Chapter 16
Bioanalysis

Sriram Subramaniam

16.1 Introduction

Bioequivalence (BE), pharmacokinetic (PK), and toxicokinetic (TK) studies involve assessment of drug exposure data that are vital to understand drug safety and efficacy. Generation of drug exposure data involves quantitation of the drugs and/or its metabolite(s) in biological matrix samples collected after drug administration. Therefore, quantitation of drugs and/or metabolites in biological matrices plays a vital role in the assessment and interpretation of BE, PK, and TK studies. Bioanalysis, a term which will be often used in this chapter, refers to the process of quantitation of drug and/or metabolites in biological matrices (i.e., blood, serum, urine, and tissues). Bioanalysis involves use of reliable bioanalytical methods to quantitate drugs and/or metabolites in samples from in vivo BE, PK, and TK studies. Hence, the quality of such studies is directly related to the quality of underlying bioanalytical methods and conduct. It is therefore imperative that the bioanalytical assays used in clinical and preclinical studies are validated for their intended use, and bioanalytical conduct is consistent and objective. Bioanalytical method validation (BMV) encompasses all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix is reliable and reproducible for the intended use. This is especially important for bioanalytical methods used in clinical and nonclinical studies intended for submission to regulatory agencies, such as the United States' Food and Drug Administration ("FDA"), commonly referred to as regulatory bioanalysis. In fact, the United States' Code of Federal Regulations, Title 21 (21 CFR 320.29) require that bioanalytical methods used in BE studies are accurate, precise, and

S. Subramaniam (✉)
Center for Drug Evaluation and Research, U.S. Food and Drug Administration, 10903 New Hampshire Avenue, Silver Spring, MD 20993, USA
e-mail: Sriram.Subramaniam@fda.hhs.gov

L.X. Yu and B.V. Li (eds.), *FDA Bioequivalence Standards*, AAPS Advances in the Pharmaceutical Sciences Series 13, DOI 10.1007/978-1-4939-1252-0_16,

sufficiently sensitive so that the actual concentration of the drug or its metabolite (s) achieved in the body can be measured (FDA CFR 2013). To address the expectations on bioanalysis to the pharmaceutical industry, the FDA published a guidance on BMV ("FDA BMV guidance") in 2001 (FDA 2001). In addition, the FDA recently issued a draft guidance (FDA 2013)[1] to reflect revisions to the existing FDA BMV guidance (FDA 2001). At this point, the revised FDA guidance is issued in draft form for public comments before it is finalized.

With the advancement of bioanalytical tools and techniques, and significant gains in scientific and regulatory experience over the years, there has been a critical examination of the current bioanalytical guidelines and practices. The third American Association of Pharmaceutical Scientists (AAPS)/FDA Bioanalytical Workshop in 2006 ("2006 AAPS/FDA Workshop") evaluated the current practices and clarified the FDA BMV guidelines (Viswanathan et al. 2007). This was followed by the 2008 AAPS Workshop ("2008 ISR Workshop") which further discussed issues raised during the 2006 AAPS/FDA Workshop (Fast et al. 2009). Since then, the recommendations of the 2006 AAPS/FDA Workshop (Viswanathan et al. 2007) and the 2008 ISR Workshop (Fast et al. 2009) have been discussed in several workshops and meetings (Timmerman et al. 2009; Savoie et al. 2009; Savoie et al. 2010; Garofolo et al. 2011; DeSilva et al. 2012), and have been the basis for the recent regulatory guidelines (EMA 2011; Health Canada 2012). Also, as mentioned earlier, the FDA has recently (2013) proposed revisions to the existing FDA BMV guidance (FDA 2001) in response to advancement in technology and changes in practices relating to BMV.

The focus of this chapter is to address the current best practices for BMV as it relates to BE studies. In addition to discussing the expectations of the FDA BMV guidance, the chapter will identify and evaluate recent bioanalytical practices, and highlight the potential challenges in bioanalysis based on review of scientific and regulatory articles, and white papers published since issuance of the FDA BMV guidance (2001). The chapter is not intended to describe in detail specific assay methods and resolution of bioanalytical issues, as these issues have been discussed in detail in current literature.

16.2 Bioanalytical Methods

Bioanalytical methods can be broadly classified as chromatographic and ligand binding methods. While a detailed description of the principles and procedures for the methods are beyond the scope of this chapter, a brief outline of the methods is provided below.

[1] This draft guidance is not for implementation. Since the draft guidance is issued for public review and comment, the recommendations in the guidance may be modified when finalized.

16.2.1 Chromatographic Methods

In chromatographic methods, the analyte of interest is isolated and separated using appropriate sample clean-up procedures and chromatographic conditions, respectively, and detected using a suitable detection system. Sample extraction, chromatography, and detection techniques are briefly discussed below.

16.2.1.1 Sample Extraction

Generally, prior to chromatography, sample clean-up is performed for method sensitivity. Proteins in biological matrices may bind to analyte of interest and can clog the chromatography columns. Blood contains intra- and extra-cellular proteins, plasma contains significant proteins, and urine and cerebrospinal fluids contain relatively less proteins but still require extraction to improve reliability (Mulvana 2010). In addition to proteins, endogenous compounds such as phospholipids and fatty acids, and exogenous components in biological matrices can potentially affect separation and detection of the analyte of interest (e.g., foul high performance liquid chromatography (HPLC) columns and contaminate MS source) (Singleton 2012). The purpose of sample clean-up is to extract out the analyte(s) of interest from biological matrices to minimize interference and maximize recovery. Consequently, sample clean-up reduces variability and inconsistencies during analysis. Different sample clean-up procedures are used depending on the choice of matrix, drug, chromatography, and detection systems. Broadly, sample clean-up procedures include, protein precipitation (PP), solid phase extraction (SPE), and liquid–liquid extraction (LLE).

In PP, miscible organic solvents (e.g., methanol or acetonitrile), often modified with buffer or acid and bases, are added to biological samples to denature proteins and consequently precipitate the samples. For example, if the analyte is highly protein bound, a volatile acid (e.g., formic acid) or base (ammonium hydroxide) is used to disrupt binding and increase analyte recovery. The precipitate is removed by centrifugation or filtration, and extract injected. Although PP is simple and fast, it does not necessarily yield clean extracts, as it may not remove endogenous components such as phospholipids, fatty acids, lipids (Van Eeckhaut et al. 2009; Mulvana 2010).

More efficient sample clean-up may be obtained from LLE and SPE. In LLE, immiscible organic solvents (e.g., diethyl ether, ethyl acetate, methyl-*tert*-butyl ether (MTBE), hexane) are used to extract the analyte of interest by partitioning it into an organic layer (Singleton 2012; Nováková 2013). Therefore, LLE can mitigate or avoid matrix effects as ionized compounds, including salts or phospholipids, do not partition into the organic layer (Nováková 2013). The advantage of LLE is mainly its ease of use, and requires no special instrumentation. A major limitation of LLE is its applicability to polar compounds (Nováková 2013). To transfer an ionizable analyte to organic solvent it first needs to be converted to a

nonionic form in an aqueous medium at an appropriate pH, followed by selection of a suitable solvent to efficiently and selectively extract the analyte. Usually multiple extractions are necessary and final re-suspension in an aqueous medium at the original pH is needed, resulting in reduction in recovery of the analyte (Trufelli et al. 2011). Also, in LLE, there is a tendency to form emulsions at the interface between liquid layers (Trufelli et al. 2011; Singleton 2012; Nováková 2013). Further, LLE may require large solvent volumes. These problems have been reported to be minimized with new versions of LLE, such as supported LLE. In supported LLE, the entire sample is adsorbed on a solid support (i.e., diatomaceous earth), and an organic solvent is passed through the solid support resulting in partition of the analyte of interest into the organic solvent (Singleton 2012). Recently, LLE has been scaled down, requiring relatively low volumes of sample (50–100 μL) and organic solvent (0.6–2 mL) (Nováková 2013). Also, high throughput LLE versions using on-line extraction or 96-well plate arrangements are available. For other recent LLE techniques the reader is encouraged to refer to Singleton (2012) and Nováková (2013).

To further increase selectivity and clean-up, SPE is often employed. SPE can reduce sample volume, be easily automated, and used on-line with liquid chromatography separation. In SPE, the separation process is based on the affinity of the analyte to the stationary phase or sorbent. The sorbents are ion-exchange, normal phase, reverse phase or a combination to selectively retain the analyte of interest. The interfering matrix components either pass through unretained or are retained relatively longer than the analyte of interest. The choice of sorbent controls selectivity, affinity, and capacity (Nováková 2013), depending on the physiochemical properties of the analyte, biological matrix, and interaction between sorbent and analyte. The SPE usually involves a wash step to remove undesired components, and an elution step to extract the analyte of interest. Therefore, selection of the proper washing and elution solvents are important (Trufelli et al. 2011). It is reported that immunosorbents and molecularly imprinted polymer (MIP) sorbents can significantly increase selectivity of SPE (Nováková 2013). The drawbacks of SPE include, the time required for processing (manual SPE), expense, and lot-to-lot cartridge variability. Also, matrix effects have been reported to result from the sample pre-concentration step and the SPE procedure itself (i.e., from salts in buffers used) (Van Eeckhaut et al. 2009). However, the advantages of SPE overshadow the drawbacks. SPE remains one of the most widely used extraction techniques for routine bioanalysis. For recent SPE techniques, the reader is encouraged to refer to Mulavana (2010), Singleton (2012), and Nováková (2013).

16.2.1.2 Chromatography

The aim of chromatography is to assure that the analyte(s) of interest is adequately resolved from interfering components. Chromatographic separation is primarily based on the differences in physicochemical properties between the analyte and matrix components related to both mobile and stationary phases (Li et al. 2011).

The main factors (techniques) for chromatographic separation are hydrophobicity (reversed-phase), molecular charge (ion-exchange), and size (size exclusion) of the stationary phase (Bozovic and Kulasingam 2013). The choice of the separation technique depends on the characteristics of the analyte to be separated, and often a combination of techniques may be required.

