone for

5- α -reductase inhibitors or estrogen for selective estrogen receptor modulators.

Integration of drug metabolism and pharmacokinetics (DMPK), pharmacology, and biology studies in drug discovery can greatly accelerate an understanding of the pharmacokinetic–pharmacodynamic (PK-PD) relationships of lead compounds. The minimum effective dose observed in the pharmacology model is validated from knowledge of drug and active metabolite levels at the target site and compared with *in vitro* efficacy. Compounds known to have *in vitro* potency but are devoid of *in vivo* activity are suspected of having poor bioavailability (BA) or other DMPK properties (transport to target site, rapid clearance, etc.). Alternatively, compounds with an unanticipated high *in vivo* activity may have superior access to the site of action or form active metabolites.

LC-MS has a fundamental role in the success of many of these discovery studies. An appropriately designed, early *in vitro* study can determine intrinsic clearance in multiple species. *In vitro* assessments have improved our ability to predict systemic clearance using intrinsic clearance. However, predicting volume of distribution and tissue concentrations is far more difficult. Combinatorial approaches such as cassette dosing or coadministration of many compounds is one means of quickly assessing penetration into target sites. Typically, ~20 compounds are coadministered, but as many as 100 have been attempted (Berman et al., 1997). The specificity of MS detection allows one to simultaneously measure numerous compounds in biofluids and tissues and rapidly screen drug candidates for their ability to penetrate into the site of action (Wu et al., 2000).

1.2.3 Candidate Selection

Within a therapeutic program, a limited number of compounds may be investigated in greater detail as possible preclinical drug candidates. These include assessments at various doses in the rodent and nonrodent toxicology species. Defining the systemic and local exposures, refining PK-PD models and exploring dose-proportionality are among the objectives of this phase. Studies with both single and multiple ascending doses may be undertaken in an effort to assess accumulation, induction and toxicity. Whereas a “generic” LC-MS assay may suffice in supporting these non-GLP assessments of drug properties, one needs to be aware of the potential pitfalls, including stability of parent and metabolites and matrix effects from unknown metabolites, endogenous components, and dosing vehicles such as polyethylene glycols, a frequently used formulation for IV dosage.

As a drug candidate progresses further, translational medicine often will define biomarkers from pharmacology or metabolomics studies that can be used in clinical trials. Over the past 15 years, there has been considerable progress in the use of LC-MS to measure small biochemicals and

peptides. The ability to use biomarkers as a surrogate endpoint and to ensure a reliable PK-PD relationship is a common strategy for most drug development programs.

1.3 LC-MS BIOANALYSIS IN PRECLINICAL DEVELOPMENT OF DRUGS

1.3.1 Toxicokinetics

Drug safety assessment studies regulated under good laboratory practice (GLP) are an important part of the preclinical development activities. In a typical toxicology study, toxicokinetic evaluation is performed in order to ascertain adequate drug exposure in the study animals. To support bioanalysis of toxicokinetic samples from the GLP studies, generic LC-MS methods used during drug discovery may no longer be suitable. Modification of the generic method or redevelopment of the respective method is often needed, followed by full assay validation according to the current regulatory guidance and industrial practices (EMA, 2011; FDA 2001; Viswanathan et al., 2007). These requirements are implemented to ensure adequate sensitivity, selectivity, accuracy, precision, reproducibility, and a number of other performance related criteria for a given method.

Preclinical toxicity studies typically employ a broad dose range that could result in a wide range of circulating concentrations of the test compound. Test samples containing analyte levels exceeding the upper limit of quantification (ULOQ) need to be diluted, a step that can sometimes introduce errors. On the other hand, the lower limit of quantification (LLOQ) must be established so that the assay is sensitive enough to measure trough levels from the lowest dose, yet not too sensitive that background noise (false positives) in specimens collected from control animals is detected. A useful rule-of-thumb is to set the LLOQ at ca. 5% of the anticipated peak concentration following the low dose, which should allow accurate analyte measurement for approximately four half-lives.

