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LC-MS/MS Bioanalysis Method Development, Validation, and Sample Analysis: Points to Consider When Conducting Nonclinical and Clinical Studies in Accordance with Current Regulatory Guidances

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Abstract

Recently, there has been a heightened public awareness of drug safety across the globe. Nonclinical and clinical pharmacokinetic and toxicokinetic toxicology safety studies all require that the study samples be analyzed under the auspices of good laboratory practice (GLP) standards. GLPs are followed in order to ensure that safety studies are reliable and accurate. Several countries have issued or are in the process of issuing their own versions of bioanalytical guidance documents for performing method validation activities; however, each one is slightly different. These differences often make complying with regulatory requirements difficult and cumbersome. Several networking organizations have been working diligently to harmonize the various global bioanalytical guidance documents. This paper attempts to shed light on some of the critical points to consider when performing bioanalytical method development and GLP compliant validation activities from a scientist's perspective.

Keywords: Good laboratory practice; Regulatory guidance; Nonclinical; Preclinical; Clinical; Pharmacokinetic; Toxicokinetic; Bioanalytical method; Method development; Validation

Abbreviations: AAPS: American Association of Pharmaceutical Scientists; ALQ: Above the Limit of Quantitation; ANDA: Abbreviated New Drug Application; ANVISA: Agencia Nacional de Vigilancia Sanitaria (Brazil); BA: Bioavailability; BFG: Bioanalytical Focus Group; BE: Bioequivalence; BLQ: Below the Lower Limit of Quantitation; CHMP: Committee for Medicinal Products for Human Use; CoA: Certificate of Analysis CPMP: Committee for Proprietary Medicinal Products; CRO: Contract Research Organization; CV: Coefficient of Variation; CVG: Calibration and Validation Group (Canada); DRF: Dose Range Finding; EBF: European Bioanalysis Forum; EMA: European Medicines Agency; FDA: Food and Drug Administration (United States) GBC: Global Bioanalysis Consortium; GCC: Global CRO Council GLP: Good Laboratory Practices; ICH: International Conference on Harmonisation; IND: Investigational New Drug; IS: Internal Standard; ISR: Incurred Sample Reanalysis; JBF: Japan Bioanalysis Forum; LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry; LIMS: laboratory inventory management system; LLE: Liquid-liquid extraction; LLOQ: Lower Limit of Quantitation; MD: Method Development; MF: Matrix Factor; MHFW: Ministry of Health and Family Welfare (India); MHLW: Ministry of Health, Labor and Welfare (Japan); MSDS: Material Safety Data Sheet NDA: New Drug Application; OTC: Over the Counter drugs; OECD: Organisation for Economic Co-operation and Development; PK: Pharmacokinetic; PPT: Protein Precipitation; QC: Quality Control; R^2: Coefficient of Determination; RE: Relative Error; RSD: Relative Standard Deviation; SLE: Solid-supported liquid extraction SOP: Standard Operating Procedure; SFDA: State Food and Drug Administration (China); TGA: Therapeutic Goods Administration (Australia); TPD: Therapeutic Products Directorate (Canada); TK: Toxicokinetic; ULOQ: Upper Limit of Quantitation

Introduction

Compliance with good laboratory practices (GLPs) for conducting sample analysis of nonclinical (also known as preclinical) laboratory studies and clinical studies is intended to ensure the quality and integrity of the safety data filed in support of investigational new drug applications (INDs), new drug applications (NDAs), abbreviated new drug applications (ANDAs), supplements in developing bioanalytical method validation information used in human clinical pharmacology, bioavailability (BA), and bioequivalence (BE) studies requiring pharmacokinetic (PK) evaluation [1-7]. Current US regulations do not provide specific requirements for conducting GLP nonclinical and clinical study sample analysis. Both the US FDA and the EMA have released respective bioanalytical guidance documents which focus on bioanalytical methods used for nonclinical and clinical PK/toxicokinetic (TK) studies and studies with the EMA Draft guidance even going as far as stating such studies be performed in compliance with GLP [1,2]. The position to conduct clinical and nonclinical bioanalytical method validation and sample analysis in compliance with GLP for this reason can vary simply by the country in which the work is being performed or in which the study data will be submitted. In an effort to accommodate a global pharmaceutical market, some companies within the industry have taken a position to perform such study analyses in compliance with GLPs. The ultimate goal for any study sample analysis or method validation, regardless of whether or not GLP compliance is enforced, is to ensure the bioanalytical methods used are proven robust through

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thorough development, validation (as per a protocol, study plan or standard operating procedure [SOP]), and applied as written in order to robustly quantify the analyte in the presence of a specific matrix. Method validations are instrumental in ensuring sample analysis methodologies can consistently and accurately determine actual concentration in incurred sample within a specific matrix.

Several globally recognized regulatory authorities responsible for the regulatory oversight of clinical and nonclinical laboratory studies have provided guidance for the design and conduct of these studies: Australia's TGA, Brazil's ANVISA, Canada's TPD, China's SFDA, Europe's EMA, India's MHFW, Japan's MHLW, and the United States' Food and Drug Administration (FDA) [1,2,8-14]. Unfortunately, the guidance provided by each recognized regulatory authority cannot be applied globally as additional harmonization and collaboration would be needed to address the differences between the individual positions provided by each authority. The US FDA issued the Guidance for Industry: Bioanalytical Method Validation in 2001[1]. Since then, three AAPS/FDA Bioanalytical Workshops (also known as the Crystal City conferences) have been held to discuss this guidance and to help bring clarity to the industry recommendations that were published [15-18]. Four Calibration and Validation Group (CVG) workshops [19-22] were also held to discuss the AAPS/FDA Bioanalytical Workshops and to further increase understanding and provide an industry consensus regarding the conduct of regulatory compliant bioanalytical validation and sample analysis. A report from the 2008 AAPS workshop on GLP Bioanalysis indicates a specific focus on assay reproducibility and incurred samples [23]. EMA issued the Draft Guideline on Validation of Bioanalytical Methods in 2009 [2]. As quoted at the second CVG workshop, "Each successive event resulted in progressive discussions of relevant topics, and allowed the industry to discuss and agree on a cohesive approach to evolving regulatory guidances" [20].