In addition to adequately resolving the analyte of interest from those of other closely eluting compounds, an ideal chromatography technique should be able to measure the analyte at low levels, have short retention times, and be time and cost efficient. Reversed-phase chromatography is based on the reversible adsorption of molecules based on their polarity under conditions where the stationary phase is more hydrophobic than the mobile phase (Bozovic and Kulasingam 2013). This is the most popular and widely used liquid chromatography (LC) technique due to its robustness, efficiency, column stability and availability of several different phase chemistries that can be customized for a particular use.

In addition to LC column selection and mobile phase composition, factors such as gradient time, mobile phase pH, and column temperature need to be considered when dealing with unstable analytes (Li et al. 2011). Also, the purity of the solvent used to dissolve the analyte, and the compatibility of the solvent with mobile phase and ion source (i.e., if coupled to mass spectrometers) are important considerations. It is critical that buffers containing inorganic salts are avoided at all times, as well as inorganic acids, ion-pairing reagents, and nonvolatile buffers. Formate, acetate, and ammonia at low concentrations are frequently used additives, as they are compatible with mass spectrometric detection (Bozovic and Kulasingam 2013).

Increasing resolution efficiency, flow rate, and column temperature are some of the ways to improve run time. Gradient elution is the preferred mode of separation for small molecules, as it has a broader range of retentivity, higher peak capacity, and faster analysis compared to isocratic elution.

Over the years, development of stationary phases have evolved, including silica, phenyl, C8, or C18 columns that improve retention times, enhance column lifetime, and increase throughput (Mulvana 2010). Also, porous silica rod or MIP columns increase throughput and resolution. In addition, with the advent of columns with sub-2 μm particle size and liquid-handling systems that can operate such columns at high pressures, ultra-high performance liquid chromatography (UHPLC) has become increasingly popular in quantitative bioanalysis. UHPLC increases speed, resolution, sensitivity, and lower solvent consumption (Van Eeckhaut et al. 2009; Trufelli et al. 2011; Nováková 2013; Jemal et al. 2010). To prevent increase in back pressure and dirtying of columns, a pre-column is recommended for bioanalysis with UHPLC. Hydrophilic interaction liquid chromatography (HILIC) is another powerful, new technique for separation of small polar molecules that are weakly eluted or retained in conventional LC techniques. HILIC combines the use of bare silica or polar bonded stationary phases and mobile phase with high content of organic solvents (Van Eeckhaut et al. 2009). The higher content of organic solvents in HILIC increases selectivity, sensitivity, and efficiency of drug quantitation by effective retention of polar compounds, enhancing electrospray ionization (ESI), speeding separation under high flow rates or in columns with small particle size

(due to low back pressure), and being compatible with elution solvent used in reversed phase-SPE (Van Eeckhaut et al. 2009; Trufelli et al. 2011; Nováková 2013). Therefore, HILIC has become very popular in bioanalysis, often in UHPLC arrangements (Nováková 2013).

16.2.1.3 Mass Spectrometry

Following sample clean-up and chromatography, the analyte(s) of interest is detected and quantitated using an appropriate detection system. Currently the most commonly used detection system for analysis of small molecules is mass spectrometry (MS). Therefore, this detection system is discussed briefly. Although MS detection is generally regarded as highly selective, chromatographic separation is still recommended to avoid problems with interferences in MS that can affect quantitation (Nováková 2013).

For detection by MS, the uncharged analytes eluting from the HPLC system have to be first transformed to ions. This occurs at the ionization source. Therefore, the ionization source serves as an interface between HPLC and mass spectrometer. There are various types of ionization sources. Currently, the most commonly used ionization sources are ESI and atmospheric pressure chemical ionization (APCI). Since ionization in ESI and APCI occurs at atmospheric pressure, the ESI and APCI sources are commonly referred to as c (API) sources. The effluent containing the analyte from the HPLC is nebulized. Nebulization occurs in ESI by the high voltage field resulting in charged droplets that are focused toward the mass analyzer and get smaller and smaller as they approach the entrance to the mass analyzer. As the droplets get smaller, individual ions emerge in a process referred to as "ion evaporation" (Niessen 2003). In APCI, nebulization occurs by spraying the mobile phase (containing the analyte) with a nebulizer gas in a heated vaporizer tube (350–500 °C) and the resultant aerosol cloud is ionized by a corona discharge needle (Niessen 2003). A newer ionization source, atmospheric pressure photoionization (APPI), vaporizes HPLC eluant like APCI, but uses photons from an ultraviolet (UV) lamp to initiate the ionization process (Korfmacher 2005).

Following ionization, the mass spectrometer analyzes the ion of the analyte of interest (i.e., precursor ion) based on its mass to charge ratio (m/z). However, for bioanalytical purposes, the MS response obtained for the precursor ion alone may not be suitable for quantitative analysis. This is because there may be many molecules in the matrix that produce ions of the same m/z as the target analyte, thus making the result nonspecific and often invalid. This limitation can be surmounted by tandem mass spectrometry (MS/MS). The most commonly used MS/MS in bioanalytical assay is the triple quadrupole mass spectrometer operated in selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) mode (Niessen 2003; Korfmacher 2005).

The triple quadrupole mass spectrometer consists of three quadrupoles: the first (Q1) and third (Q3) quadrupoles are mass analyzers, and the second quadrupole (Q2) is the collision cell (Bozovic and Kulasingam 2013). When triple quadrupole

mass spectrometer is operated in SRM or MRM mode, high selectivity is achieved due to two-stage mass filtering. Briefly, in the first stage, the selected precursor ion is resolved from coeluting components in Q1 based on its *m*/*z*, and accelerated into the collision cell, Q2, where it fragments by collision with a neutral inert gas (e.g., nitrogen or argon) in a process referred to as collision induced dissociation (CID). In the second stage, the analyte is further differentiated from interfering components in the third (Q3) quadrupole by monitoring unique fragment ion(s) (a.k.a., product or transition) of the precursor derived in Q2. This two-stage mass filtering of SRM or MRM increases the level of detection specificity, sensitivity, and throughput.

Selection of fragment ion(s) can be realized by careful tuning of the critical MS/MS parameters, such as collision energy, collision gas pressure, and cone voltage. Generally, to identify the precursor ion, a diluted solution of a pure compound can be directly introduced into the instrument (by flow injection analysis or split infusion) while the first quadrupole (Q1) is set to scan over a defined *m*/*z* range. The most abundant peak visible in the mass spectrum produced in this operating mode should represent the precursor ion (Bozovic and Kulasingam 2013). Precursor ions should be identified and the source parameters tuned to achieve the maximum peak intensity, without compromising signal-to-noise. Usually, once the precursor ion of the target analyte is identified, the mass spectrometer's ion optics and quadrupoles are tuned for the product ions. For selection of SRM transitions, Jemal et al. (2010) propose that at least two SRM transitions are utilized during method development as a coeluting metabolite or an endogenous compound may interfere with one or more of the selected SRM transitions.

LC coupled by an API source to MS/MS detection is currently considered the method of choice for quantitative analysis of small molecules in biological matrices. For more information on the factors to consider in development of LC-MS/MS bioanalytical methods, the reader can refer to excellent articles by Jemal and Xia (2006), Jemal et al. (2010), Mulvana (2010), and Li et al. (2011).

16.2.2 Ligand Binding Assays

Ligand binding assays (LBA) are immunoassays where an antigen–antibody reaction is used to capture the analyte of interest. Due to the advantages of LC-MS/MS methods to quantify small molecules, currently LBAs are not frequently used for low molecular weight compounds. However, LBAs are still the method of choice for quantitation of macromolecules and antibodies in complex biological matrices due to their high sensitivity and specificity. LBAs also play an important role in the detection and quantitation of biomarkers in clinical and nonclinical studies.

Immunoassays are broadly classified as homogeneous or heterogeneous assays (Findlay and Das 2006). In a homogenous assay all reagents are in solution, whereas in a heterogeneous assay at least one key reagent is immobilized and involves at least one washing step to remove excess analyte. Enzyme-linked immunosorbent assay (ELISA) is an example of heterogeneous assay. ELISA can

be in a competitive or noncompetitive format. In a noncompetitive ELISA, the primary antibody to the analyte of interest is immobilized on microtiter or multi-well plate, and biological sample is introduced and incubated to facilitate binding of analyte to the immobilized antibody, and excess analyte is removed by washing. The immobilized antigen–antibody complex is then detected by directing an enzyme-labeled antibody specific to the analyte followed by addition of an enzyme-specific substrate probe. The resulting reaction is quantitated using an appropriate detection system depending on the type of the substrate probe. In the competitive ELISA, antigen is immobilized and competition is established between immobilized antigen and antigen in solution (i.e., analyte of interest) for fixed binding sites on the primary antibody in solution. After incubation and washing, an enzyme-labeled secondary antibody, directed against immunoglobins for the same species from which the primary antibody was created, is added. Following incubation and washing, an enzyme-specific substrate is added to generate a signal which is then quantitated.

The differences in regulatory requirements for chromatographic assays versus LBAs, and challenges involved in the conduct of the assays will be highlighted in subsequent sections.

16.3 Expectations for Validation of Bioanalytical Methods

According to the FDA BMV guidance (2001), validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of the method are suitable and reliable for the intended use. Method validation provides assurance that the bioanalytical method will perform reliably when used to analyze study samples. Therefore, during method validation, it is imperative that all the stress conditions and potential problems expected during analysis of the study samples are addressed to assure that the assay will perform as intended. This section describes the best practices for validation of bioanalytical methods.

16.3.1 Reference Standards

Reference standards are used to prepare stock solutions that are in turn used for the preparation of spiked samples (i.e., calibration standards and quality controls). Routinely, blank biological matrices are spiked with known concentrations of stock solutions to prepare calibrators and quality controls (QC). The calibrators and QCs are used to validate the performance of the method (see Sect. 16.3.4). Therefore, knowledge of the identity, purity, and stability of the reference standards is essential for reliable estimation of the analyte.

The FDA BMV guidance (2001) recommends that when possible reference standards are identical to the analyte of interest. Otherwise, an established chemical form (i.e., free acid/base, salt, or ester) of known purity can be used.

