Handbook of LC-MS Bioanalysis

Best Practices, Experimental Protocols, and Regulations

Wenkui Li · Jie Zhang · Francis L. S. Tse

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HANDBOOK OF LC-MS BIOANALYSIS

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PREFACE

Bioanalysis is the most heavily regulated area within the discipline of drug metabolism and pharmacokinetics, which supports a large sector of drug development. The health authorities have very specific requirements regarding quality and integrity of bioanalytical results, and different customers usually have additional expectations on the performance of bioanalytical assays.

Much has happened in recent years toward faster, cheaper, and better ways of providing quality bioanalytical results. Both the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) have renewed or are renewing their guidances on bioanalytical method validation (e.g., 21July2011/EMEA/CHMP/EWP/192217/2009) with the ultimate goal of improving the quality of bioanalytical results. Novel approaches to bioanalytical method development as well as advents of new liquid chromatographic (LC) techniques and mass spectrometric (MS) instruments have been reported. Various automatic laboratory procedures, electronic laboratory notebooks, and data management systems are now available. All of these culminate in a remarkable improvement in the quality, speed, and cost-effectiveness of bioanalytical work and contribute to the value we deliver to the patient.

Given the rapid changes within the field of bioanalysis and also in the larger area of drug development in which we operate, it is timely to conduct a broad overview of our discipline. This book is the first comprehensive handbook for LC-MS bioanalysis and provides an update on all important aspects of LC-MS bioanalysis of both small molecules and macromolecules. It not only addresses the needs of the bioanalytical scientists on the pivotal projects but also features perspectives on some advanced and emerging technologies including high-resolution mass spectrometry and dried blood spot (DBS) microsampling.

The 51 chapters of the book are divided into four parts. Part I provides a comprehensive overview on the role of LC-MS bioanalysis in drug discovery and development and therapeutic drug monitoring (Chapter 1), the key elements of a regulated bioanalytical laboratory (Chapter 2), and the current international regulations and quality standards of bioanalysis (Chapter 3).

In Part II, the global regulations and quality standards related to LC-MS bioanalysis are reviewed and compared. Chapter 4 highlights the current regulations governing bioanalytical method validations from a number of countries and regions including Brazil (ANVISA), Canada, China, the European Union (EMA), India, Japan, and the United States (FDA). This is followed by two in-depth reviews on the topics of assay reproducibility (Chapter 5) and method transfer (Chapter 6). Chapter 7 presents the current practices and regulatory requirements on Metabolites in Safety Testing (MIST). The guidances of regulatory bodies worldwide on bioanalysis for bioequivalence (BE)/bioavailability (BA) studies are compared in Chapter 8, whereas Chapter 9 concerns the specific topic of good laboratory practice (GLP) and its interpretation and application by different agencies, countries, and regions. Of special interest is the rapid evolvement of regulations on bioanalytical data management, which is discussed in Chapter 10. Chapter 11 concludes Part II by giving a detailed analysis of regulatory inspections including health authority expectation, reported inspectional trends, citations, and regulatory followup letters. Recent FDA 483 observations as well as other "hot topics" in bioanalysis compliance that have raised concerns about data integrity are reviewed. Applicable best practices in LC-MS bioanalysis are portrayed in Part III. From this section of the book, the reader will find sound scientific rationale and helpful practical instructions on the assessment of whole

blood stability and blood/plasma distribution (Chapter 12), and on biological sample collection, processing and storage (Chapter 13). Chapter 14 introduces various sample preparation techniques for LC-MS bioanalysis, while the best practices in LC separation and MS detection are discussed in Chapters 15 and 16, respectively. A good bioanalytical method must be sensitive, specific, selective, reproducible, high-throughput, and fundamentally robust. Many factors that can contribute to the success of an assay are reviewed including the choice of internal standard (Chapter 17), evaluation of system suitability (Chapter 18), sensitivity enhancement via derivatization of analyte(s) of interest (Chapter 19), evaluation and elimination of matrix effect (Chapter 20), evaluation and elimination of carryover and/or contamination (Chapter 21), and robotic automation (Chapter 22). Chapters 23–29 describe the bioanalysis of drugs, biomarkers, and other analytes of interest in various body fluids and tissues, and Chapter 30 is devoted to DBS sampling and related bioanalytical issues. Chapter 31 offers some useful strategies for enhancing MS detection, and Chapter 32 shows the proper use of statistics as a tool for ensuring adequate method performance in LC-MS bioanalysis. The simultaneous quantitative and qualitative LC-MS bioanalysis of drugs and metabolites are discussed in Chapter 33.