In the last decade, tremendous efforts have been expended by industry and regulatory authorities alike to establish guidances for bioanalytical method validation and sample analysis, resulting in several key publications and opinion papers on the topic [24-33]. Instrumental to these guidance efforts was the formation of several global networking groups: the American Association of Pharmaceutical Scientists Bioanalytical Focus Group (AAPS BFG), the Canadian Calibration and Validation Group (CVG), the European Bioanalysis Forum (EBF), the Global Bioanalysis Consortium (GBC), the Global Contract Research Organization Council for Bioanalysis (GCC), and the Japan Bioanalysis Forum (JBF) [34]. Each of these groups continues to actively pursue better guidance initiatives through various discussion groups and workshops: the 5th CVG Bioanalysis Workshop held recently in Montreal, Canada; the 2nd GCC Meeting for Bioanalysis held recently in Montreal, Canada; the 3rd EBF gathering held recently in Barcelona, Spain; and the planned 2011 first GCC European Meeting for Bioanalysis which will be held in London, UK.

The efforts of these global networking groups are greatly appreciated by many in the industry; however, a global approach to bioanalytical method validation and sample analysis will require regulatory authorities (around the world) to compromise their differences.

Many positions exist as to why one would or would not choose to perform PK/TK clinical method validation, PK/TK clinical analytical sample analysis, and nonclinical method validation studies in compliance with GLPs. The decision is specific to the particular business, geographical location, and program goals. This paper presents a general overview of LC-MS/MS method development, method validation, and sample analysis and includes a number of points to consider from several bioanalytical guidance references. Also presented are additional scientific points to consider when taking on nonclinical and clinical LC-MS/MS bioanalysis method development.

Study set-up and background information

This section provides an overview of all supporting processes and information that should be assembled and in place to support the conduct of bioanalytical studies.

Most critical to the success of any bioanalytical method development and validation study is initial communication with all appropriate personnel for the purpose of gaining a thorough understanding of the targeted development and validation project needs prior to initiating any experiments. Toxicology programs often have a campaign of studies in the pipeline that are intended to lead to a successful IND filing or needed to support a response to a regulatory hold. Toxicology programs will often require multiple species, multiple matrices per species, and several routes of dose administration [2,3,9-11,14]. On the clinical side, a clinical study protocol may involve analysis of several matrices and may include quantitation of the parent analyte and several metabolites [2,4-10,2,13]. In order to mitigate risk, an advanced knowledge of the toxicology or clinical study start dates and additional collaborative efforts to complete core method validation experiments prior to toxicology or clinical study dosing and sample collection, should be gathered. The entire method validation effort that will support the toxicology or clinical program should use consistent methodologies based on sound scientific judgment. Efficient study starts and successful study completion will often depend on the type and quality of information obtained from all initial stakeholders (CRO's should specifically consider consulting the sponsor and the sponsor's chemist) in addition to electronic searches and literature reviews. It is most desirable that as much information as possible be collected prior to beginning the wet chemistry. Documentation for any other supporting bioanalytical methods that have been previously developed and validated for the analyte of interest should also be considered. Information regarding physical chemical information on pKa, solubility, tendencies to adhere to glass and plastic, photodegradation, and stability during preparation and storage should also be obtained.

The toxicology or clinical study excipients, concentrations, volumes, and routes of dose administration may have an adverse effect upon method selectivity and may impact selection of the sample preparation strategy. The analyte reference standard must be clearly defined by the sponsor and must be appropriately characterized (source, lot number, identity, strength / purity, composition, and expiration or retest date) through means of a certificate of analysis or other traceable quality statement which appropriately characterizes the test or control article by defining where possible the preferred nomenclature, salt form, chemical structure, and formula weight. A material safety data sheet (MSDS) or documentation for appropriate storage and safe handling should also be available. For the internal standard (IS), a certificate of analysis (CoA) and expiration dating are not required due to the ability to monitor and track analytical performance of the IS with the analyte; however, safe handling information must be supplied. A stable isotope labeled IS or an appropriate analog should be used whenever possible. For nonclinical studies, the selection of the species and/or matrix for which the initial full validation is to be performed is often dependent on susceptibility of the analyte to be unstable and/or to enzymatically degrade. Method validation matrices should be clearly defined by species and strain for nonclinical studies or special clinical study populations (for example: BALB/c mouse, Sprague-Dawley Rat, New Zealand White Rabbit, Beagle Dog, Göttingen Minipig, Cynomolgus

Monkey, human, etc.) and should match the toxicology or clinical program/study needs. For clinical studies, often genetics, gender and age must be clearly defined. The blank matrix should be obtained from a reputable source and must have proper identification of species and matrix type, lot number, storage information and expiration dating. The matrix physiological properties (lipemic, hemolyzed, etc.) can also be important to note. If an anticoagulant is used, it should be selected based on the known properties of the analyte, should not impart undesirable properties such as significant changes in pH, instability, precipitation or gel formation, and should be consistent for the entire toxicology or clinical program. If a stabilizer or enzyme inhibitor is required, it should not interfere with quantitation of the analyte. A priori acceptance criteria must be documented and can be and are often driven by SOPs, but sound scientific judgment, based upon empirically derived method development data should dictate when modifications are warranted prior to finalization of the study protocol/study plan and execution of validation experiments.