Different strains of rats such as Sprague Dawley, Wistar Hannover, and Fischer are used in toxicology studies. The LC-MS assay method should be validated using the matrix from the same strain. The beagle dog is generally the default nonrodent species. Nonhuman primates, such as cynomolgus, rhesus, or marmoset monkeys, are occasionally used. The most common use of nonhuman primates is when assessing immunogenicity of large molecule drugs or when the metabolic profiles of dogs differ significantly from human. Drug metabolizing enzymes, such as aldehyde oxidase, can have pronounced differences across species. Matching metabolic profiles to human assures good safety coverage for all metabolites. When metabolism differs across species, metabolism-mediated toxicity can result in

6 ROLES OF LC-MS BIOANALYSIS IN DRUG DISCOVERY, DEVELOPMENT, AND THERAPEUTIC DRUG MONITORING

sensitivity within one species relative to others. For this reason, there may be a need to measure metabolites in GLP preclinical studies. Although metabolite measurement in those toxicokinetic (TK) samples might be exempt from full GLP compliance due to various reasons, for example, absence of purity certification of reference metabolites and lack of full validation of the intended LC-MS assays, care must be taken to ensure the integrity of the results generated. Often, an assay separate from the parent measurement may be set up for the occasional metabolite quantification. New guidance requires that steady-state exposures of significant metabolites in all species are obtained (Anderson et al., 2010). Non-GLP or tiered assays allow these decisions to be made without extensive validation of multiple assays (Viswanathan et al., 2007).

In parallel with clinical drug development is the continued testing of the compound in animal toxicology studies. This includes extending the safety in primary toxicology animals with longer study durations. Dose range-finding studies are conducted in preparation for the 2-year carcinogenicity studies in mouse and rat. Phototoxicity studies are performed in mice. Reproductive toxicology is performed in rats and rabbits. Bioanalytical assays need to be validated in these additional species. Again, metabolites unique to these species need to be considered.

The bioanalyst should be prepared to support LC-MS bioanalysis of tissue samples for certain programs. Extensive validation and stability determinations might be needed, sometimes for both parent drug and metabolites. Having a stable isotope labelled internal standard can help avoid problems such as differences in extraction recovery and compensate for variability due to sample processing, transfer and analysis of study tissue samples. Homogenization prior to freezing is also preferred. Nevertheless, one can never fully ensure consistent analysis from tissue samples since the spiked quality control (QC) samples cannot fully mimic the incurred tissues. The most definitive approach would be to compare tissue results obtained using LC-MS to those from LC analysis in a radiolabeled study.

1.3.2 Preclinical ADME and Tissue Distribution Studies in Animals

Preclinical studies to elucidate the absorption, distribution, metabolism, and excretion (ADME) of drug candidates are usually conducted before and during the clinical phase. Radiolabeled drug is often needed for the animal ADME or tissue distribution (quantitative whole body autoradiography) studies, although with today's LC-MS instrumentation, much information can be gathered without the use of radiolabeled isotopes. Parent drug absorption and elimination can be readily assessed using LC-MS assays. Metabolites can be determined using LC-MS under unit or high resolution conditions. Blood-to-plasma partitioning and protein binding, once

done exclusively using radiolabeled drug can now be performed using highly sensitive LC-MS assays. The question of whether radiolabeled mass-balance studies in laboratory animals are still needed today has generated much discussion (Obach et al., 2012; White et al., 2013). The advance in LC-MS technology was the catalyst for this change.

1.4 LC-MS BIOANALYSIS IN CLINICAL DEVELOPMENT OF DRUGS**1.4.1 First-in-Human Studies**

Upon successful completion of the preclinical safety assessment of drug candidates, the investigational new drug (IND) submission is prepared. Traditionally, first-in-human (FIH) studies have included separate single and multiple ascending dose (SAD and MAD) studies. Today, adaptive studies can include a combination of SAD and MAD. To ensure safety, a sufficiently low starting dose is selected, and the supporting bioanalytical assay usually requires an LLOQ much lower than that used in toxicology studies. For a drug candidate with a wide safety margin, a bioanalytical method with a similar dynamic range will be needed. While it might be difficult to obtain a full PK profile on the earliest doses of an ascending dose study, a full PK profile will be required when an efficacious dose is reached. In addition to defining the maximum tolerable dose and possibly biological effect, the DMPK objectives in FIH studies include defining drug absorption, dose proportionality, and systemic clearance. Metabolite profiling and measurement will also be conducted to make sure unique human metabolites do not exist and major circulating metabolites at or above 10% of total drug-related exposure at steady state are also present at comparable or greater exposure levels in at least one of the main preclinical toxicology species (FDA, 2008).

A bioanalytical LC-MS method should be developed and validated prior to completion of the study protocol. Important information such as conditions for blood sample collection, plasma harvest, sample storage, and transfer must also be verified. If samples need to be stabilized because of the presence of labile parent or metabolites, the information should be provided well in advance so that the clinical staff can be properly trained in the required sample handling procedures.