Part IV aims to provide detailed instructions with representative experimental protocols for the LC-MS bioanalysis of various types of drug molecules commonly encountered in the bioanalytical laboratory today (Chapters 34–49). Chapter 50 describes a typical procedure using microflow LC-MS for the quantitative analysis of drugs in support of microsampling. Finally, a protocol on the quantification of endogenous analytes in biofluids without a true blank matrix is given in Chapter 51.

Our purpose in committing to this project was to provide scientists in industry, academia, and regulatory agencies with not only all the "important points to consider" but also all "practical tricks to implement" in LC-MS bioanalysis of various molecules according to current health authority regulations and industry practices. In this book, we are confident that we have accomplished our goal. The book represents a major undertaking, which would not have been possible without the contributions of all the authors and the patience of their families. We also thank the terrific editorial staff at John Wiley & Sons and give a special acknowledgment to Michael Leventhal, Associate Editor, and Robert Esposito, Associate Publisher, at John Wiley & Sons for their premier support of this project.

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ABBREVIATIONS

AAPS	American association of pharmaceutical scientists
AAS	Atomic absorption spectroscopy
AC	Absolute carryover
Ach	Acetylcholine
ACUP	Animal care and use protocol
ADC	Antibody–drug conjugate
ADME	Absorption, distribution, metabolism, and excretion
AFA	Adaptive focused acoustics
ALQ/AULOQ	Above the upper limit of quantification
ANDA	Abbreviated new drug application
ANVISA	National health surveillance agency (in Portuguese, Agência Nacional de Vigilância Sanitária)
AP	Analytical procedure
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
APPI	Atmospheric pressure photoionization
ASE	Accelerated solvent extraction
ASEAN	Association of southeast asian nations
AUC	Area under the curve
BA	Bioavailability
BDMA	Butyldimethylamine
BDMAB	Butyldimethylammonium bicarbonate
BE	Bioequivalence
BIMO	Bioresearch monitoring
BLQ/BLLOQ	Below the lower limit of quantification
BNPP	Bis(4-nitrophenyl)-phosphate
BSA	Bovine serum albumin
CAD	Charged aerosol detection
CAD	Collision-activated disassociation
CAPA	Corrective and preventive action
CD	Compact disc
CDC	Centers for disease control and prevention
CDSCO	Central drugs standard control organization
CE	Collision energy
CFR	Code of federal regulations
CID	Collision-induced dissociation
CL	Clearance
CNS	Central nervous system
CoA	Certificate of analysis
COV	Compensation voltage
CPGM	Compliance program guidance manual
CR	Concentration ratio
CRO	Contract research organization
Cs	Calibration standard
CSF	Cerebrospinal fluid
CSI	Captive spray ionization
CV	Coefficient of variation
CXP	Collision exit potential
CZE	Capillary zone electrophoresis
DBS	Dried blood spot
DDI	Drug–drug interaction
DDTC	Diethyldithiocarbamate
DDVP	2,2-Dichlorovinyl dimethyl phosphate
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DIFP/DFP	Diisopropyl fluorophosphate
DM	Drug metabolism
DMS	Differential mobility spectrometry
DMS	Dried matrix spot
DNA	Deoxyribonucleic acid
Dns-Cl	Dansyl chloride
Dns-Hz	Dansyl hydrazine
DP	Declustering potential
DPS	Dried plasma spot
DPX	Disposable pipette extraction