Method development

Nonclinical and clinical bioanalytical method development (MD) activities are not required to be performed in compliance with GLPs but should be adequately documented to support a reproducible method document for validation. Method development is intended to define the method and provide sound scientific evidence for method design and suitability for its intended purpose. If the MD experiments outlined in Table 1 are incorporated into the MD plan and properly executed, then the resultant method design should be deemed suitable to proceed with validation. The following aspects should also be considered during MD:

- "Carryover should be addressed and minimized during method development" [2]. If carryover is inevitable or unavoidable, it should be noted in the method and a non-randomized sequence should be used with extra blanks inserted after the high calibration standard and high quality control (QC) injections.
- Precautions should be taken to eliminate any possibility of contamination of the mobile phases, diluents, wash buffers, stocks, and working stocks. Use of disposable glassware, pipet tips with filters, and automation, whenever possible, is highly recommended.
- Analyte stability (freeze/thaw and use of stabilizer agents) should be assessed during MD to help avoid delays in validation activities due to QC (analyte in matrix) stability issues.
- Selection of the anticoagulant should be made by the MD scientist and should remain consistent throughout the entire toxicology program (nonclinical and clinical). If the anticoagulant or counter-ion is changed for a PK/TK study, at a minimum an equivalency experiment must be performed to show that the existing method can be used for the new anticoagulant. Additional proof of stability may also be necessary.
- Whole blood stability and collection process stability are not often performed as a method development activity; however, assessment may provide valuable information for both nonclinical and clinical studies. The analyte may adsorb to cellular or proteinaceous components during the time period between collection and sample processing.
- An impact assessment on quantitation of analyte interference in matrix containing lipids and hemolysis is not required by the FDA Bioanalytical Guidance; however, considerations should be made for this assessment if the desired submission will target Brazil or an EMA country [2,9].
- Effect of the dosing vehicle on the matrix should also be evaluated during MD, particularly for nonclinical studies, depending on the dose route. The dosing vehicle can cause potential interference or matrix ionization effects, as the vehicle may be present at relatively high levels for nonclinical samples. This may not be an issue for the clinical method, as the dosing vehicle is typically present at low levels in clinical study samples. Depending on the excipient, this is a requirement within the EMA draft guidance [2].
- If metabolites are known and standards are available, then an evaluation of metabolite impact on quantitation of analyte is required. In nonclinical studies, these are not often known, and if they are, there may be no reference standards available or characterized.
- During MD, specific instrument parameters must be clearly defined to ensure a consistent parameter approach can be set and remain fixed during validation: mobile phase composition, gradient profile, ionization mode, HPLC column identity. Minor parameters may be optimized to account for instrument to instrument variability and improve response (i.e., voltage and gas settings, precursor and product ion mass adjustment to optimize mass center).
- Once the sample preparation parameters are refined, they must remain fixed during validation. Changes in aliquot volume or sample preparation parameters necessitate at least partial revalidation. The type of sample preparation procedure utilized must be fit for purpose, and is dependent upon the analytical range, study size (i.e., small nonclinical study versus large clinical study), and matrix type. Several types of sample preparations may be evaluated during MD: simple dilution in diluent, protein precipitation (maximum 100 μL sample volume to get an efficient precipitant), liquid-liquid extraction, supported-liquid extraction or solid phase extraction.
- During MD, phospholipid removal should be evaluated and the impact on the assay mitigated by using different sample preparation techniques, different chromatographic gradients, or by employing a diversion valve to shunt the phospholipids to waste before they enter the HPLC column.
- Once the MD experiments have been satisfactorily completed, a

Overview of Method Development Exercises
Analyte Information
• Structure
• Properties
Method Optimization
QC Preparation Appropriate to Range
Evaluation of Extraction Strategies (Protein Precipitation, LLE/SLE, SPE)
• Phospholipid impact evaluation
• Recovery
• Matrix Stability
• Extract Stability
• Initial Carryover Evaluation
Post-column infusion experiment
Mock Validation Run
• Accuracy and Precision
• Carryover
• Freeze / Thaw Stability
• Bench Top (pre-processed) Stability
• Long Term Stability
• Recovery
• Matrix Effect
• Selectivity
Length of run should mimic longest expected sample set, padded with blanks

Table 1: Overview of Method Development Exercises.

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"mock validation run" should be performed to test the draft method design prior to proceeding with validation.

- It is also recommended to use a pilot study or dose range finding samples to evaluate vehicle effects, selectivity, curve range, etc., before proceeding to method validation.

Method validation

Regulatory guidances for bioanalytical validation studies suggest the following validation parameters be defined in a protocol, study plan or SOP along with applicable acceptance criteria prior to execution of the experiments [1,2]: selectivity, matrix effects, accuracy, precision, reproducibility, sensitivity, linearity and range, lower limit of quantitation (LLOQ), upper limit of quantitation (ULOQ), and stability evaluation (stock solution, freeze/thaw, short term, long term, and post-processed). Method validation may involve a single analyte, multiple analytes, or a parent and metabolites. In any case, validation should be performed using the same matrix as targeted for study sample analysis. As per the AAPS BFG comment [34]: "During method validation, a blank biological matrix will be spiked with the analyte(s) of interest using solutions of reference standard(s) to prepare calibration standards, QC and stability samples. In addition, suitable internal standard(s) is/are added during sample processing in chromatographic methods." "During validation, carryover should be assessed by injecting two blanks following a high QC or calibration standard" [2]. The current validation experiments performed at MPI Research for clinical and nonclinical studies are defined in Table 2 and Table 3.