Reference standards can be broadly classified as (1) certified (e.g., U.S. Pharmacopeia (US)), (2) commercially available from a reputable source, and (3) in-house or custom-synthesized. Information for reference standards should include lot numbers, source, purity, storage, stability, handling, and expiration or recertification dates (Viswanathan et al. 2007). Usually, certificates of analysis (CoA) with the above information are available for reference standards. When CoAs are unavailable (e.g., rare metabolites) or reference standards are used beyond their expiration, FDA's recent draft guidance (FDA 2013) recommends that the purity and stability of the reference standards are demonstrated. CoAs or purity information is preferable for reference standards for internal standards (Sect. 16.3.2), however, lack of interference with the analyte of interest (Viswanathan et al. 2007), consistency between lots (e.g., when multiple lots are used) (DeSilva et al. 2012), or other suitability information may be demonstrated for internal standards. Also, sometimes the assays used by the vendors of reference standards may not be sensitive to assess purity (e.g., thin layer chromatography) and impurities (LC with ultraviolet detection). In such cases, purity determination using rigorous analytical methods may be necessary. Additional factors, including light sensitivity and moisture content may also need to be established for reference standards depending on the analyte.

Contrary to small molecules, macromolecules are usually not well characterized due to the nature of production. Macromolecular reference standards are often heterogeneous (Viswanathan et al. 2007), and therefore, lot-lot variability in purity and potency between preparations can be expected. It is therefore critical to use appropriate reference standards to validate an assay for macromolecules compared to the macromolecule used to dose the subjects.

In addition to reference standards, selection of reagents including ligand agents (e.g., antibody, antibody pairs), binding proteins, conjugated antibodies, and radioligands are critical in the development and validation of LBAs (Kelley and DeSilva 2007). Also, it is important that the reagents in LBAs have suitable specificity and selectivity for the intended use, and stable binding characteristics. Some reagents, including, conjugated antibodies and radioligands, have lot-to-lot variations. Therefore, for long-term studies, availability of a sufficient quantity of the reagents is necessary. Similar to the reference standards, reagents in LBAs are also macromolecules, hence assay sensitivity and robustness can be adversely affected due to instability. Therefore, appropriate storage and handling are paramount in maintaining the integrity of the reagents.

16.3.2 Internal Standards

To correct for analyte loss or variation during sample processing (e.g., extraction, evaporation, reconstitution), chromatographic separation, and instrumental performance (e.g., injection volume, ion suppression/enhancement), an internal standard (IS), which has the same or similar physical and chemical properties as the analyte, is added prior to sample processing to both spiked and study samples in equal

concentrations. By using ratios of the response of analyte and IS in samples, variations in recovery and instrumental response can be corrected to improve the precision and accuracy of the methods. ISs are commonly used in chromatographic assays. ISs are less common in LBAs as sample clean-up is not as common as chromatographic methods.

Selection of IS is generally based on the following factors: (1) the physical and chemical properties (e.g., hydrophobicity, ionization properties) of the IS closely mimics the analyte during the analytical procedure, (2) purity of the IS is adequate, and (3) IS is stable during bioanalytical conduct.

There are two main types of IS: structural analogues and stable isotope labeled (SIL). The SIL ISs are compounds where atoms in the analyte are replaced with stable isotopes such as deuterium (^{2}H), ^{13}C, ^{15}N, or ^{17}O. For this reason, SIL ISs closely resemble the analyte to be measured and therefore are most effective to track variations in analyte response. SIL ISs are commonly used depending on availability and cost. Due to nearly the same physicochemical properties as the analyte of interest, SIL ISs, in theory, minimize the influence of matrix effects (Sect. 16.3.3) as the degree of ion suppression/enhancement caused by the coeluting matrix components must theoretically be the same for SIL ISs and its normal analyte counterpart (Viswanathan et al. 2007).

The selection of ISs depends on the extraction procedure, chromatographic separation, and analyte detection systems used. Also, the selection of ISs depends on which stages of analysis are critical for tracking the analyte. For example, if sample extracts are not clean, then tracking the analyte during MS detection is crucial to correct for matrix effects (Tan et al. 2012). Excellent articles by Tan et al. (2009, 2012) discuss the intricacies of IS selection.

Since ISs are used to correct for variations in analyte response, variations in IS response are expected. While excessive variations in IS response may affect quantitation, a high variation does not necessarily equate to unreliable data. Therefore, assessment of the impact of IS variations on quantitation is vital. There is no consensus on what constitutes an "excessive" IS response that affects quantitation. However, it is commonly accepted that monitoring IS response variations during sample analysis is a good practice. While the current FDA BMV guidance (2001) does not discuss IS variations, the recent FDA draft guidance (2013) recommends monitoring IS variations and establishing an objective, a priori criteria for abnormal IS variations. One of the common acceptance criteria for monitoring IS variations is setting a fixed percentage (e.g., ±50 %) of mean IS response of spiked samples (i.e., calibrators and quality controls) within an analytical batch as an acceptable IS response range for the batch. Any sample with IS response outside the acceptable range in the batch will be flagged for reanalysis.

16.3.3 Matrix Effects

Although LC-MS/MS systems are generally considered to be very selective and sensitive, such methods do not automatically guarantee highly selectivity.

The transformation of uncharged molecules of the analyte to its charged components (i.e., ions) plays a key role in the detection of the analyte in LC-MS/MS systems. However, the efficiency of the formation of the desired ions is often perturbed by undetectable components in the incurred sample[2] matrix that coelute with the analyte(s) of interest. Hence, the efficiency of the formation of the analyte ions is matrix dependent. This phenomenon is referred to as "matrix effect" and results in reduction or enhancement of the ion intensity(ies) of the analyte(s) of interest, commonly referred to as "ion suppression" or "ion enhancement." Ion suppression or ion enhancement frequently is accompanied by a significant loss of precision and accuracy. Matuszewski et al. (2003) demonstrated that imprecision increased when the same method was validated with five different sources of plasma compared to a single source of plasma. Therefore, matrix effects may significantly affect assay performance. Appropriately, the FDA BMV guidance (2001) recommends that matrix effects are investigated and eliminated in LC-MS/MS methods. Excellent articles on matrix effects and its evaluation are available (Matuszewski et al. 2003; Van Eeckhaut et al. 2009; Trufelli et al. 2011). Estimation of matrix effect is discussed in Sect. 16.6.1.

Matrix effects can also arise in LBAs from interferences from unrelated compounds (from binding proteins, endogenous analogues, concomitant drugs, immunoglobulins) originating in the matrix (DeSilva et al. 2003; Kelley and DeSilva 2007). Therefore, validation of matrix effects in LBAs is extremely important when switching biological matrices.

16.3.4 Calibration Curve and Assay Performance

The minimum and the maximum known analyte concentrations used in an assay represent the lower limit of quantitation (LLOQ), and upper limit of quantitation (ULOQ), respectively, of the bioanalytical method. The LLOQ and ULOQ also describe the quantitation or calibration range of the bioanalytical method. In addition, the LLOQ describes the sensitivity of a bioanalytical assay (Sect. 16.3.4.2). Assessment of assay performance includes validation of the following components:

16.3.4.1 Calibration Curve

A calibration (or standard) curve describes the relationship between instrument response and known concentrations of the analyte. This relationship is essential to estimate the concentrations of the unknown samples. The FDA BMV guidance

[2] Samples collected from an animal or human dosed with drugs during drug development.

(2001) recommends that a calibration curve is prepared in the same biological matrix as the samples in the intended study (exception see Sect. 16.6.5) by spiking the matrix with known concentrations of the analyte. Also, it is recommended that the calibration range is based on the anticipated analyte concentration range in the BE study or studies (FDA 2001).

A calibration curve usually consists of a blank sample (i.e., matrix sample processed without analyte and internal standard), a zero standard (i.e., matrix sample processed without analyte but with internal standard), and at least six non-zero standards (i.e., matrix samples processed with analyte and internal standard) covering the expected range, including LLOQ and ULOQ (FDA 2001). The number of non-zero standards (or calibration standards) used is a function of the dynamic range and nature of the concentration-response relationship. A sufficient number of non-zero standards are often used to adequately define the relationship between concentration and response. The calibration standards can contain more than one analyte. Generally, it is good practice to use freshly prepared calibrators during validation to support that the method is sufficiently rugged.

Unlike chromatographic assays for small molecules, the standard curves for LBAs used to measure macromolecules are inherently nonlinear and therefore more non-zero standards may be recommended for LBAs. While the FDA BMV guidance (2001) recommends a minimum of six non-zero standards in duplicates, using additional calibrators is a good practice for LBAs. Kelley and DeSilva (2007) suggest including eight non-zero standards in duplicate. Also, due to the nonlinear response function, selection of non-zero standards to completely describe the calibration response becomes important for LBAs. In addition to non-zero standards, the FDA BMV guidance (2001) recommends anchoring points (above and below the established LLOQ and ULOQ: DeSilva et al. 2003) for LBAs to improve overall curve fit. While there is no consensus for acceptance criteria for anchor points, rejection of anchor points to force batch acceptance is discouraged (Savoie et al. 2010).

The FDA BMV guidance (2001) recommends that, except for the LLOQ, the back-calculated concentrations of the non-zero standards should be within 15 % of their nominal (theoretical) concentrations (20 % at LLOQ). For LBAs, the recent FDA draft guidance (2013) recommends that the back-calculated concentrations of the non-zero standards are within 20 % (25 % at LLOQ). Also, the recent FDA draft guidance (2013) recommends that at least 75 % of the non-zero standards are accurate, including the LLOQ, and the standards are excluded only for failure to meet the above acceptance criteria, or assignable causes (e.g., poor chromatogram, documented processing errors).

Usually, the standard curve fitting is determined by applying the simplest model that adequately describes the concentration-response relationship. The FDA BMV guidance (2001) recommends that selection of weighting and use of a complex regression model be justified. Also, it is important to assure that exclusion of an individual standard does not change the model used (FDA 2013). Since calibration

response for LBAs shows nonlinear behavior, and their response-error relationship is not constant (i.e., highest precision does not always coincide with highest sensitivity), a weighted, nonlinear, least squares method with sufficient non-zero standards is recommended for LBAs (i.e., 4- or 5-parameter logistic model) (FDA 2001).