DQ	Design qualification
DTNB	5, 5'-Dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
EB	Endogenous baseline
EBF	European bioanalysis forum
EDMS	Electronic data management system
EDTA	Ethylenediaminetetraacetic acid
EFPIA	European federation of pharmaceutical industries associations
EHNA	Erythro-9-(2-hydroxy-3-nonyl) adenine
ELN	Electronic laboratory notebook
ELSD	Evaporative light scattering detection
EMA	European medicines agency
EP	Entrance potential
EPA	Environmental protection agency
ESI	Electrospray ionization
FAIMS	Field-asymmetric waveform ion mobility spectrometry
FDA	Food and drug administration
FIH	First-in-human
FOIA	Freedom of information act
FP	Focusing potential
FTICR	Fourier transform ion cyclotron resonance
FWHM	Full width at half maximum
GAMP	Good automated manufacturing practice
GBC	Global bioanalytical consortium
GC-MS	Gas chromatography-mass spectrometry
GCP	Good clinical practice
GLP	Good laboratory practice
GMP	Good manufacturing practice
GPhA	Generic pharmaceutical association
HAA	Hexylammonium acetate
HCT	Hematocrit
HETP	Height equivalent of a theoretical plate
HFIP	Hexafluoroisopropanol
HILIC	Hydrophilic interaction liquid chromatography
HMP	2-Hydrazino-1-methyl-pyridine
HP	2-Hydrazinopyridine
HPFB	Health products and food branch
HPLC	High pressure liquid chromatography or high performance liquid chromatography
HRMS	High resolution mass spectrometry
HSA	Human serum albumin
HTLC	High-turbulence liquid chromatography
IA	Immunoaffinity
IACUC	Institutional animal care and use committee
ICH	International conference on harmonization
ICP-MS	Inductively coupled plasma–mass spectrometry
ID	Inner diameter
IDMS	Isotope dilution mass spectrometry
IEC	Ion-exchange chromatography
IMS	Ion mobility spectrometry
IND	Investigational new drug
IP	Ion-pairing
IQ	Installation qualification
IS/ISTD	Internal standard
ISA	Incurred sample accuracy
ISR	Incurred sample reanalysis or incurred sample reproducibility
ISS	Incurred sample stability
IV	Intravenous
KFDA	Korea food and drug administration
LC-MS	Liquid chromatography–mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LIMS	Laboratory information management system
LLE	Liquid–liquid extraction
LLOQ	Lower limit of quantification
LOD	Limit of detection
LUV	Large unilamellar vesicle
MAD	Multiple ascending dose
MAX	Mixed mode anion exchange
MCD	Maximum concentration difference
MCX	Mixed mode cation exchange
MD	Method development
MDF	Mass defect filter
MEPS	Microextraction by packed sorbent
MF	Matrix factor
MFC	Microfluidic flow control
MHFW	Ministry of health and family welfare
MHLW	Ministry of health, labour and welfare
MHRA	Medicines and healthcare products regulatory agency
MIP	Molecularly imprinted polymers
MIST	Metabolites in safety testing
MLV	Multilamellar vesicle
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MTBE	Methyl tert-butyl ether
MV	Method validation
MVS	Multichannel verification system
MWCO	Molecular weight cutoff
NCCLS	National committee for clinical laboratory standards
NCE	New chemical entity
NDA	New drug application
NHS	National health service
NIH	National institutes of health
NL	Neutral loss
NME	New molecular entity
NMR	Nuclear magnetic resonance
NPLC	Normal phase liquid chromatography

NRTI	Nucleoside reverse transcriptase inhibitor
NSB	Nonspecific binding
NSI	Nanospray ionization
OC	Oral contraceptive
OECD	Organization for economic cooperation and development
OEM	Original equipment manufacturer
OOS	Out-of-specification
OQ	Operational qualification
ORA	Office of regulatory affairs
OSI	Office of scientific investigations
PB	Protein binding
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCT	Pressure cycling technology
PCV	Packed cell volume
PD	Pharmacodynamics
PDA	Photodiode array
PDF	Portable document format
PEEK	Polyether ether ketone
PEG	Polyethylene glycol
PGC	Porous graphitic carbon
PI	Principal investigator
PK	Pharmacokinetics
PM	Preventive maintenance
PMP	Pressure monitoring pipetting
PMSF	Phenylmethylsulfonyl fluoride
PNPA	p-Nitrophenyl acetate
PoC	Proof of concept
PPE	Protein precipitation extraction
PPT	Protein precipitation
PQ	Performance qualification
PTM	Posttranslational modification
QA	Quality assurance
QAS	Quality assurance statement
QAU	Quality assurance unit
QC	Quality control
QqQ	Triple quadrupole
QqQ_{LIT}	Hybrid triple quadrupole-linear ion trap
QqTOF	Hybrid quadrupole time-of-flight
Q-TOF	Quadrupole time-of-flight
RAD	Radioactivity detection
RAM	Restricted access material
RBC	Red blood cell
RC	Relative carryover
RE	Recovery
RED	Rapid equilibrium dialysis
RF	Response factor
RFID	Radiofrequency identifier
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RT	Retention time
RT	Room temperature
RT-qPCR	Real-time reverse transcription polymerase chain reaction
SAD	Single ascending dose
SALLE	Salting-out assisted LLE
SAX	Strong anion ion exchange
SBSE	Stir bar sorptive extraction
SCX	Strong cation ion exchange
SD	Standard deviation
SDMS	Scientific data management system
SE	Standard error
SFC	Supercritical fluid chromatography
SFDA	State food and drug administration
SIL-IS	Stable isotope labeled internal standard
SIM	Selected ion monitoring
siRNA	Small interfering RNA
SLE	Supported liquid extraction
S/N	Signal-to-noise
SOP	Standard operating procedure
SPE	Solid phase extraction
SPME	Solid phase microextraction
SRM	Selected reaction monitoring
SSBG	Sex steroid binding globulin
STD	Standard
SUV	Small unilamellar vesicle
SV	Separation voltage
TADM	Total aspirate and dispense monitoring
TDM	Therapeutic drug monitoring
TEA	Triethylamine
TEAA	Triethylammonium acetate
TEAB	Triethylammonium bicarbonate
TFA	Trifluoroacetic acid
TGA	Therapeutic goods administration
THU	Tetrahydrouridine
TIC	Total ion chromatogram
TK	Toxicokinetics
TMA	Trimethyl ammonium
TPD	Therapeutic products directorate
TSCA	Toxic substance control act
TTFA	Thenoyltrifluoroacetone
UHPLC	Ultra high performance liquid chromatography
ULOQ	Upper limit of quantification
UPLC	Ultra Performance liquid chromatography
URS	User requirement specification
UV	Ultraviolet
WAX	Weak anion exchange
WBC	White blood cell
WHO	World health organization