A full validation is performed when implementing methods for the first time, or when additional analytes or metabolites are added for quantitation. A partial validation is performed when the method is transferred to a different lab, the instrumentation / detection system / software is changed, the matrix is changed within species, the species is changed within matrix, the sample preparation procedure is modified, the standard curve concentration range is changed, or if there is a need to evaluate selectivity in the presence of metabolites or concomitant medications [1,18]. A partial validation is required when the anticoagulant counter ion changes from K to Na, but not when the counter ion stoichiometry changes from K_2 to K_3 [20]. System suitability tests are not a requirement, but are recommended [17,20].

- Nonclinical methods (non-human species)

For nonclinical methods, a full validation is usually performed first in the rat due to the need for enhanced care regarding known enzymatic effects. A partial validation is performed for the second species unless the method has been altered, and then a full validation is highly recommended.

- Clinical methods (human)

For clinical methods, full validations are required. When validating

Overview of Validation Exercises and Criteria		
Validation Exercise	**Minimum Experiments**	**Performance Criteria**
Selectivity	Matrix blanks: 6 lots, n=1 for each lot Matrix blank fortified with IS: 6 lots, n=1 for each lot LLOQ Selectivity Sample: 6 lots, fortified with analyte at LLOQ level and IS. n=3 for each lot	At least 5 out of the 6 lots must meet the following criteria: Response for the analyte in matrix blanks or matrix blank fortified with IS must be ≤20% of the mean analyte response in the acceptable LLOQ calibration standards Response for IS in matrix blanks must be ≤5% of the mean IS response in the acceptable LLOQ calibration standards At least two-thirds of the selectivity LLOQ replicates for each lot must meet accuracy acceptance limit, and the mean accuracy must be within ±20.0% of the nominal concentration
Cross-analyte Interference	Each analyte at ULOQ evaluated separately. IS at the level of use evaluated separately	Interference must be ≤ 20% of the mean analyte peak response or ≤ 5% of the mean IS response of the acceptable LLOQ calibration standards
Carryover	Minimum of 2 blank samples after the injection of each ULOQ standard	Analyte response ≤20% of the mean analyte response in the acceptable LLOQ standards, IS response ≤5% of the mean IS response in the acceptable LLOQ standards
Linearity	Minimum of 6 non-zero calibration standard (CS) levels	(R^2) ≥0.985
Calibration Standards: Accuracy	Injected at the beginning and end of the analytical run	Minimum 6 non-zero (or 75% of total) CS must be within ±15.0% RE of nominal (exception: LLOQ within ±20.0 %RE)
QC Samples – Core Validation	Three concentration levels: Low, Mid, High; n=6 at each level	Minimum 50% of the QC replicates at each level and 66.7% of all QCs must be within 15.0% RE of nominal Mean inter- and intra-assay accuracy within ±15.0% RE of nominal; precision ≤15.0% RSD
QC Samples – Ancillary Runs	Three concentration levels: Low, Mid, High; n ≥ 2 at each level	≥66.7% of all QCs and at least 50% at each level must be within 15.0% RE of nominal
LLOQ Samples (Sensitivity)	n=6, ≥ 1 run	Mean accuracy within ±20.0%RE of nominal; precision ≤20.0% RSD
Recovery	Analyte at low, medium and high levels, and IS at the level of use: pre-extraction spiked samples (n=6) are compared with mean response of post-extraction spiked matrix samples (n=6)	Recovery for analyte and IS must be relatively consistent across all QC levels
Dilution (Parallelism)	One level (minimum 10 fold dilution); n=6	Mean accuracy within ±15.0% RE of nominal; precision ≤15.0% RSD
Matrix Effect	Post-extraction spiked samples (n=6, at each QC low, mid and high level) are compared with mean response of 6 injections of analyte or IS in solvent	MF will be calculated and reported for the analyte and for the IS
Ruggedness	Minimum of two variables over the course of validation (e.g. different column, instrument and/or analyst)	Mean inter- & intra-assay accuracy within ±15.0% RE of nominal; precision ≤15.0% RSD

Table 2: Overview of Validation Exercises and Criteria.

Overview of Validation Stability Exercises and Criteria		
Validation Exercise	**Minimum Experiments**	**Performance Criteria**
Stock Solution	n ≥6; long term at typical storage conditions; bench top at conditions representing typical processing conditions for ≥6 hours	Precision of area response or relative response must be ≤15.0% RSD; RD within 7.0% for analytes, 20.0% for internal standards
Bench top	≥ 4 hours n ≥6 at QC Low and High levels	Mean accuracy within ±15.0% RE of nominal; precision ≤15.0% RSD
Freeze/Thaw	3 freeze/thaw cycles n ≥6 at QC Low and High levels	Mean accuracy within ±15.0% RE of nominal; precision ≤15.0% RSD
Long term	n ≥ 6 at QC Low and High levels at -10 to -30°C or -50 to -90°C for at least **1** and 4 months.	Mean accuracy within ±15.0% RE of nominal; precision ≤15.0% RSD
Reinjection Reproducibility	Calibrations standards (CS) and QCs (n=6 at each level) re-injected from an acceptable validation batch run, maintained at autosampler temperature for ≥72 hours.	Mean accuracy within ±15.0% RE of nominal; precision ≤15.0% RSD; calculated using calibration standards from re-injected run
Extract Stability	Stored extracts at QC Low, Mid, and High levels (n=6) maintained at autosampler temperature for ≥72 hours.	Mean accuracy within ±15.0% RE of nominal; precision ≤15.0% RSD; calculated using freshly extracted curves or back calculated using the original curves from the batch the aged extracts were originally extracted and injected

Table 3: Overview of Validation Stability Exercises and Criteria.

between matrices (i.e., human plasma to human urine), a full validation is recommended to evaluate precision, accuracy, selectivity, and stability. For minor changes to a method within the same matrix and calibration range, a partial validation may be performed. For example, if the internal standard for a plasma assay that has been fully validated is changed, it is a full validation is not required. This is because ancillary experiments that are independent of IS identity such as matrix stability are not be affected by a change in internal standard. Ancillary experiments that are impacted by IS identity such as reinjection reproducibility or extract stability must be repeated. The changes being made to the method must be evaluated to determine which experiments should be repeated during the partial validation, keeping in mind the purpose of each experiment and the effect the change would have on each experiment.