The SAD/MAD study may also include an arm to study the food effect (fasted vs. fed) on the BA of the drug. Some drugs bind to food resulting in decreased absorption. In contrast, food can stimulate bile acid secretion that helps to dissolve less soluble drugs, making them more bioavailable. A bioanalytical LC-MS method should, therefore, be evaluated in both normal and lipemic plasma. The assay should be insensitive to changes in phospholipid concentration, a

common issue in electrospray ionization that requires attention during method development and validation.

Drug concentrations in urine are also typically measured to assess renal clearance. Unlike plasma, blood or serum, urine does not normally contain significant amounts of proteins and lipids. The lack of proteins and lipids in urine samples can be associated with the issue of nonspecific binding or container surface adsorption of drug molecules, especially those lipophilic and highly protein bound, in quantitative analysis of urine samples. The issue is often evidenced by the unusually low extraction recovery of the analytes of interest and/or nonlinearity of the calibration curves or highly variable QC sample results. Quick identification and effective prevention of analyte loss due to nonspecific binding or container surface adsorption must be conducted by bioanalytical scientists prior to the study so that the correct collection and storage condition can be provided (Li et al., 2010).

1.4.2 Human ADME Studies

Comprehensive information on the ADME of a drug in humans can be obtained from mass-balance studies using a radiolabeled compound, and this should be an early objective in clinical drug development (Pellegatti, 2012). Information on drug tissue distribution in rodents (e.g., rat) and the anticipated therapeutic dose are needed for planning a human ADME study. Some knowledge of the drug metabolism *in vitro* and in animals can help to select the position and desired specific activity of the radiolabel. Quantitative whole body autoradiography is a common tool for tissue distribution studies. Disposition of radioactivity into specific organs is quantified and scaled to human. Dosimetry calculations are performed to ensure safe radioactivity exposure limits in dosing of humans. Typically the maximum exposure limit is 1 mSv (ICRP 103, 2007). Traditional ADME studies generally use liquid scintillation counting and doses of $\sim 100 \mu\text{Ci}$ of ^{14}C labeled drug mixed with unlabeled drug. LC-MS for measuring unlabeled drug is often used in human ADME studies to differentiate the parent compound from its metabolite(s). For studies employing microdoses ($< 100 \mu\text{g}$) or doses of low radioactivity ($< 1 \mu\text{Ci}$), accelerator mass spectrometry may be needed to measure the ^{14}C labeled drug (Garner, 2005), whereas high sensitivity LC-MS methods have been used to determine unlabeled drug concentrations (Balani et al., 2005).

ADME studies, though limited by their single dose nature, do illuminate what is important to measure in toxicology and clinical studies to satisfy Metabolites in Safety Testing requirements (Anderson et al., 2010). Obach et al. (2012) have advocated deferring the cost of this study until after proof of concept (POC) and relying on pharmacokinetic information derived from nonradiolabeled studies, namely SAD and MAD. The risk of delaying the human ADME study is that unique human metabolites may be uncovered

after POC. The surprise of having significant metabolites found late in drug development can expose a lack of safety coverage or protection of intellectual property if the metabolite is active. The advancement of more powerful high resolution mass spectrometry for metabolite identification in LC-MS bioanalysis of early stage study samples helps to mitigate the risk.

1.4.3 Human Drug–Drug Interaction Studies

A drug–drug interaction (DDI) is a situation where a drug affects the activity or toxicity of another drug when both are coadministered. Interactions can be found where saturable or inducible enzymes or transporters are expressed and play a role in the absorption and disposition of the drug. DDI can increase or decrease the activity of the drug or a new effect can be produced that neither produces on its own. This interaction can occur between the drug to be developed and other concomitantly administered drugs, foods, or medicinal plants or herbs. During clinical development, DDI studies are normally conducted for the drug candidate in healthy volunteers or patients to confirm any significant observations seen during *in vitro* DDI studies.

From the perspective of LC-MS bioanalysis, assay specificity against the coadministered medicines and their significant metabolites needs to be demonstrated. In the case of metabolites that are difficult to obtain, interference could be discounted based upon MS detection (e.g., differing MW or MRM). On the other hand, possible interference due to drug candidates and/or their major metabolites on the accuracy of determination of DDI compounds and their significant metabolites must be checked to ensure the quality of LC-MS bioanalytical results for the DDI assessment.