PART I

OVERVIEW OF LC-MS BIOANALYSIS

1

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

Steve Unger, Wenkui Li, Jimmy Flarakos, and Francis L.S. Tse

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 μg/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

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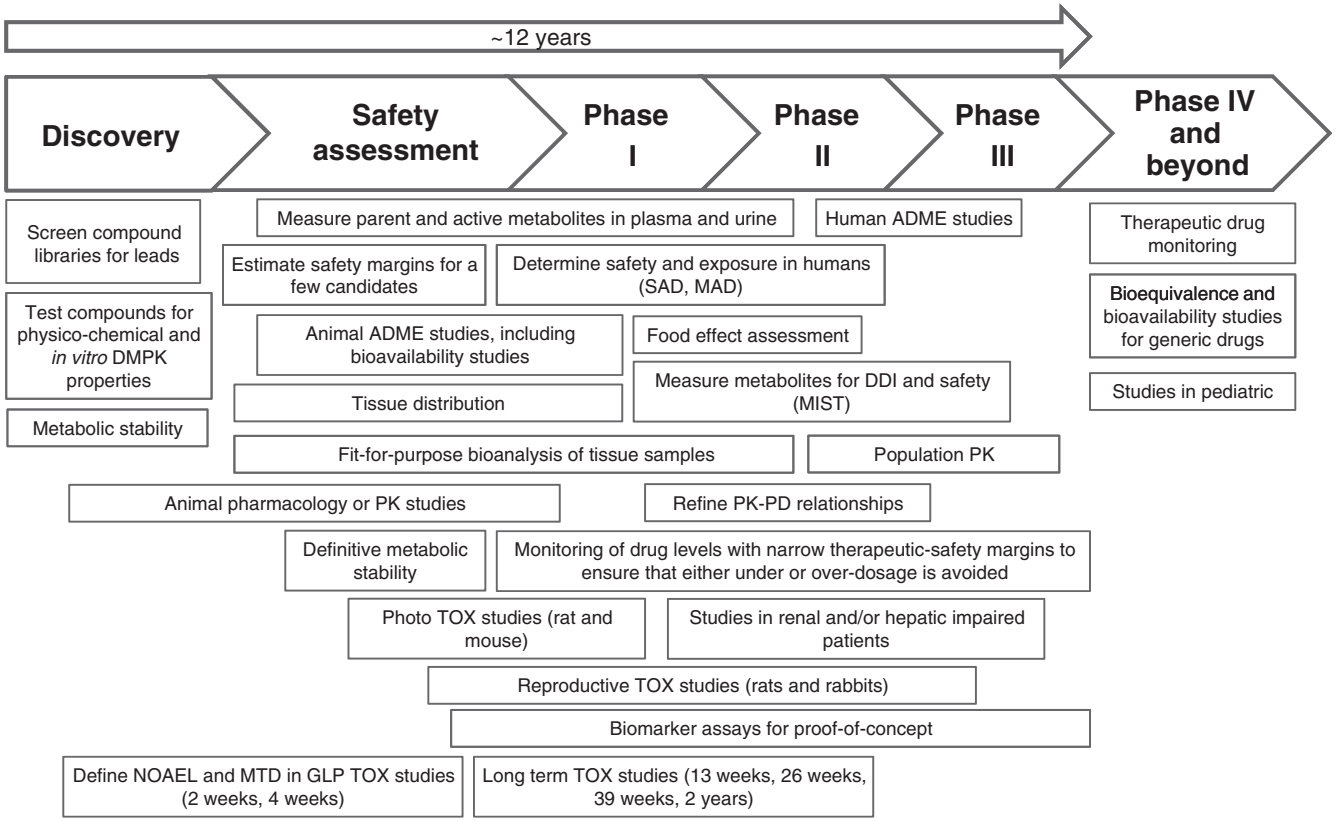

FIGURE 1.1 A flowchart of drug discovery and development, and postapproval studies of drugs where LC-MS bioanalysis plays important roles.

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

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$100 μCi of ^{14}C labeled drug mixed with unlabeled drug. LC-MS for measuring unlabeled drug is often used in human ADME studies to differentiate the parent compound from its metabolite(s). For studies employing microdoses ($<$100 μg) or doses of low radioactivity ($<$1 μCi), 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.

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 emtracitabine (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