Regulation and regulatory guidances

Bioanalytical method validation activities in the US are not required to be performed in accordance with GLP regulations; however, there are a number of regulatory guidances available to steer method validation design requirements and in the case of EMA even GLP study conduct. Although compliance to GLP regulations is not required for US method validations, the framework of the GLP regulations when applied to a method validation or clinical sample analysis can provide a better quality system structure which, in return, can bolster documentation details and can enable advanced planning as well as provide for better investigational references should the need arise.

Australia's [8] Guidance states that, "The Bioanalytical methods used to determine the active moiety and/or its biotransformation product(s) in plasma, serum, blood or urine or any other suitable matrix must be well characterized, fully validated and documented to yield reliable results that can be satisfactorily interpreted. The main objective of the method validation is to demonstrate the reliability of a particular method for the quantitative determination of an analyte(s) concentration in a specific biological matrix. The characteristics of a Bioanalytical method essential to ensure the acceptability of the performance and the reliability of analytical results are: (1) stability of the stock solutions and of the analyte(s) in the biological matrix under processing conditions and during the entire period of storage; (2) specificity; (3) accuracy; (4) precision; (5) limit of quantitation and (6) response function."

Brazilian Guidance requires selectivity to be performed using six sources of matrix, four normal, one lipemic and 1 haemolysed [9,22]. Certification for the IS required. The curve must have 67% of the standards meet the acceptance criteria.

Canada's [10] guidance states that, "The principles and procedures for analytical validation described in the summary document "Analytical Methods Validation: Bioavailability, Bioequivalence, and Pharmacokinetic Studies," V.P. Shaw et al. (1992), Journal of Pharmaceutical Sciences 81(3) and "Workshop/Conference report – Quantitative Bioanalytical methods validation and implementation: Best practices for chromatographic and ligand binding assays," C.T. Viswanathan et al (2007) The AAPS Journal 9 (1) Article 4, should be followed. In addition to pre-study validation, appropriate performance characteristics (accuracy, precision, quality control) should be documented for each analytical run during a study."

China: The SFDA issued a GLP guidance for nonclinical pharmacokinetic (PK) studies for investigational new drug submission in 2005 [11]. The status of bioanalytical guidance in China is discussed in the article by Tang and Zhong [31]. The article includes a detailed comparison table of method validation guidelines from the SFDA, US FDA, and EMA draft. "The scope of the SFDA guidelines for full validation is the same as the US FDA and the EMA. For GLP TK studies in which multiple species are used, both the SFDA and the FDA guidelines state that only partial validation is required when different species are used.China requires that nonclinical PK and TK studies be conducted in compliance with GLPs. Partial validations are required when multiple species are used" [31]. The notable areas are as follows: 1) the LLOQ must be sufficiently defined to quantify the drug at 3 to 5 times T1/2, or 1/10 to 1/20 of C_{max} with acceptable accuracy and precision; 2) there is no requirement for the curve regression model; and 3) recovery must be performed at the QC Low, Mid, and High levels. The SFDA guidelines do not specify acceptance criteria for many of the evaluations.

EMA: No Bioanalytical guideline currently available, new bioequivalence guideline with a section on Bioanalytical methods, use ICH/FDA/current scientific knowledge. Draft CHMP "Guideline on Validation of Bioanalytical Methods" [2] is in progress now. It is noted that the EMA states that "The validation of Bioanalytical methods and the analysis of study samples should be performed in accordance with the principles of Good Laboratory Practice (GLP), and that "A full validation should be performed for each species concerned" [2]. This draft guidance covers the following parameters: selectivity (6 sources blank matrix, interference of metabolites, degradation products, co-meds, back conversion to parent), carryover (high QC or curve std, then blank), LLOQ, calibration curve (6 stds, >75% comply, fresh curves), accuracy and precision (LLOQ QC, Low QC, Mid QC, High QC; RE <15% / <20% LLOQ QC; 3 runs/2 days), dilution integrity (>ULOQ, <15%), matrix effect (6 lots, haemolysed, lipemic, special populations,

MF <15%, specific excipients (poly(ethylene glycol), polysorbate)), stability –vs.- fresh curves (Freeze/Thaw, Short Term, Long Term, Stock Solution Stability, Post-preparative, Bench Top, Autosampler; <15%). "It is noted that the EMA requires a full validation for species changes" [31].

Japan has GLP regulations, but no GLP guidance for bioanalytical method validations.

OECD: "GLP is a quality system concerned with the organizational process and the conditions under which non-clinical health and environmental safety studies are planned, performed, monitored, recorded, archived and reported" [14] OECD countries adhere to the Mutual Acceptance of Data practices.

Sensitivity

The lowest standard on the calibration curve is used to determine the limit of quantitation (LLOQ). The LLOQ is the lowest assessed concentration which can reproducibly give an analyte response that is both accurate (100±20% recovery) and precise (≤20% RSD). The Bioanalytical Guidance [1] states that the LLOQ should be at least 5 times the response compared to the blank response. The EMA Guidance [2] states that "the LLOQ should be adapted to expected concentrations and to the aim of the study". Typically ng/mL levels are utilized for pre-clinical Bioanalysis; for example, 1-1000 ng/mL range. Clinical bioanalysis methods often require lower LLOQ, down to pg/mL level, which may limit the choice of sample preparation techniques and instrument platforms.