16.3.4.2 Assay Sensitivity

Assay sensitivity is often described by the LLOQ of the assay. It refers to the lowest concentration of the analyte that can be reliably quantitated by an analytical method, with acceptable accuracy and precision.[3] The FDA BMV guidance (2001) recommends that LLOQ is established using at least five QC samples at the LLOQ concentration in validation batches (see Sect. 16.3.4.4). The recent FDA draft guidance (2013) recommends that the accuracy does not deviate by more than ±20 % (±25 % for LBAs) of the theoretical concentration and the precision around the mean value does not exceed 20 % of the CV (25 % for LBA). The signal-to-noise ratio (S/N) at the LLOQ is recommended to be at least 5 (in other words the analyte response at the LLOQ is at least five times the response compared to blank response). Therefore, peak response in blanks or zero standards will be less than 20 % of LLOQ response. Peak response in blanks or zero standards greater than 20 % of LLOQ response is often referred to as interference and may affect accuracy and precision at the LLOQ. In addition, to control method error in LBAs, the consensus of the 2006 AAPS/FDA workshop was that total error[4] be less than ±40 % at the LLOQ (Viswanathan et al. 2007).

16.3.4.3 Selectivity

The terms "selectivity" and "specificity" are often mentioned in bioanalytical validation, sometimes interchangeably. Selectivity is a measure of extent while specificity is an absolute measure. In other words, specificity is the upper limit of selectivity, i.e., a method is specific when it is perfectly selective for an analyte or group of analytes (Rozet et al. 2011). For this reason, selectivity is used in this chapter.

According to the FDA BMV guidance (2001), selectivity is the ability of an analytical method to differentiate and quantify the analyte(s) of interest in the presence of interfering components in the matrix. Potential interfering

[3] The *accuracy* of an analytical method describes the closeness of mean test results obtained by the method to the actual concentration of the analyte. The *precision* (or imprecision) of an analytical method describes the random error of measurement, i.e., dispersion of the results around average value, often expressed as relative standard deviation (RS) or coefficient of variation (CV).

[4] Sum of absolute values of % accuracy and % precision.

substances in a biological matrix include endogenous matrix components, metabolites, decomposition products, concomitant medication, and other xenobiotics.

For selectivity, the FDA BMV guidance (2001) recommends analyses of blank samples of the appropriate biological matrix (plasma, urine, or other matrix) from at least six sources. It is recommended that each blank sample is tested for interference, and selectivity is assured at the lower limit of quantification (LLOQ). Routinely, interference is defined as peak response in blanks or zero standards equal to or greater than 20 % of LLOQ response.

The FDA BMV guidance (2001) recommends evaluating cross-reactivity of metabolites, concomitant medications, or endogenous compounds individually and in combination with the analyte of interest. This includes evaluation of expected concurrent medications that may potentially interfere with the analyte of interest. In certain situations, the European Medical Agency (EMA 2011) has proposed that the potential for interconversion of metabolite and parent drug during sample analysis be investigated, and its impact on quantitation determined (see Sect. 16.6.3).

Nonspecific binding should be determined for LBAs. Nonspecific binding can result from cross-reactivity with related compounds (e.g., metabolites, concomitant medications, or endogenous compounds), and interferences from matrix components. The guidance also recommends evaluation of parallelism for LBAs to detect matrix effect (FDA 2013). Parallelism shows that sample dilution response is parallel to standard concentration-response curve. It is important to note that parallelism is not the same as QC dilution linearity, as parallelism requires the use of incurred samples (DeSilva et al. 2012).

16.3.4.4 Precision and Accuracy

QCs at known concentrations are used to validate the precision and accuracy of a bioanalytical method. QCs are prepared by spiking known concentrations in the same blank biological matrix as intended for the study. It is a good practice to prepare QCs from an independent stock solution compared to the calibrations standards. When calibrators and QCs, are prepared from the same stock solution, it is a good practice to establish the accuracy of the stock solution against an independent stock solution.

In addition to LLOQ QC (see Sect. 16.3.4.2), QCs at a minimum of three concentrations, representing the entire range of the standard curve are recommended: one within 3× LLOQ (low QC sample), one near the center (middle QC), and one near the upper boundary of the standard curve (high QC) (FDA 2001). A minimum of five replicates per QC concentration is recommended (FDA 2001). It is recommended that the QC concentrations reflect the expected concentrations in the study (FDA 2013). A minimum of three to six validation batches are routinely used in method validation to assess assay precision and accuracy. Each validation batch usually consists of at least one set of calibration curve (i.e., blank, zero and non-zero standards) and a minimum of five QC replicates at each QC concentration.

Intra- and inter-batch precision and accuracy are determined based on the QC results. For acceptable performance, it is recommended that the assay accuracy be within 15 % of the nominal (theoretical) QC concentrations and the assay precision not exceed 15 % of the coefficient of variation (CV) at each QC concentration, with the exception of the LLOQ (for LLOQ criteria see Sect. 16.3.4.2) (FDA 2001). Due to greater variability for LBAs, the recent FDA draft guidance (2013) recommends acceptable accuracy and imprecision of ±20 % (±25 % at the LLOQ) for LBAs. In addition, to control method error in LBAs, recent FDA draft guidance (2013) recommends that the total error be less than ±30 % for LBAs (±40 % at the LLOQ; Viswanathan et al. 2007). It should be noted that precision and accuracy estimation requires inclusion of all QC data, including outlier data. Only data from QC samples with documented assignable causes (e.g., poor chromatogram, broken tube) can be excluded for precision and accuracy estimation.

In general, QC data from all precision and accuracy validation batches are necessary to provide a reliable estimation of precision and accuracy. Exclusion of batches not meeting QC acceptance may not be appropriate as it may bias precision and accuracy estimation (FDA 2013). Only validation batches with an assignable cause for failure are suitable for exclusion from precision and accuracy estimation (FDA 2013).

When multiple batches fail without an assignable cause, it is a good practice to investigate and resolve the reason for failure. In such situations, the nature of the batch failures (i.e., minor or major) should determine whether it is prudent to continue with method validation or return to method development.

In addition to precision and accuracy, recovery[5] of analyte(s) in a bioanalytical method needs to be validated. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. It is recommended that recovery experiments are performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100 % recovery (FDA 2001). Recovery of the analyte need not be 100 %, but the extent of recovery of an analyte and of the internal standard must be consistent and reproducible (FDA 2013). Alternatively, to avoid matrix effect, recovery is also measured by comparing analyte extracted from matrix against analyte spiked to extracted blank matrix (Matuszewski et al. 2003).

16.3.4.5 Stability

The stability of the analyte must cover the expected storage and handling conditions of the samples during the study, including storage and handling conditions at the clinical site and during shipment. The storage and handling conditions include long-term (e.g., frozen) and short-term (e.g., bench-top, refrigerated) storage, and

[5] The extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method.

freeze–thaw stability in the intended biological matrix, and stability of sample following extraction (e.g., extract stability). When the storage and handling conditions established in method validation are exceeded during the study, stability must be established under the actual study conditions. The consensus at the 2006 AAPS/FDA Workshop was that stability assessments are conducted in unaltered matrix intended for the study with same type of anticoagulant (Viswanathan et al. 2007). If a stabilizer is employed in the study, it is a good practice to evaluate stability with and without stabilizer in stability samples. It is recommended that all stability determinations be made using freshly prepared calibrators and/or QCs (FDA 2013). The FDA BMV guidance (2001) recommends stability evaluation at low and high QC concentrations, with at least three replicates at each QC concentrations. Stability acceptance criteria need to be established a priori, and are recommended to be within 15 % of nominal concentrations (FDA 2013).

Since it is difficult to predict the number times study samples will be frozen and thawed, it is recommended that freeze–thaw stability should be determined for at least three freeze–thaw cycles (FDA 2013). Also, freeze–thaw samples are recommended to be frozen at the intended frozen storage conditions of the study samples (e.g., −20, −70 °C), and completely thawed prior to freezing during freeze–thaw cycles (FDA 2013).

Long-term stability evaluations typically cover the expected time between the date of first sample collection and the date of last sample analysis (FDA 2013). If samples are stored at different temperatures during the course of the study, it is a good practice to assure stability at the different temperatures (Viswanathan et al. 2007). It is recommended that conditions used in long-term stability experiments reflect the same storage conditions intended for the study samples. For example, the long-term stability at higher temperature (e.g., −20 °C) may not be necessarily extrapolated to a lower temperature (e.g., −60 or −70 °C) at which study samples are stored (Andersson and Ehrsson 1995; Viswanathan et al. 2007). Although most compounds may show no difference in stability at different frozen storage temperatures, some may be more stable at a particular temperature.

While validation of stability using QC samples provides useful stability information, analysts need to be aware that this information sometimes may be limited as the complexities of incurred samples may not always be reflected in QCs (see Sect. 16.6.3).

Stability of analyte in stock solutions needs to be evaluated (FDA 2001). Typically, stock solutions of the analyte for stability evaluation are prepared in an appropriate solvent at known concentrations. When stock solution exists in a different buffer composition, the recent FDA draft guidance (2013) recommends that the stability of this stock for the duration of storage is demonstrated. The stock solutions for comparison against an older stock solution need to be prepared fresh from the reference standard.

For LBAs, assessments of analyte stability are recommended to be conducted in the matrix intended for the study (e.g., should not use a matrix stripped to remove endogenous interferences) (FDA 2001). Reagents including ligand agents (e.g., antibody, antibody pairs), binding proteins, conjugated antibodies and radioligands

are critical in developments and validation of LBAs (Kelley and DeSilva 2007). Therefore, reagent stability is important for LBAs (Viswanathan et al. 2007). In addition to the reagents exhibiting specificity and selectivity, the stability binding characteristics are important (Kelley and DeSilva 2007). It is a good practice to store reagents under the designated conditions of the manufacturer, or at conditions for which stability data has been generated (Viswanathan et al. 2007).