1.4.4 Renal Impaired and Hepatic Impaired Studies in Human

Kidney (or renal) failure is a medical condition in which the kidneys fail to adequately filter toxins and waste products from the blood. Similarly, liver (or hepatic) failure is the inability of the liver to perform its synthetic and metabolic function as part of normal physiology. Either can be acute or chronic. Drug elimination may occur by filtration in the kidney or metabolism in the liver. When impacted by disease, drug accumulation can result in toxicity. Depending on the properties of metabolism and excretion of a drug candidate, clinical studies in renal impaired or hepatic impaired patients need to be conducted. In addition to conventional plasma samples, urine samples may be collected and analyzed. Some drugs may be metabolically activated, resulting in idiosyncratic liver toxicities. Therefore, it is important to understand both the impact of an impaired liver on the normal pharmacokinetic properties of a drug as well as the potential of a drug to impact liver function.

8 ROLES OF LC-MS BIOANALYSIS IN DRUG DISCOVERY, DEVELOPMENT, AND THERAPEUTIC DRUG MONITORING

From the perspective of LC-MS bioanalysis, assay dynamic range must be suitable to measure exposures from any given dose, or assay integrity of sample dilution must be checked to ensure data integrity for samples with unexpected high analyte concentrations due to the impaired liver or kidney function.

1.4.5 Phase II and Phase III Studies

Moving beyond preliminary safety studies to POC studies is a milestone goal for clinical drug development. A successful program will demonstrate POC before the end of phase II studies. Therefore, moving from healthy subjects to the intended patient population is an important transition. However, patients might take more medications or are under treatment with drug combinations. With this regard, the robustness of the intended LC-MS assay should be validated free from possible interference of combination drugs and their metabolites.

Phase II and III studies are larger and more expensive. In order to support the bioanalysis of a large number of samples from these large multicentered trials, automation is an important consideration. For long-term, multicentered studies, the assay must be rugged enough to ensure storage stability. A well-planned stability assessment of drug candidate and its metabolite(s) of interest is critical as stability must cover all reported results. Any significant assay bias must also be well characterized. The entire bioanalytical work is represented in the new drug application (NDA) submission. This includes tabular and written summaries of assay validation performance of both nonclinical and clinical assays. Given that the development process of a drug may last more than a decade, it is important to maintain institutional knowledge to avoid gaps at filing.

1.4.6 “Fit-for-Purpose” Biomarker Measurement Using LC-MS in Clinical Samples

As drug candidates progress through POC studies, there is great need for LC-MS assays to measure biomarkers in clinical studies. There are numerous examples, including steroids, lipids, nucleotides, and peptides, which are directly amenable to LC-MS. Due to the endogenous nature of biomarkers, bioanalysis of those compounds usually encounters a series of challenges in maintaining analyte integrity from collection to analysis, achieving specificity, and obtaining sufficient sensitivity, especially when endogenous concentrations are downregulated. Those challenges entail special consideration and meticulous experimental design in method development, validation, and study conduct. Among the four common approaches to the preparation of standards, i.e. (1) authentic analyte in authentic matrix, (2) authentic analyte in surrogate matrix, (3) surrogate analyte in authentic matrix, and

(4) charcoal or chemical stripping and immunodepletion, the last three are the ones most often applied.

Currently, there is no regulatory guidance specifically for biomarker bioanalysis. Therefore, whatever needs to be done in LC-MS bioanalysis of biomarkers should fit for the purpose of the intended use of the data. This approach has gained consent within the bioanalysis community. The term “fit for purpose” reflects flexible inclusion/exclusion of validation experiments, experiment design, and acceptance criteria. In general, assessment of accuracy, precision, and stability is considered the essential part of assay validation, while others, for example, matrix effect and recovery, are considered optional, especially when a stable isotope labeled IS is used.

Another emerging trend in biomarker quantitation is the LC-MS bioanalysis of peptide or protein biomarkers although ligand-binding assays, for example, enzyme-linked immuno sandwich assay (ELISA), still play an important role. Compared to ELISA, LC-MS assay development is relatively fast with no need to raise antibodies. More importantly, LC-MS assays can measure proteins as peptide surrogate with similar sensitivity and specificity to many immunoassays. However, introduction of stable label protein internal standard can be very challenging and costly.

1.4.7 Other LC-MS Assays Needed for Clinical Development of Drugs

As clinical drug development progresses, there can be other needs for LC-MS assays. Metabolism-mediated toxicity or adverse events often trigger these requests. In toxicology studies, this can include an assessment of parent and metabolite concentrations in various tissues from the most sensitive species. In man, penetration or distribution questions may be difficult to answer. For blood–brain barrier penetration, only cerebrospinal fluid surrogate sampling may be possible.