Selectivity

Selectivity is an important component of method validation. "The method must be able to quantify the analyte in the presence of endogenous compounds, degradation products, other medicines likely to be present in study samples, and metabolites of the medicine(s) under study" [8]. There are several items to be considered when evaluating the selectivity of the assay. Evaluation of a minimum of 6 different lots or sources of matrix must be performed as matrix blanks (containing no analyte or IS). Additional lots beyond the six required lots should be added when needed in order to test each of the expected selectivity scenarios. For nonclinical studies with large animals, six individual lots are recommended, while studies with small animals (i.e., rat or mouse) may use pooled lots. For clinical assays, lots from individual donors are suggested. Choice of the matrix lots should be based on the expected composition of study samples. For example, if specific constraints are placed on the study samples, then the choice of selectivity lots should reflect these constraints. Some analytes generate dissimilar results in different genetic populations, different genders, or different age groups. For clinical studies in which there are fasted and fed components, the choice of selectivity lots during validation should include a choice of lots from fasted subjects and fed subjects in order to evaluate the potential impact of fed/fasted state on the matrix. In addition, hemolytic and lipemic plasma should be evaluated to determine the impact on quantitation. For evaluation of hemolytic and lipemic lots, it is suggested that QC samples be prepared at high and low levels of hemolysis/lipemia and prepared per the analytical method. Acceptance criteria for these samples should mimic that of the assay (relative error ±15%, relative standard deviation ≤15%).

Evaluation of the impact of co-administered drugs, over the counter drugs (OTCs), and metabolites should be performed using matrix blanks in at least one lot of control matrix. Interference from the co-meds or metabolites must be ≤ 20% of the analyte response in the LLOQ. For clinical studies, specificity in the presence of OTCs and metabolites of OTCs should be performed. If the co-meds are known and are stated in the clinical protocol, then those specific chemical entities should be evaluated. While it is a good practice, we cannot always know what OTCs the patients may be taking – and the Sponsors do not always want to pay for the additional experiments. Metabolite profiling is required. Any metabolite >10% of the parent should be quantitated (dependent upon activity or toxicity). Limited drug and metabolite method information prior to preclinical method development is a challenge. For clinical analysis, metabolite information is often available from existing preclinical methods. However, human metabolite profiles can be different from animal species. For human use, the EMA requires an impact assessment for possible back-conversion to minimize incurred sample reanalysis (ISR) problems.

Matrix effects (Ion suppression, ion enhancement)

"Matrix effects should be investigated to ensure that precision, selectivity, and sensitivity will not be compromised" [1]. "Matrix effect is the suppression or enhancement of ionization of analytes by the presence of matrix components in the biological samples. Quantitative measurement of matrix effect provides useful information in validation of MS-based bioanalytical methods. The quantitative measure of matrix effect can be termed Matrix Factor (MF) and defined as a ratio of the analyte peak response in the presence of matrix ions to the analyte peak response in the absence of matrix ions" [17]. Stable isotope labeled IS can compensate for matrix effects on quantification of the target analyte.

Recovery

Recovery of the analyte and internal standard must be evaluated to determine loss during sample preparation and matrix ionization. Recovery can be defined as the detector response from a sample of a given concentration of analyte added to the biological matrix prior to sample preparation compared to the detector response from a sample of the same concentration of analyte added to the biological matrix after sample preparation. A stable isotope labeled IS may compensate for any loss that occurs during sample preparation or due to matrix effects. However, due to the cost of these compounds, a stable isotope labeled IS may not be available during the pre-clinical phase.

As with selectivity, recovery of the analyte may be affected by the presence of metabolites, co-administered drugs, and the lack of population controls. Some metabolites, such as acyl glucuronide, N-oxide, and lactone drug, are unstable and can convert to the parent drug during sample preparation [20]. Pre-clinical studies are often conducted using naïve animals; thus, these co-administered drug products to not pose a concern. However, human subjects are exposed to a variety of different drug substances over a lifetime and possibly during the study. Additionally, population controls such as diet or environment do not exist for most clinical trials, meaning that the effects of these influences may need to be evaluated for their effect on the recovery and quantitation of the analyte.

Calibration curves

Fresh calibration curves must be used for method validation. Frozen calibration curves may only be used after stability has been determined. Each run should contain two curves, one at the beginning and one at the end of the injection sequence. Calibration curves should be prepared in the same biological matrix as the intended study samples; however, they may be prepared in a surrogate matrix if the study matrix is rare. Validation experiments that show the proxy

matrix does not impact the assay should be performed if a proxy matrix is employed. Per FDA guidance, the simplest regression model that adequately describes the concentration-response relationship should be used. A linear regression with 1/x or $1/x^2$ weighting is typically sufficient. The use of a quadratic fit is allowed but should be limited, and the cause for non-linearity should be evaluated. The selection of final regression model and weighting factor should be based on all of the validation data, and evaluation should be documented.

Matrix stability

Stability of the analyte in matrix must be evaluated at ambient conditions (bench top), through at least three freeze/thaw cycles, and for storage stability (short term and long term). Stock solution and working stock solution stability for the analyte and for the IS must be performed. Post-processed or autosampler stability on the instrument must also be performed. A detailed discussion on the assessment of processed sample stability in bioanalytical methods is presented in the CVG 3 white paper [21]. The chosen stability intervals and durations must be selected to support the actual needs of sample analysis. For clinical studies, stability at both frozen (-10°C to -30°C) and ultralow frozen (-50°C to -90°C) should be evaluated, due to the lack of availability of ultralow frozen storage at many clinics. Evaluation of several days of ambient stability or refrigerated stability may also be needed to support samples generated at the clinics. Stability samples must be within ±15% relative error of the nominal concentration and have a precision of ≤15% RSD in order to show acceptable stability. Qualifier QC samples must be included with stability QC samples in order to ensure run integrity. Evaluation of stability in the presence and absence of coadministered drugs should be evaluated. If using an altered matrix due to endogenous levels or matrix interference (i.e., charcoal stripped), evaluation of stability in both the altered and unaltered matrices must be performed. An aliquot of the lot of unaltered matrix that was used to prepare the stability QC samples should be stored with the stability QC samples. This lot will be analyzed in replicates along with the stability QC samples in order to determine the baseline endogenous concentration in the matrix, and the average concentration in the blank replicates may be used to correct for the endogenous levels in the unaltered matrix stability QC samples. Alternatively, the unaltered matrix stability QC samples may be compared to the average time-zero concentration in order to determine stability.