16.3.4.6 Dilution

The FDA BMV guidance (2001) recommends that dilutions, if expected during the study, are validated by diluting QC samples with the same biological matrix as the study samples (FDA 2001). The dilution factor(s) intended for study sample analysis should be tested during validation. If dilution factors used during sample analysis are greater than those tested during validation, then validation of additional dilution factors may be necessary during sample analysis (Viswanathan et al. 2007). No within-study dilution QC samples are necessary if dilution is tested during validation and if the dilution of study samples is conducted with like matrix (human plasma for human plasma) (FDA 2001). The dilution integrity is demonstrated by accuracy and precision parameters during validation. While no specific criteria for dilution are recommended in the FDA BMV guidance (2001) or the recent FDA draft guidance (2013), the general consensus is that the dilution acceptance criteria do not exceed the assay accuracy criteria (see Sect. 16.3.4.4). The EMA (2011) has proposed that the accuracy and precision of the dilution QC samples be within ± 15 %. However, one needs to be cautious that dilutions of QC samples may not always reflect dilution of incurred samples (DeSilva et al. 2012). Also, it is a good practice to dilute samples treated with enzyme inhibitors or stabilizers with enzyme inhibitor- or stabilizer-treated blank matrix.

16.3.4.7 Cross-Validation

Inter-bioanalytical method or inter-laboratory reliability needs to be established when two or more bioanalytical methods are used within the same study or across different studies, or when two or more laboratories are used for bioanalysis within a study. This is commonly referred to as cross-validation comparison. The FDA BMV guidance (2001) recommends conducting cross-validation with spiked matrix standards and subject samples at each site or laboratory when data within the same study are generated by two or more bioanalytical methods, or two or more laboratories. Cross-validation is also important when data are generated using different analytical techniques (e.g., LC-MS-MS versus ELISA) in different studies. While no specific criteria for cross-validation have been proposed in the FDA BMV guidance (2001) or the recent FDA draft guidance (2013), the EMA (2011) has

proposed that the accuracy of QCs in two different methods is within 15 % (wider, if justified), and the difference in sample concentrations obtained from both methods is within 20 % of the mean value for at least 67 % of the repeats.

16.3.4.8 Partial Validation

Partial validation is recommended when changes are made to an already validated bioanalytical method (FDA 2001). Partial validation can range from one intra-assay accuracy and precision determination to a nearly full validation. The extent of partial validation depends on the type of modification to a bioanalytical method. The FDA BMV guidance (2001) provides examples of bioanalytical method changes that may require partial validation, including method transfers between laboratories or analysts, and changes in analytical methodology, anticoagulant in biological fluid, matrix within species or species within matrix, sample processing procedures, concentration range, instruments and/or software platforms, and sample volume. Also, partial validation may be necessary for demonstration of selectivity of an analyte in the presence of concomitant medications or specific metabolites.

16.3.4.9 Carry-Over

Carry-over can be related to autosampler or LC column. Carry-over can affect the reliability of quantitation, hence needs to be addressed during method validation (Viswanathan et al. 2007). Carry-over is commonly analyzed by injecting one or more blanks or zero standards immediately after a single or multiple injection of ULOQ calibrator or high QC samples (Viswanathan et al. 2007; Savoie et al. 2010). If carry-over exists, it is recommended that the source of carry-over is identified and eliminated. If carry-over is inevitable (e.g., highly retained compounds) or cannot be eliminated, it is a good practice to assess the extent of carry-over and its impact on quantitation, ascertain specific procedures to handle carry-over, and analyze study samples in their PK profile sequence without randomization (Viswanathan et al. 2007; Savoie et al. 2010). While the EMA (2011) has proposed carry-over criteria, at present there are no acceptance criteria for carry-over in the FDA BMV guidance (2001) and the recent FDA draft guidance (2013), and at the 2006 AAPS/FDA Workshop (Viswanathan et al. 2007).

16.3.4.10 Others

16.3.4.10.1 Multi-analyte

The recent FDA draft guidance (2013) recommends that samples involving multiple analytes should not be rejected based on the data from one analyte failing the

acceptance criteria. The data from rejected batches need not be reported, but the FDA draft guidance (2013) recommends to document rejected batches and the reason(s) for failure. When samples are reassayed only for one analyte, the consensus is to collect and retain raw data collected for the other analytes (Viswanathan et al. 2007). Matuszewski et al. (2003) reported that matrix effect issues in LC-MS/MS methods simultaneously analyzing multiple analytes can be complex, consequently the absence of matrix effect for all individual analytes may need to be demonstrated.

16.4 Application of Validation Methods to Study Sample Analysis

16.4.1 Analytical Batch

Study samples are analyzed in analytical batches. Each analytical batch includes: (a) a calibration curve, consisting of blank sample, a zero standard, and at least six non-zero standards spanning the validated assay range, (b) at least duplicate QCs at three concentrations, and (c) study samples. The same regression model used in assay validation is employed for the calibration curve in all analytical batches. Also, similar to method validation, the three QC concentrations are selected based on the calibration range: one within 3× the LLOQ (low QC sample), one near the center (middle QC), and one near the upper boundary of the standard curve (high QC) (see exceptions in Sect. 16.4.3). It is important that the QCs in the analytical batches represent the concentrations expected in the study.

The minimum number of QCs per batch recommended to ensure proper control of the analytical batch is at least 5 % of the number of study samples analyzed or a total of six QCs (i.e., duplicates at low, medium, and high QCs), whichever is greater (FDA 2001). In each analytical batch, it is imperative that calibrators and QCs are processed (preferably interspersed during processing) along with the subject samples under the same processing conditions (see Sect. 16.4.3 for special cases). It is recommended that all study samples from a subject be analyzed in the same batch when feasible. Study samples from multiple subjects may be analyzed in an analytical batch depending on the number of samples collected per subject, acquisition time, and a host of other factors. The storage of sample extracts prior to analysis need to be within the storage period validated for extract stability. Extrapolation of concentrations in study samples either below the LLOQ or above the ULOQ of the standard curve is not recommended (FDA 2001).

16.4.2 Acceptance Criteria

The criteria for batch acceptance should be established a priori and be objective. This includes criteria for acceptance of calibration standards, QCs, and interference. The FDA guidance (2001) recommends that 75 % of calibration standards in analytical batches are within 15 % of nominal value (20 % for LBAs), except at the LLOQ where the mean value is within 20 % of nominal value (25 % for LBAs). Only calibration standards outside the above-mentioned acceptance criteria or with documented assignable causes can be excluded. Based on extrapolation of the FDA BMV guidance's QC acceptance criteria, the recent FDA draft guidance (2013) recommends QC acceptance when at least 67 % of the total QCs and 50 % of the QCs at each level are within 15 % of their nominal concentrations in each analytical batch. The above QC acceptance criteria are independent of the number of QC levels and number of replicates at each QC level. Also, peak response in blanks or zero standards are recommended to be less than or equal to 20 % of LLOQ response to minimize interference.

Although the same QC acceptance criteria were recommended for LBAs in the FDA BMV guidance (2001), the LBAs are reported to have higher imprecision due to nature of the reagents and antibody–antigen reaction. Therefore, the FDA's recent draft guidance (2013) recommends that at least two-thirds of the total QCs and 50 % of the QCs at each level are within 20 % for LBAs, and any exception to this criteria is justified (Viswanathan et al. 2007; Kelley and DeSilva 2007; FDA 2013). This criteria has also been adopted by EMA (2011) for LBAs.

Typically, accuracy and precision of QC concentrations at each level from all successful analytical batches are evaluated to determine inter-batch accuracy and precision during the study. In-study assay performance is considered acceptable if the inter-batch accuracy and precision of QCs from successful runs are within 15 % (20 % for LBAs) of their nominal concentrations and 15 % CV (20 % CV for LBAs), respectively (FDA 2013). To understand the true assay performance, it is necessary to include inaccurate QC concentrations without any assignable causes in precision and accuracy estimation.

16.4.3 Analytical Conduct: Special Cases

Typically, the calibration range validated pre-study should be used in the analytical batches. However, in some situations, at the start of analysis, the study sample concentration range may be narrower than the expected concentration range. Consequently, the validated calibration range is too broad and QC concentrations may not be reflective of the study sample concentrations. In such instances, the recent FDA draft guidance (2013) recommends: (1) to narrow the calibration curve and modify QC concentrations, or (2) retain the original standard curve but include additional QC or new QC concentrations to reflect the study sample concentrations.

In either case, partial validation of the modifications is necessary. It is not necessary to reanalyze samples analyzed prior to modifying standard curve and/or QC concentrations as long as the partial validation is acceptable.

There may be situations when the bioanalytical method necessitates separation of an analytical batch into distinct processing batches (FDA 2013). Distinct processing batches include, but not limited to, extraction of finite samples due to limited capacity of SPE manifold, and processing of subject samples by multiple analysts due to large sample size. In such cases, the recent FDA draft guidance (2013) recommends that each distinct processing batch includes at least duplicates QCs at all QC levels (e.g., low, middle, and high) that are processed along with the study samples. Also, QC acceptance criteria are recommended for the analytical batch as a whole as well as the distinct processing batches (FDA 2013; also refer to Sect. 16.6.4).

For LBAs, replicate measurements during study sample analysis may not be necessary when replicate samples are used in validation and the method is demonstrated to be robust (DeSilva et al. 2012). Also, when using duplicate or triplicate determinations for samples in LBAs, exclusion of one or more of replicate determinations, if exercised, is based on pre-established, objective criteria.

Selection of samples for reanalysis and reporting of final values are recommended to be based on a priori, objective criteria (FDA 2013). It is a good practice to restrict sample reanalysis to samples with assignable causes that will invalidate the data (e.g., poor chromatogram, instrument failure, documented processing errors, samples below LLOQ or above ULOQ). Reanalysis of possible outliers (including PK, suspected, and confirmatory repeats) is discouraged, and when necessary needs to be justified with appropriate pre-established criteria.

It is not a good practice to re-inject failing analytical batches to bring them to acceptance. A high frequency of analytical batch failures needs to be investigated and resolved prior to continuing sample analysis. Also, following batch interruptions, the decision to continue analysis of the remaining samples or re-inject all the samples depends on the cause, duration, and resolution of the interruption. Generally, it is a good practice to have objective, pre-established criteria for analysis following batch interruption. Also, before re-injecting batches, it is important to establish re-injection reproducibility to determine whether an analytical batch can be reanalyzed.