For antiinfective drugs, penetration studies are critical to ensuring that trough concentrations greater than IC_{50} levels are maintained where needed. When this is not achieved, resistance can develop. A similar objective to define cellular penetration can be achieved by analyzing peripheral blood mononuclear cells (PBMCs) after dosing with virology drug candidates. Plasma concentration is a poor indicator of drug activities in the cell since the activation of the drug (nucleoside) to its triphosphate involves multiple enzymatic processes that may vary by individual (Rodman et al., 1996). The pharmacokinetics of the intracellular triphosphate is also very different from that of the nucleoside. For example, the intracellular triphosphate form of emtricitabine has a much longer half-life than the plasma half-life of emtricitabine (Wang et al., 2004). Analysis of drug concentrations at the target site is often fundamental to prove target engagement and can serve to build the clinical PK-PD model. For instance, both intracellular penetration and phosphorylation is needed

to activate nucleoside reverse transcriptase inhibitors. Therefore, determination of the active phosphorylated drug in PBMCs can illustrate a drug candidate's limitation as a substrate for kinase activation (Shi et al., 2002).

Drugs that have synergistic effect, such as oncology and virology agents, are generally dosed as a combination therapy. Developing an efficient LC-MS assay to simultaneously measure dose combinations is a worthwhile investment for both clinical drug development and later therapeutic drug monitoring (TDM) (Taylor et al., 2011). As many as 17 antiretroviral agents in human plasma have been measured in highly active antiretroviral therapy (HAART) (Jung et al., 2007).

Performing cost-effective LC-MS monitoring of combination therapeutics helps to ensure that safety margins of each therapeutic agent are maintained. Assays can be challenging due to the potential effect of one drug on the other. Inhibition of clearance may elevate parent concentrations while reducing those of the metabolites. Likewise, induction may reduce parent drug concentrations while elevating systemic metabolite levels. In either case, a wider range of assay or higher dilutions would be needed. The assay must also be demonstrated not to interfere with the dosed combination as well as other common medications or their metabolites. Testing of interfering drugs is readily accomplished in validation; however, testing metabolites from such complex mixtures is difficult. Using plasma from subjects receiving the concomitant drug therapies may be the best approach in method development.

1.4.8 LC-MS Bioanalysis in Postapproval Studies (Phase IV) of Drugs

Following drug approval, additional studies are often undertaken at the request of the regulatory agency. Pediatric studies can require a reduction of the sample volume or additional work to obtain a lower LLOQ. Antiinfective drugs may require an assessment in special fluids such as otitis media before being approved for ear infections. Furthermore, a regulatory agency may require the routine monitoring of drugs with a narrow therapeutic index such as many oncology or immunology drugs in larger patient populations.

1.4.9 LC-MS Bioanalysis in BE and BA Studies for Generic Drugs

At patent expiration of a brand drug, generic versions that demonstrate BE to the innovator's product may be marketed via the Abbreviated New Drug Application (ANDA) process. In the United States, a first-to-file company enjoys 6-month exclusivity for its generic product. In order to demonstrate the BE of two proprietary preparations of the same drug molecule, studies must be conducted to show an equivalent rate and extent of BA of the two products. The rate is usually measured in terms of the peak plasma concentration

(C_{\max}), whereas the extent is represented by the area under the concentration–time curve. Having a robust assay that can provide precise and accurate results is important in supporting BE trials (Shah and Bansal, 2011), and LC-MS assays have eclipsed GC-MS or HPLC methods for this purpose (Marzo and Dal Bo, 2007).

BE studies may also be required for a change in drug substance, drug product, or manufacturing site. When comparing two treatments the study design must include a sufficient washout between treatments. To eliminate inter-run bias, all treatments for a given subject must be analyzed within the same run. Dilutions are to be avoided, thus the dynamic range may need to be adjusted to accommodate the anticipated concentrations. The number and placement of QC samples in BE studies must mimic study sample concentrations. Due to the need for high accuracy and precision to assess formulations, many countries have instituted special guidances for BA and BE studies. These rules are in addition to normal bioanalytical requirements that have been established over the past two decades (FDA, 2001; Viswanathan et al., 2007; EMA, 2011).