Post-preparative stability

Post-preparative stability is established through "reinjection reproducibility" and "extract stability". Reinjection reproducibility is performed to define the period of time in which an entire run can be reinjected and produce the same reportable results. In this experiment, the originally extracted standards and QC samples are reinjected after storage at autosampler conditions. Extraction stability defines the period of time in which the extracted samples are representative of fresh extracts. Extract stability may be evaluated by comparing the results from injection of stored extracted QCs versus freshly extracted curves and QC. An alternate approach is to quantitate reinjected QC extracts against the results of the initial injection of the curves associated with the reinjected QCs. It is recommended that both types of post-preparative stability be executed during validation.

Accuracy and precision

The purpose of validation is to ensure that the methods developed are sufficiently accurate and precise to quantify the actual concentrations of analyte which will be present in the study samples. Accuracy is defined as the actual back calculated concentration versus the expected nominal concentration of the QC samples. The US [1] and Brazil [9] both require a minimum of 5 determinations at each level. The QC samples are prepared by spiking analyte into matrix at Low (three times the LLOQ), Mid (around 50% of the logarithmic curve range) and High (at about 75% of the upper limit of quantitation (ULOQ)) concentrations [2]. Dilution QC samples are prepared at 5 to 100 times ULOQ for assessment of the ability of the method to accurately quantitate study samples which are initially above the limit of quantitation (ALQ) during sample analysis (this is also known as dilution integrity). QC samples are also prepared at the LLOQ validate the sensitivity of the assay. Samples with concentration below the LLOQ are below the limit of quantitation (BLQ). "The QC samples are analyzed against the calibration curve, and the obtained concentrations are compared with the nominal value" [2]. An accuracy value of not more than 100±15% should be attained for Low, Mid, and High QC samples, and not more than 100±20% for QC samples at the LLOQ [1,2,10], with at least 50% at each level meeting acceptance [1]. Precision is the degree of reproducibility and is usually reported as a %RSD. A value of not more than 15% for Low, Mid, and High QC samples, and not more than 20% for the QC samples at the LLOQ is acceptable. Accuracy and precision should both be compared within independent runs (i.e., intra-run), and between at least two different runs (i.e., inter-run). It is highly recommended that at least one of the runs contains the same number of samples as will be expected in the longest anticipated sample run.

Tracking the IS response variation is also critical to consistent assay performance. There is an assumption that the IS will correct for variability and compensate for differences between different matrix sources. The minimum IS response and the maximum IS response should be monitored during a run sequence.

Chromatographic re-integration

Chromatographic reintegration (which would include manual modifications) is addressed in the third AAPS/FDA Bioanalytical Workshop, in the EMA draft guidance, and the CVG 3. [2,17,21]

"Regarding chromatographic methods, source documentation should include original and reintegrated chromatograms for accepted runs, along with the reason for changingintegration parameters across a run or for individual samples within a run."

This statement is representative of the current industry view on reintegration modifications; however, current practices may still exhibit some variability across the industry. The following guidelines are offered.

Strive to use good scientific judgment to set the integration parameters optimally for all samples in a run. Integration should be consistent across runs. Significant differences may be an indication of a lack of method ruggedness. At best, manual modification should be an infrequent occurrence; although, there are some applications where higher frequencies of sample variability are expected to occur. These may require manual modification or reintegration. A means of ensuring that data is produced without undue introduction of bias is required in those cases where manual modification or reintegration within a run is used. Consideration should be given toward establishing and implementing a policy to define the following:

- Circumstances under which it may be necessary to use manual modification or within run reintegration
- The acceptable procedures to be employed

- The documentation process; what will be recorded, when and by whom
- The approval or oversight process; management oversight to be included, what level and when
- Follow existing guidelines for acceptance criteria and consider defining the number or percentage of runs at which it is no longer acceptable to employ manual modification. This approach may also include a procedure to conduct an investigation into method for optimization.

Determine the company position on manual modifications in either a standard operating procedure or formal written policy and actively follow it.

Carryover

Carryover can adversely affect accuracy and precision [35-38]. During validation carryover is evaluated by injecting blank extracts after the highest calibration standard or QC High sample [2]. If the peak response in the carryover blank exceeds 20% of the LLOQ response, carryover is considered significant. When carryover is inevitable, it needs to be noted in the method. The method should also contain recommendations for minimizing the impact of the carryover during analysis of study samples, such as placing extra blanks throughout the sequence or injecting the toxicology samples in a specific order. Unexpected carryover must be investigated. Major sources of carryover include inappropriate sample diluent, inappropriate mobile phases and adsorption of the analyte to instrument components.

Contamination

Contamination can adversely affect accuracy and precision [37,38]. If the peak in blank samples is greater than 20% of the LLOQ response, contamination is significant and should be investigated. Major sources of contamination include spills, aerosols, splashing, mixing and drip during liquid transfer steps. For solid-phase extraction, the possibility of contamination is highest during dilution, elution and evaporation steps. For liquid-liquid extraction and protein precipitation sample preparations, contamination may occur during dilution, mixing of organic solvents, supernatant transfer, and evaporation steps. As a preventive measure, disposable secondary containers should be used whenever possible. Glassware should be cleaned thoroughly before use. Workbenches, pipettes, vacuum manifolds, and evaporation needles, etc., should be cleaned prior to use. Gloves should be changed frequently during sample preparation.