Integration of chromatograms must be objective and consistent. When re-integration of chromatograms is normally discouraged, however, when performed the FDA's recent draft guidance (2013) recommends that the rationale for the re-integration is clearly described and documented, and audit trails maintained. It is recommended that objective procedures are established that specify the situations when re-integration is necessary and how it needs to be performed (FDA 2001). While modification of integration parameters may be necessary in some situations, it is a generally good practice to use the same integration parameters for all analytical batches on the same instrument for a given study provided the integration is valid and consistent.

The 2008 ISR Workshop (Fast et al. 2009) and the FDA's recent draft guidance (2013) recommends conduct of ISR for all BE studies (refer to Sect. 16.7 for details). This recommendation has also been adopted by the EMA (2011).

16.5 Documentation

The goal of documentation for regulated bioanalysis is to retrospectively construct events that transpired during method validation and study sample analysis. Therefore, contemporaneous recording of events is vital to good documentation. In addition to meeting the requirements of regulatory agencies, contemporaneous documentation is helpful to the firm to identify isolated problems or systemic issues retrospectively. The FDA BMV guidance (2001) recommends documenting summary (e.g., summary of methods, protocol, validation reports), method validation (e.g., complete method description, validation report of assay performance and stability, established procedures, and chromatograms), and study sample analysis (analytical report of in-study assay performance, reanalysis, deviations, unexpected events, chromatograms, and established procedures) information. A frame work for expected documentation at the analytical site for method validation and sample analysis, and essential information for validation and analytical reports, is provided in a tabulated format for easy reference in the 2006 AAPS/FDA Workshop whitepaper (Viswanathan et al. 2007). The paragraphs below highlight some important considerations for bioanalytical documentation.

The FDA BMV guidance (2001) recommends that analytical laboratory have established standard operating procedures (SOPs) that cover all aspects of analysis, from the time the samples reach the laboratory until the results of the analysis are reported. This includes SOPs for record keeping, security and chain of sample custody, sample preparation, and analytical tools such as methods, reagents, equipment, instrumentation, and procedures for verification of results. All study related communication within the analytical facility and between analytical facility and the sponsor or the clinical sites are part of study records, therefore are recommended to be retained (FDA 2013).

Records of contemporaneous entry of events constitute source records, and such records are recommended to be retained (FDA 2013). Source records for bioanalysis include, but are not limited to, laboratory notebooks, analysts' notes, receipt and storage of reference standards and samples, freezer log books, sample processing entries, instrument usage log and maintenance records, batch summary sheets, chromatograms, and audit trails. The recent FDA draft guidance (2013) recommends that sufficient information must be included in source records to re-construct the events described in the records. Acceptable data entry procedures include identifying the analyst recording the events, and the dating the entries. Investigation and resolution of all unexpected events are recommended to be documented (FDA 2013).

The recent FDA draft guidance (2013) recommends that records of all validation and analytical batches analyzed, including unsuccessful batches, batch summary sheets (should include sample IDs, analyte and IS response, response ratio, back-calculated concentrations, and record modification), chromatograms, and audit trails, are retained. When samples are reassayed for one analyte in a multi-analyte method, it is a good practice to retain the raw data collected for the other analyte(s) (data need not be processed). Also, it is good practice to retain records in the format it was acquired (i.e., electronic, paper).

Re-integration of chromatograms must be explicitly identified. The FDA's recent draft guidance (2013) recommends that the reason for re-integration and mode of integration are clearly documented, and re-integration is based on pre-established criteria. Also, the consensus from the 2006 AAPS/FDA Workshop was that original and re-integrated chromatograms, and audit trail of events during data processing are retained in the format it was acquired, and audit trail feature is enabled in the laboratory information management systems (LIMS) (Viswanathan et al. 2007).

The FDA's recent draft guidance (2013) recommends that the reason for rejecting batches should be clearly documented with supporting evidence. Also, reanalysis of analytical batches or samples is expected to be clearly identified, and based on pre-established procedures.

16.5.1 Validation and Analytical Reports

An outline of the necessary information in validation and analytical reports are provided below. Validation and analytical reports routinely include a brief description of the protocol and analytical method (analyte, IS, sample pretreatment, method of extraction and analysis) used, and identify the method SOP (and version). The FDA BMV guidance (2001) recommends that the reports indicate the lot number, purity, source, and expiration or retest dates of reference standards for drug and/or metabolites, and internal standards. Also, the guidance recommends the reports describe the procedures for preparation and storage of stock solutions, QCs, and calibrators, including preparation dates, and source and lot of blank matrix and reference standards used.

Summary tables listing all validation or analytical batches (successful and unsuccessful), dates of analysis, and reason for rejection are recommended for validation and analytical reports (FDA 2013). While unsuccessful batches need to be identified, reporting summary data for the batches is not required. Tabulation of the back-calculated concentrations of calibrators and QCs with inter-batch precision and accuracy information is recommended. In addition, validation reports are expected to include intra-batch accuracy and precision, necessary stability, extraction recovery, selectivity, and matrix effect information (FDA 2013).

Analytical reports are expected to include dates of study sample receipt, shipment temperature, sample integrity at the time of receipt, sample accountability,

and storage location and temperature at the analytical site (v). Also, analytical reports are supposed to clearly identify the samples reanalyzed, the reason for reanalysis, and reporting of final values. All deviations from the protocol or procedures, and its impact on the study need to be detailed. The FDA's recent draft guidance (2013) recommends that ISR results, including samples reanalyzed, original and reanalyzed sample concentrations and their % difference, and the acceptability of ISR data, are included in the report. The assay procedure, protocol, and SOPs for re-integration, reanalysis, and acceptance criteria should be attached to the report. For pivotal BE studies for marketing, chromatograms from 20 % of subjects are recommended to be included in the report. Addendum to validation (e.g., partial validation, long-term stability) and analytical reports (e.g., investigations) if any, needs to be attached.

16.6 Challenges

One of the main issues in assuring adequate performance of the assays during study sample analysis stems from the challenges imposed by the matrix complexities of incurred samples. Although, for the most part, the contents of the matrix used to prepare QCs are the same as incurred samples, it is important to note that the matrix for QCs may not behave the same as incurred samples for several reasons (see Table 16.1). For example, QCs may not contain the same drug metabolites as

Table 16.1 Matrix differences between spiked (CS and QC) and incurred samples. (Reproduced from Tan et al. 2009)

	CS/QC	Incurred sample
Screening criteria for matrix sources	Usually loose	Usually specific and strict dependent on the objectives of a study, such as age 40–50 and nonsmoker
No. of lots/sources	Usually more than one source (pooled)	One single source
pH	Averaged due to pooling	More variable
Extra components associated with medication	None	Metabolite(s), co-medication and non-active ingredients in formulation
Amount collected	Usually large, e.g., 200 mL per collection	Usually small, e.g., 7 mL per sampling time
No. of freeze/thaw cycles prior to being extracted	Usually 2 or more	Usually 1
Storage tube and pre-use storage	Usually stored at −20 ° C and without special protection until being selected for a specific study	Could be collected under sodium light and stored at −80 ° C immediately after collection
Amount of anticoagulant	May be different because of different amounts collected	

CS calibration standard, *QC* quality control

incurred samples, which could be important as plasma metabolite concentrations generally are an order of magnitude higher compared to their parent drug. Also, compared to incurred samples, matrix used to prepare QCs may not contain drug isomers, have the same enzyme activity, or contain the same co-administered drugs or coeluting components. In addition, other factors may also affect assay performance during study sample analysis. This section highlights some of the factors that may affect quantitation during bioanalysis.

16.6.1 Matrix Effects

While the current FDA BMV guidance (2001) recommends that matrix effects be investigated and eliminated in LC-MS/MS methods, it does not specify procedure (s) to detect matrix effects. The consensus at the 2006 AAPS/FDA Workshop was that matrix factor (MF) can be used as a quantitative measure to ascertain matrix effect (Viswanathan et al. 2007). MF can be defined as ratio of analyte response in the presence of matrix ions to analyte response in the absence of matrix ions. In the absence of matrix effect, MF should be 1, while values below or above 1 may indicate ion enhancement or suppression. While absolute MF value is useful, it does not provide information of the variability in response in different incurred sample matrices (Viswanathan et al. 2007). Therefore, it has been proposed that the variability of MF be determined in six different matrix lots with an acceptable variability (as measured by the coefficient of variation) of <15 % (Viswanathan et al. 2007). Variations of MF include IS-normalized MF (i.e., ratio of MF of analyte to MF of IS, or analyte to IS ratio in the matrix extracts divided by analyte to IS ratio in the absence of matrix extract) (Viswanathan et al. 2007). The EMA guidance (2011) recommends the variability of the IS-normalized MF from six lots of matrix should not be greater than 15 % at low and high QC concentrations. Although matrix effects may extend to LC methods coupled to other detection systems (UV, fluorescence, electrochemical), matrix effects are usually linked to LC-MS/MS methods with simplified extraction procedures and minimal chromatographic separation, as such methods are popular for their high throughput. Also, the contribution of matrix effect for LC-MS methods vary depending on the ionization source (e.g., APCI versus ESI) of MS systems (Matuszewski et al. 2003). For bioanalytical methods that simultaneously analyze multiple analytes, it may be necessary to demonstrate lack of matrix effect for all individual analytes (Matuszewski et al. 2003). Table 16.2 provides a summary of various measures to eliminate or minimize matrix at different stages of bioanalytical methods (Nováková 2013). Recently, plasma phospholipids have been associated with matrix effects, which can be avoided by removing phospholipids during extraction and resolving the analyte from phospholipids during chromatography (Jemal et al. 2010). Excellent discussion of matrix effects and the various measures to eliminate or reduce matrix effects can be found in current literature (Matuszewski

Table 16.2 Approaches to minimize matrix effects (ME) at different stages of bioanalytical methods. (Reproduced from Nováková 2013)