1.4.10 LC-MS Bioanalysis in Therapeutic Drug Monitoring

TDM is needed for drugs with a narrow therapeutic index or when used in impaired populations. While TDM is mostly used to avoid overdosing drugs, it may also help to avoid underdosing or to monitor compliance. TDM is useful in many therapeutic areas, from antiinfectives to immunosuppressants (Adaway and Keevil, 2012). For many antibiotics with a good safety margin, underdosing is of primary concern. On the other hand, antibiotics including the aminoglycosides, vancomycin, and colistins are associated with nephro- or neurotoxicity, thus overdosing is a primary concern.

Antiinfective medications can rapidly develop drug resistance so mono-therapy of HIV medicines is not likely. Comedication or HAART is the norm. There can be significant DDI in combination therapy. Ritonavir is both a HIV protease inhibitor as well as a potent cytochrome P450 CYP3A4 inhibitor used to intentionally boost the exposure to other drugs. Knowing individual drug concentrations in plasma will, therefore, aid therapy. A TDM LC-MS method measured 21 antiviral drugs from four drug classes in human plasma (Gehrig et al., 2007). Protein precipitation was needed to achieve high recovery of all diverse drugs.

Maintaining an effective level of exposure can be improved by determining drug concentrations at the site of action. As many as 10 antiretroviral drugs were determined in human PBMCs (Elens et al., 2009). Intracellular activation of reverse transcriptase inhibitors by phosphatases to their mono-, di-, and active triphosphate plays a critical role in the effectiveness of the administered prodrug.

10 ROLES OF LC-MS BIOANALYSIS IN DRUG DISCOVERY, DEVELOPMENT, AND THERAPEUTIC DRUG MONITORING

Free drug concentrations can often provide a better correlation to drug effect than total (free and protein bound) drug levels. While ultrafiltrate or dialysate may be prepared from plasma and assayed, a simpler solution is to assay saliva. Saliva can be obtained in a noninvasive manner and saliva concentrations represent the free drug since protein-bound drugs cannot enter saliva. Other noninvasive matrices such as hair may be useful to establish patient compliance. Slightly more invasive is finger-prick sampling. Assay sensitivity is always critical when working with these low sample volumes. However, a finger-prick sample may be useful in pediatrics and subjects with compromised vascularization, including the elderly and drug addicts.

Dried blood spots (DBS) with LC-MS bioanalysis have gained popularity in TDM for a wide spectrum of drug molecules. Because DBS samples can be collected by patients themselves or their guardians with very minimum training, it opens up the possibility of collecting clinical pharmacokinetic samples not only from various in-patients but also from out-patients, especially those from remote areas (Burhenne et al., 2008). This is especially important when there is a need to monitor drugs with a narrow therapeutic index, for example, tacrolimus and cyclosporine A, with a wide variation in intra- and interpatient pharmacokinetics. DBS samples can be promptly taken whenever a concentration-related side effect appears (Li and Tse, 2010).

TDM is often limited by the difficulty in securing a representative sample and the cost of its analysis. Immunoassays, while highly cost-effective in multiplexed formats, may not provide the specificity required for combination therapies in diverse patient populations. Assay bias may result from not being able to distinguish parent drug from inactive metabolites and endogenous components. Due to its high specificity and sensitivity, LC-MS is evolving as a key player in TDM for various drugs, including anticancer and antiinfective drugs and immunosuppressants (Saint-Marcoux et al., 2007). Since LC-MS has the potential to simultaneously determine multiple analytes, its cost-effectiveness is beginning to rival immunoassays.

Within the field of immunosuppressants, TDM using LC-MS is well established to measure drugs targeted to the mammalian target of rapamycin (mTOR) such as temsirolimus or everolimus. Assays often measure combination therapies that include cyclosporine A, mycophenolic acid, everolimus, sirolimus, tacrolimus, and their metabolites (Saint-Marcoux et al., 2007). LC-UV assays can be used for some of these drugs, but the specificity, speed, and ability to measure all within a single assay makes LC-MS the preferred approach. Due to its preferential partitioning into red blood cells, cyclosporine A and the mTOR inhibitors sirolimus and everolimus are measured in blood. Mycophenolic acid is highly bound to albumin and therefore analyzed from plasma. Monitoring of both the ether and acyl

glucuronides of mycophenolic acid may also be needed (Yang and Wang, 2008). While individual extracts from blood and plasma may be needed, a common LC-MS method can be used. Sampling a few hours after the last dose and at trough is common, requiring an assay with a wide dynamic range (Sallustio, 2010).

For mTOR inhibitors, fragmentation may be limited. When monitoring the deprotonated molecule in negative ion mode, methanol loss is a commonly used transition. In positive ions, cationization with Na^+ , K^+ , or NH_4^+ is preferred over the MH^+ ion. Less specific transitions such as ammonia loss are compensated by the greater specificity of a high mass parent. Stable label internal standards improve assay performance, particularly for blood assays or when cationization adducts are monitored.