Incurred sample reanalysis

In most instances, ISR is required for pre-clinical and clinical bioanalysis, to demonstrate assay accuracy and reproducibility [39,40]; however, Health Canada maintains that ISR is not a requirement at this time [21]. ISR discrepancy may indicate stability or specificity problems, specific to incurred samples, which were not apparent with QCs during validation. After the 2006 AAPS/FDA Workshop Report [17] was issued, most studies began using repeat of individual samples to include both C_{max} and elimination phases. The acceptance criterion is two-thirds of reportable values must be within 20% of initial values. The ISR data is presented separately and not as study values.

ISR verifies that variables that could affect the analytical results are adequately controlled when the method is applied to study samples. The assessment should be conducted at least once for each matrix for each species used for GLP toxicology studies.

In practice, ISR may not be feasible for pre-clinical studies with very small collected sample volume, or when the method requires a large aliquot size that consumes the collected sample volume. For clinical studies, the extent and nature of ISR is left to the analytical investigator; however, it is recommended that ISR be assessed for every clinical study. Factors such as concentration, patient population, special population, concomitant medication, and metabolites, should all be considered during ISR sample selection. First in human, proof of concept in patients, special population, and bioequivalence studies are examples of factors to be considered for ISR.

In instances of ISR failure, investigation is mandatory. ISR failure can be caused by contamination after initial analysis, drug instability, metabolites conversion to the parent drug, protein binding differences, concomitant medication interference, variable recovery, sample inhomogeneity, matrix effects, etc. ISR needs to be conducted in a timely manner after the initial analysis to avoid potential complication from drug instability and potential metabolite conversion.

Investigations

A well-documented study will include investigations that must be performed using sound scientific judgment and an SOP driven process. The investigations must be “timely, unbiased, well documented and scientifically sound” [20]. The ultimate goal is to identify the root cause of the failure. The possible outcomes of an investigation are an assignable cause, a possible assignable cause, or no assignable cause [21]. “When the cause is uncovered, corrective action must be implemented and the potential impact on previously generated data evaluated” [20]. Investigations should be performed if inconsistent replicate analyses occurs, when samples are obtained outside of the assay range (ALQ or BLQ), when sample processing errors are noted to occur, when equipment failures occur, when power outages occur, when the software malfunctions, if poor or inconsistent chromatography is observed, and if the study data is inconsistent (not allowed for EMA human PK studies). Investigations should occur for peaks in the blank chromatograms, or inconsistent IS response. If peaks are observed in the pre-dose or control samples, then the facility responsible for sample collection may need to be notified. The SOP must clearly define how to consistently address failed runs for multi-analyte assays, including how to report the results when analysis for one analyte met the acceptance criteria and the other(s) did not meet the acceptance criteria. Investigations must be initiated whenever anomalous results occur, whenever unexpected trends occur, and whenever out of acceptance criteria results occur. When an investigation is required a validation should be suspended and no samples should be analyzed until the investigation is concluded and corrective actions have been implemented. Investigation results should be maintained in the study data.

Project logistics

The project logistics for conducting bioanalysis for a clinical study require different considerations than those for a pre-clinical study. For a pre-clinical study, the method development, validation, and sample analysis are often handled by the same analyst or same group due to the relatively small study size. This format reduces handoff and variability. Because clinical studies are typically larger, multiple individuals, sites or instruments may be employed. During validation, ruggedness of the assay may be evaluated to demonstrate equivalence of multiple analysts, instruments, and or LC columns. If multiple sites are involved, method transfer validations are required. Method performance across sites and communication may pose challenges in these cases.

The 4th CVG workshop emphasized the importance of sample chain of custody, sample shipment, sample storage, inventory, temperature monitoring, and LIMS system compliance. "The workshop consensus is that a well controlled procedure must be established with good documentation and a defined chain of custody" [22].

Given the large number of study samples for a clinical study, it is desirable to employ automation as much as possible. During the pre-clinical stage, it may not be cost-effective to generate the automated processes. Additionally, the preclinical species may pose issues with automation due to limited sample volumes. However, the benefits of automation for high throughput are desired and the efficiency and savings offset the expense and time investment for the large clinical studies.

In both types of studies, pre-clinical and clinical, a laboratory inventory management system (LIMS) is beneficial for maintenance of study samples. The LIMS system can incorporate chain of custody and temperature monitoring for all samples of both study types. A LIMS that can generate individualized barcodes for sample identification enhances the quality of sample handling, by allowing the location of individual samples to be tracked throughout the laboratory. Proper location tracking can provide supporting evidence of the proper storage location and even temperatures for all samples. A LIMS may also be able to monitor and track the temperature of the sample storage units. These considerations are important for both types of studies, but the benefits are enhanced for a clinical study because of the number of samples, shipments, analysts, and possible laboratory sites.

Conclusions

There have been tremendous strides towards international harmonization of bioanalytical method validation requirements and how one can apply GLP compliance. This harmonization endeavor has been achieved through the efforts of good documentation practices. internationally recognized experts and regional discussion groups. The information presented in this document is intended to assist laboratory scientists achieve compliance for nonclinical and clinical bioanalytical studies using a systematic approach starting with information gathering, followed by thorough method development and systematic validation. We have attempted to emphasize the need for exercising good scientific judgment.

Of note: The EMA "Guideline on Bioanalytical method validation" (EMA/CHMP/EWP/192217/2009) document was adopted by CHMP on 21 July 2011 while this article was in press and will come into effect 1 February 2012. Descriptions of several validation parameters were significantly revised from the draft document (2).

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