A step of bioanalytical method	ME reduction approach	Examples of realization
Sample preparation	More extensive clean-up	SPE-based approaches with extensive and well optimized washing steps, RAM
		LLE-based approaches—ionized species do not partition into the organic layer
	Higher selectivity	SPE, MIP, immunoaffinity SPE
	Protein precipitation prior to SPE/LLE	
	Dilution of sample	
Chromatography	Higher separation efficiency	Fast/high resolution LC approaches, 2D-LC
	Nano-LC	Nano flow-rates, smaller droplets formed
	Change in selectivity	HILIC or other orthogonal chromatographic mode, change in mobile or stationary phase
	Gradient elution	Change in selectivity, enhancement of efficiency and also elution of highly retained interfering compounds
Mass spectrometry	Higher selectivity	Negative ion mode
	Ionization technique less susceptible to ME	APPI, APCI, EI-MS
Calibration data processing and other strategies	Appropriate calibration approach	Internal standard method, standard addition method, matrix-matched calibration
	Use of SIL-IS	13 C SIL-IS should be preferred over deuterium labeled compounds
	Echo peak strategy [122]	Elution very close to t_R of analyzed compounds ~ the same ME

LC liquid chromatography, *SPE* solid phase extraction, *LLE* liquid–liquid extraction, *RAM* restricted access materials, *MIP* molecularly imprinted polymers, *HILIC* hydrophilic interaction liquid chromatography, *APPI* atmospheric pressure photoionization, *APCI* atmospheric pressure chemical ionization, *EI-MS* electron ionization mass spectrometry, t_R retention time

et al. 2003; Jemal and Xia 2006; Van Eeckhaut et al. 2009; Jemal et al. 2010; Mulvana 2010; Li et al. 2011; Trufelli et al. 2011; Nováková 2013).

Nonspecific binding determination is important for LBAs. The guidance also recommends evaluation of parallelism for LBAs to detect matrix effect. Parallelism shows that sample dilution response is parallel to standard concentration-response curve. It is important to note that parallelism is not the same as QC dilution linearity as parallelism requires the use of incurred samples (DeSilva et al. 2012). Kelley and Desilva (2007) proposed testing for matrix effects in LBAs by comparing the concentration-response relationship of both spiked and unspiked samples of at least ten lots of the biological matrix to a comparable buffer solution.

16.6.2 Internal Standard

In addition to physicochemical factors, the concentration of IS is important (Tan et al. 2012; Mulvana 2010). Selection of optimum IS concentrations assures that the signal-to-noise ratio is adequate to obtain good sensitivity and precision, and minimizes or eliminates potential interference from unlabeled impurities in the reference standards of the IS or the analyte of interest.

While the use of IS acceptance criteria based on IS response range of spiked samples is a good practice for reanalyzing samples with abnormal IS response, it has to be used with caution in certain situations. For example, when IS variations in unknown samples and spiked samples are similar, IS variations do not affect the accuracy of the calibrators and QCs. In such situations, the need for reanalysis for IS variations may be moot. Also, in cases where IS variations in study samples are abnormally different from those in spiked samples, the IS acceptance criteria based on spiked samples may not be meaningful. In such cases, investigation should be conducted to confirm whether IS compensates for matrix effects (Tan et al. 2009; Savoie et al. 2010).

Abnormal variations in IS may occur for a number of reasons, including human errors (spiking twice or not spiking IS), imprecision of pipettes used to spike samples with IS (repeater pipettes), partial or complete blockage of autosampler needle (Table 16.3). Trends or patterns in variations in IS response may need to be investigated. Trends or patterns in IS variation include, but not limited to, contamination of the orifice or rods of MS due to incomplete or inadequate sample clean-up, matrix effects due to coeluting components, improper IS selection, incomplete solubility of IS in stock solution or extraction solvent, or inadequate mixing of IS (Tan et al. 2009). Therefore, it is a good practice to evaluate IS variations across an analytical batch, and investigate any abnormal patterns IS response in terms of its impact on the quantitation of unknown samples.

Although SIL ISs are preferred to develop a robust and accurate assay, the use of SIL ISs does not automatically guarantee accurate quantitation (Mulvana 2010). For example, it was shown that a deuterated IS may have a slightly different retention time compared to its normal counterpart, and thus may result in different degrees of matrix effects between the two analogues (a.k.a., deuterium isotope effect) (Wang et al. 2007). This may significantly affect analyte to IS ratio consistency. Also, the presence of normal analyte in SIL IS and its impact need to be assessed (EMA 2011).

16.6.3 Stability Issues

While assessment of freeze–thaw, short-term and long-term stabilities using QCs is useful to understand the stability of analyte in biological matrix, one should be aware that this information may be limited as QCs may not always mimic incurred

Table 16.3 Examples of abnormal internal standard (IS) response, reason for the response, and their impact on quantitation. (Reproduced from Tan et al. 2009)

Case	Observations	Root cause identified	Effect on quantitation or comments
1	Zero or nearly doubled IS response	Missed or double addition of IS	Yes
2	Random and sharp drop in IS response	Autosampler needle blockage	Usually no, unless S/N is too low
3	Gradual decrease of IS responses	Charging of mass spectrometer	Not in this case, but it usually depends on how well an IS follows an analyte
4	Random, sharp drop, and overall downward trend in IS response	Autosampler needle blockage plus charging of mass spectrometer	It depends, but batch should be reinjected
5	Low IS responses for most of the extracted samples	Mixed usage of right and wrong caps in LLE	It depends, but samples should be reassayed by using correct materials
6	High IS responses observed for incurred samples only (usually a whole subject)	Relatively less ion suppression in subject samples than in CS/QC	It depends on how well an IS follows an analyte
7	High IS responses observed for incurred samples only (usually a whole subject)	Recovery variation plus relatively less ion suppression in subject samples than in CS/QC	It depends on how well an IS follows an analyte
8	Low IS responses for incurred samples only (usually a whole subject)	Transfer of salt-containing intermediate layer in LLE	It depends, but samples should be reassayed
9	Less IS response variation with analogue IS than with deuterated IS	Analogue IS did not follow analyte well	Quantitation affected with analogue IS and it should be changed
10	Gradual increase of IS responses	Insufficient mixing	Not in this case, but should be evaluated case by case
11	Randomly scattered low IS responses for incurred samples only and not repeated during reanalysis	Not conclusive, but speculated as due to ascorbic acid and different cycles of F/T	Not in this case, but should be evaluated case by case
12	Deuterated IS not following the analyte and re-injection results not matching those of 1st injection	Not conclusive, but speculated as due to differential matrix effect between analyte and its deuterated IS	Yes in this case, but should be evaluated case by case

IS internal standard, *CS* calibration standard; *QC* quality control; *LLE* liquid–liquid extraction, *F/T* freeze and thaw

samples (Table 16.1). Therefore, during development of bioanalytical assays, a good understanding of the differences between QCs and incurred samples, the bioanalytical methods under consideration, and the physio-chemical and

Table 16.4 Examples of sources of instability and approaches to overcome instability

Causes of instability	Strategies to avoid instability	Examples of affected analytes
Enzymatic hydrolysis	Addition of enzyme inhibitors and/or freezing samples immediately after collection, or harvesting plasma at reduced temperature followed by immediate frozen storage	Olmesartan medoxomil, Capecitabine
Hemolysis	Depending on the analyte, testing the impact of different degrees of hydrolysis during method development. Factoring sample hemolysis during stability evaluations	Nitroglycerin, Fluvoxamine
Temperature	Lowering temperature during sample collection, processing, storage, extraction, reconstitution and analysis	Aspirin, Cisplatin, Acyl glucuronides
pH	Controlling pH within the desired range during sample collection, processing, storage, extraction, reconstitution and analysis	Cisplatin, Acyl glucuronides
Light	For photo-sensitive compounds, protection from light during sample handling is necessary, e.g., wrapping tubes in foil, using amber glass vials, or sample processing under yellow light or UV-filtered light	Nifedipine, Nisoldipine
Autooxidation	Addition of antioxidants to samples, e.g., ascorbic acid, sodium metabisulfite and ethylenediaminetetraacetic acid (EDTA)	Rifampin, Levodopa
Lactone/hydroxy acid interconversion	Decreasing pH and sample processing temperature or time	Atorvastatin, Simvastatin, Pravastatin
Adsorption to container walls	Using appropriate containers for sample collection, extraction, storage and analysis, e.g., silanized glass tubes. Addition of surfactants	Sufentanil, Tetrahydrocannabinol
In-source fragmentation/ transformation	Selecting suitable analyte-specific MS tuning of ionization conditions, assuring adequate chromatographic separation	Clozapine, Carboxylic acid metabolite

For references of the cited examples, refer to the appropriate sub-sections in Sect. 16.6.3

pharmacokinetics properties of the analyte(s) interest is essential (Jemal and Xia 2006; Mulvana 2010; Jemal et al. 2010; Li et al. 2011). Stability of the analyte of interest may be affected at different stages, from sample collection to sample analysis. Instability of analyte during bioanalysis can arise due to chemical or biological process following sample collection. These factors include photosensitivity, temperature, chemical reactivity, enzymatic degradation, hydrolysis of conjugated metabolites, interconversion under certain conditions (pH, enzymes, temperature), autooxidation, and transformation at the MS source (Table 16.4) (Jemal and Xia 2006; Briscoe and Hage 2009; Jemal et al. 2010; Silvestro et al. 2010; Yadav and Shrivastav 2011; Li et al. 2011). Several articles are available that discuss various factors that may impact instability (Chen and

Hsieh 2005; Jemal and Xia 2006; Briscoe and Hage 2009; Jemal et al. 2010; Mulvana 2010; Silvestro et al. 2010; Yadav and Shrivastav 2011; Li et al. 2011). A brief discussion of some of the factors is provided below.