The high risk of organ transplant rejection warrants TDM. Clinical laboratories are becoming increasingly populated with LC-MS instrumentation and moving away from immunoassays. In addition to validating assays, proficiency tests from the College of American Pathologists or UK National External Quality Assessment Service require pooled samples from patients to be analyzed at regular intervals. Results from many hundreds of laboratories are compared to ensure standardized results are achieved.

1.5 LC-MS BIOANALYSIS OF LARGE MOLECULE DRUGS AND BIOPHARMACEUTICALS

Peptide and oligonucleotide drugs have special requirements for their bioanalysis (Nowatzke et al., 2011). Neither is generally a substrate for CYP enzymes but rather cleaved by normal proteases or nucleases. Stabilization of peptides, thus extending the half-life *in vivo*, can be accomplished using non-native enantiomers and sterically hindered amino acids or by attaching fatty acids and polyethylene glycols. The latter also serves to reduce immunogenicity. Bioanalysis using LC-MS can be difficult due to the adsorptive nature and instability of the analytes. Sensitivity loss due to lack of distinguished fragmentation is another issue. Pegylated peptides represent particular challenges due to their heterogeneous nature and large molecular weight. Larger peptides or protein therapeutics may need to be selectively reduced in size to make them tractable to LC-MS.

Due to their size, proteins (>10 kDa) are less amenable to low level, direct quantification. The broad isotope distribution and presence of multiply charged species distributes their ion abundance among several response peaks thereby “diluting” the overall characterizing signal. Direct measurement of intact proteins may be made more specific using high resolution instrumentation such as time-of-flight or Orbitraps. Data processing algorithms can plot a summation of ions resulting from individual isotopes and charged forms. However, the individual ions need to be free of interference to

maintain specificity. Furthermore, many proteins have abundant heterogeneity due to post-translational modifications (PTMs). LC-MS of enzyme digests have been increasingly used for quantification of proteins using a stable label or surrogate internal standard. However, one needs to be aware of the potential loss of selectivity of this approach due to the similarity between intact and post-translational modified proteins. When the protein therapeutic has extensive PTMs, an immunoassay which responds to all forms may be better at assessing its overall exposure.

Toxins attached to antibody–drug conjugates (ADC) are tractable to LC-MS. The antibody is immunocaptured and the toxin released by hydrolysis. In the case of a peptide toxin, enzymatic hydrolysis is used and the peptide measured using LC-MS. A PK profile of the toxin load versus time is established. Immunoassays of differing specificity can be used to assess antibody concentrations (Xu et al., 2011). ADC development is now showing the long sought promise of antibodies for drug targeting. LC-MS has a critical role in characterizing the “payload” of these drug delivery agents.

1.6 GUIDANCE AND REGULATIONS FOR LC-MS BIOANALYSIS

There is no doubt that LC-MS bioanalysis in support of regulated preclinical safety assessment of drug candidates submitted to the FDA must be conducted in compliance with the Good Laboratory Practice Regulations (Fed. Reg., Vol.43, 21CFR Part 58, 22-Dec-1978) and all subsequent amendments to these regulations. Submissions to European Medicines Agency (EMA) require compliance with the Organization for Economic Cooperation and Development (OECD) Principles on Good Laboratory Practice (ENV/MC/CHEM(98)17) and all subsequent OECD consensus documents. Other regulations include the Directive 2004/10/EC of the European Parliament and of the Council of Feb 11, 2004 on the harmonization of laws, regulations, and administrative provisions relating to the application of the principles of Good Laboratory Practice and the verification of their applications for tests on chemical substances (OJ No. L 50 of 20.2.2004), and other country specific regulations, for example, State Food and Drug Administration of China (SFDA).

The majority of bioanalysts around the world have become familiar with the procedures and requirements of bioanalysis as provided by the FDA guidance (FDA, 2001) and EMA guidance (EMA, 2011), and a series of Crystal City whitepapers (Viswanathan et al., 2007). Both the FDA and EMA guidances cover all aspects of bioanalysis from method validation to samples analysis from all nonclinical and clinical studies.

1.7 GENERAL CONSIDERATIONS OF A ROBUST LC-MS BIOANALYTICAL METHOD

All requirements on what should be performed in validating a LC-MS assay method in support of preclinical or clinical studies have been highlighted in the major health authority guidances (EMA, 2011; FDA, 2001). The items that need to be validated include but are not limited to: (1) selectivity and specificity, (2) sensitivity, (3) linearity, (4) intra- and interday precision and accuracy, (5) stability (stock/spiking solution stability, stability in QC samples that undergo freeze–thaw cycles and at room temperature, stability in the reconstituted sample extract stored under autosampler condition, long term stability under intended storage condition, stability in blood), (6) dilution integrity, (7) carryover, and (8) assay batch size.

The initial assessment of assay calibration regression model will include the slope or sensitivity of the calibration curve and weighting. Once established, all future analysis must be performed according to this model. Whenever possible, standards and QC samples must be prepared in the identical matrix to study samples. Acceptance criteria is three-fourth of all standards and two-thirds of all QC samples are within 15% (20% at LLOQ) of their nominal value and that 50% of QC samples at each concentration are acceptable. Tests for detection specificity and assay selectivity will employ either blank matrix or samples spiked at the LLOQ, respectively, in matrix from numerous individuals. Lipemic and hemolyzed plasma should be tested to ensure that the assay is insensitive to sample condition. Establishing the assay range by testing at the LLOQ and analytical QC levels (low, mid, and high) is required in replicates ≥ 5 for at least three batches. These accuracy and precision tests are critical to a validation.

The numerous stability campaigns needed to support sample analysis are performed using either a bracketed range of stock solutions or QC samples, minimally tested at low and high QC concentrations. Each part of the sample collection and storage process must be tested. Plasma assays can be used to assess blood stability by harvesting and analyzing plasma at different time points (e.g., 0, 60, and 120 min). Multiple dilutions are generally tested during validation to ensure that the most diluted study sample can be reproducibly analyzed. While tested in validation, it is important to also demonstrate performance during study sample analysis by including dilution QC samples within runs. Carryover must be minimized in method development and controlled in validation or sample analysis to less than 20% LLOQ. Tests must include an assessment of ruggedness by ensuring that the maximum batch used in sample analysis is tested during validation.

During study sample analysis, incurred sample reproducibility must also be demonstrated (Fast et al., 2009). One should recognize that incurred samples are much more complex in their composition than the QC samples. QC samples

12 ROLES OF LC-MS BIOANALYSIS IN DRUG DISCOVERY, DEVELOPMENT, AND THERAPEUTIC DRUG MONITORING

do not contain the various drug metabolites, drug isomers/epimers, coadministered drug(s) and their metabolites, and/or dosing vehicles as often seen in the incurred samples. In some cases, the plasma concentration of metabolites can reach an order of magnitude greater than that of the parent drug. Heightened awareness among bioanalytical scientists to the differences between QC samples and incurred samples will reduce the risk of subsequent bioanalytical failure (Li et al., 2011). The integrity of individual samples can also impact this assessment, as seen for hemolyzed plasma or inhomogeneous urine samples. The requirements of conducting incurred sample reanalysis (ISR) have been established over the past decade (Fast et al., 2009; EMA, 2011). The concentration obtained for the initial analysis and the concentration obtained by reanalysis should be within 20% of their mean for at least two-thirds of the repeats. ISR can be performed during next run (rolling ISR) or analyzed in separate batch at the end of the study. Testing at the end of the study examines both stability and imprecision in the assessment. Testing at the end of the study is also more reflective of any repeat analysis and can better understand issues related to interference from unstable concomitant medications or its metabolites. ISR on the following day's run not only examines assay reproducibility but also will give an earlier diagnosis of any problems. For this reason, some laboratories perform a mixture of both early rolling and later batch ISR. The assay should be tested for ISR in each clinical trial, as new populations or new clinics can spawn new problems.

Prior to transferring bioanalytical work for an ongoing study from one laboratory to another, for example, from the sponsor's laboratory to a contract research organization, cross-validation or conformance testing using QC and incurred samples must be conducted. The success of an LC-MS assay method transfer is critical to ensure the quality and integrity of subsequent PK-PD assessments.

1.8 CONCLUSIONS

LC-MS bioanalysis has become a primary tool in every stage of drug discovery, drug development, and postapproval TDM. It helps in not only selecting better drug candidates but also improving our understanding of safety and pharmacokinetics of the drugs. Furthermore, LC-MS bioanalysis of toxicology biomarkers will no doubt help to avoid the selection of poor drug candidates that would fail in longer term toxicology studies. LC-MS is increasingly being used to assay clinical biomarkers and ensure that the drug effects observed in animal models translate into success in human proof-of-concept studies.

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