16.6.3.1 Hydrolysis, pH, Temperature, Interconversion

The instability of analytes in biological fluids can be caused by enzymes. Esterases are the most prominent among hydrolases in plasma. Esterases catalyze the hydrolysis of esters and amide to their corresponding carboxylic groups (Chen and Hsieh 2005; Izhizuka et al. 2010). In addition to playing a vital role in the conversion of pro-drug to active drug, esterases can hydrolyze pro-drug or drug during sample collection, handling, and storage (Li et al. 2011). For compounds that are unstable in a biological matrix, taking adequate precautions during sample collection and/or handling are necessary to avoid instability of the analyte. These may include immediate freezing following sample collection, reduction in temperature during sample processing followed by immediate frozen storage at a very low temperature, thawing samples on wet ice, stabilizing samples by addition of enzyme inhibitors, or special treatment of samples such as acidification or protein precipitation (Guan et al. 2003; Besnard et al. 2008; Briscoe and Hage 2009; Mulvana 2010; Li et al. 2011). When an analyte of interest is unstable during blood sample collection, it is a good practice to confirm whole blood stability under the sample collection conditions. Also, when samples treated with enzyme inhibitors need to be diluted, the use of enzyme inhibitor-treated blank matrix is recommended. Also, one needs to be aware that stabilizers and inhibitors can cause interference or affect sample integrity (Mulvana 2010). Some enzyme inhibitors can be more prone to hydrolysis than the analyte of interest. In such cases, adding a relatively large amount of an analogue that is more sensitive to enzymatic degradation will prevent degradation of the analyte of interest (Li et al. 2011).

In general, lowering of temperature can substantially reduce degradation in biological matrix or solution (Chen and Hsieh 2005). For example, cisplatin is unstable at −25 °C, but stable at −70 °C (Andersson and Ehrsson 1995). The hydrolysis of acetylsalicylic acid (aspirin) can be controlled by thawing samples on ice, followed by extraction and analysis within 2 h after thawing. Storage at −20 °C for 11 days resulted in 20 % degradation of aspirin (Briscoe and Hage 2009).

The control of pH is also important for the analysis of most unstable analytes during sample collection, processing, storage, extraction, reconstitution and analysis, as pH within a narrow window is essential for most acid/base-catalyzed enzymatic and nonenzymatic reactions. pH can increase to 8.8 for unprocessed and untreated plasma samples stored at room temperature or at 37 °C, and to 9.5 during sample preparation (Fura et al. 2003). Therefore, maintaining pH in biological matrices and during sample processing is essential to prevent degradation of pH-sensitive compounds. Analytical methods for pH-sensitive compounds may

need to include procedures to stabilize the pH of biological matrices and sample extracts at the desired pH range. Cisplatin is highly susceptible to pH changes. Andersson and Ehrsson (1995) showed that cisplatin rapidly degrades at pH 7.4 in plasma, blood and ultrafiltrate, but is stable at pH 5.5 in plasma.

Ether- or ester-glucuronides are formed by glucuronidation via oxygen, while *N*-glucuronide or N^+-glucuronide arises due to glucuronidation via a primary, secondary, or tertiary amine. While there is no general rule to predict the instability of ether- or ester-glucuronides, ester-glucuronides (acyl glucuronides) tend to be less stable than ether-glucuronides (Li et al. 2011).

Acyl glucuronides tend to be unstable, especially under alkaline conditions (~pH 7.4) and elevated temperature, resulting in back-conversion to parent form (Jemal and Xia 2006; Jemal et al. 2010). Therefore, for acyl glucuronides forming compounds including, telmisartan, clopidogrelat (metabolite of clopidogrel), and ibuprofen, control of pH is important. Hydrolysis of acyl glucuronides can be minimized under mildly acidic conditions (pH 3–5). Although this is true for most acyl glucuronides, there are exceptions (Li et al. 2011). Therefore, necessary evaluation to understand pH-dependent stability of acyl glucuronides should be conducted during method development.

For compounds like clopidogrel and enalapril with methyl and ethyl ester groups, respectively, use of methanol and ethanol are not preferable during sample extraction as acyl glucuronides can react with methanol or ethanol to back convert to the parent form under basic conditions (Jemal and Xia 2006; Briscoe and Hage 2009; Jemal et al. 2010; Li et al. 2011). This may lead to overestimation of the parent drug. In some situations, underestimation of the parent drug is also possible. For example, drugs containing ethyl ester group, like enalapril, can react with methanol to produce methyl ester analogue of the drug (Jemal and Xia 2006; Jemal et al. 2010).

For *N*-glucuronides, back-conversion of the parent drug under acidic/basic and/or physiological pH condition or at an elevated sample processing temperature is largely compound dependent.

The ex vivo interconversion between the lactone metabolite and hydroxyacid drug has been observed for statins, like atorvastatin, simvastatin, and pravastatin, with the two forms exhibiting different pharmacological activities. Increasing pH and sample processing temperature or time promotes the ex vivo conversion of the lactone metabolite to the drug. It was demonstrated for statins that interconversion can be minimized by lowering sample preparation temperature (e.g., storage on ice-bath) and pH (e.g., pH ~4.5) (Kearney et al. 1993; Jemal and Xia 2000; Jemal and Xia 2006; Jemal et al. 2010; Zhang et al. 2010). Also, under the right conditions, including physiological or extreme pH and temperature, all chiral compounds can undergo interconversion. The *S* and *R* isomers of thalidomide have different pharmacological activities and PK properties.

16.6.3.2 Chemical Instability, Photolability, Autooxidation

Many *N*-oxides are thermally labile, photo-sensitive, and unstable in solutions and/or biological matrices during sample extraction, especially under strong acidic or basic conditions (Li et al. 2011).

The photochemical sensitive moieties include carbonyl, nitroaromatic structures (as electrophilic radicals), *N*-oxide function, carbon–carbon double bonds (liable to *E* to *Z* isomerization and autooxidation), and aryl chloride groups (liable to hemolytic and/or heterolytic dechlorination) (Jemal and Xia 2006; Briscoe and Hage 2009; Jemal et al. 2010). For example, nisoldipine and its metabolite are extremely photolabile both in organic solvent and in plasma, with degradation half lives of 6.3–6.7 min in dichoromethane-pentane and 10.7–11.3 min in plasma (Van Harten et al. 1987). This photodegradation was prevented by handling samples under sodium light. Also, in the presence of light, nifedipine degrades by 15 % in whole blood after 1 h, but only by 5 % in plasma after 2 h (Abou-Auda et al. 2000). Light protection is necessary when handling photo-sensitive compounds, e.g., tubes wrapped in foil, use of amber glass vials, or sample processing under yellow light or UV-filtered light.

Many small molecules, especially those containing phenol (e.g., catechol) or alcohol groups, can be readily oxidized in biological samples or reconstituted sample extracts (Saxer et al. 2004). A simple addition of antioxidants has been found to be very effective for stabilizing those analytes. Addition of ascorbic acid (vitamin C), a potent antioxidant, can be used to prevent degradation of rifampin-spiked plasma samples. It is recommended that the sample be supplemented with ascorbic acid at the time of blood sampling to stabilize rifampin (Le Guellec et al. 1997). Autooxidation of levodopa and 3-methyldopa in human plasma samples was prevented by the addition of sodium metabisulfite and ethylenediaminetetraacetic acid (EDTA) (Saxer et al. 2004). In the presence of such additives, levodopa and 3-methyldopa samples were stable for 16 weeks at −70 ºC. It is has been reported that a combination of an antioxidants, such as EDTA, fluoride oxalate, sodium citrate, heparin, may work better than a single agent for stabilizing labile compounds in a biological matrix or biological sample extracts (Li et al. 2011).

16.6.3.3 In-Source Fragmentation and/or Transformation

In-source fragmentation refers to fragmentation of molecules during the ionization process prior to entry into the Q1 chamber of the MS/MS. This is frequently observed for *N*-oxides, *S*-oxides, and glucuronide- or sulfate-conjugated metabolites of the analytes of interest that are present in sample extract. In-source fragmentation of such molecules spontaneously produces ions identical to precursor ions of the analytes of interest (Tan et al. 2012). Also, certain analytes can transform to others in MS source via in-source transformation (Jemal and

Xia 2006). For example, lactonization of a carboxylic acid metabolite can generate the same precursor ion as the original lactone. Without proper chromatographic separation, the lactone may be over-estimated.

To maximize the ionization of the analyte of interest and minimize the in-source fragmentation, suitable analyte-specific MS tuning of ionization conditions is needed (Kruger et al. 2010). For example, with the APCI interface, clozapine-*N*-oxide produces two major "fragment" ions at the same *m*/*z* value as clozapine and its *N*-demethylation metabolite. However, these ions were not found with the ESI interface (Niederlander et al. 2006).

16.6.3.4 Anticoagulant

Anticoagulant and/or anticoagulant counter ions can have impact on compound stability (Li et al. 2011). Heparin and EDTA are two commonly used anticoagulants: heparin inactivates thrombin while EDTA chelates calcium ions and interrupts the clotting cascade at multiple points. EDTA can prevent the activity of calcium dependent phospholipases and ester hydrolases, while heparin may not be inhibitory. In general, EDTA is preferred to heparin as an anticoagulant in plasma samples (Sadagopan et al. 2003). Matrix-related irreproducibility appeared to be more pronounced with heparin as the anticoagulant than with EDTA (Smeraglia et al. 2002; Yue et al. 2008).

16.6.3.5 Nonspecific binding

Nonspecific binding or container surface adsorption of drug molecules in biological samples can occur. For example, tetrahydrocannabinol blood concentration level in the glass containers was reported unchanged after 4 weeks of storage at −20 °C but not in the plastic polystyrene container (Christophersen 1986). Also, sufentanil concentrations in plasma decreased in nonsilanized glass tubes but were stable in silanized glass tubes (Dufresne et al. 2001). Addition of Tween-80 or CHAPS to the matrix may prevent nonspecific binding or container surface adsorption (Li et al. 2010).

16.6.3.6 Hemolysis

Hemolysis (i.e., lysis of red blood cells) results in release of their contents (e.g., enzymes, hemoglobin, inorganic ions). There are instances where hemolysis may affect the stability of the drug (DeSilva et al. 2012; Bérubé et al. 2011). Therefore, accuracy of PK data can be compromised when several samples in BE studies are hemolyzed. Therefore, the impact of hemolysis should be investigated depending on the analyte of interest and the method. Also, study samples should be carefully monitored for hemolysis. The FDA BMV guidance (2001) or the FDA's recent

draft guidance (2013) does not discuss hemolysis, and there is no established standard procedure to treat hemolyzed clinical samples. Also, the standard evaluation of degree of hemolysis in samples (i.e., visual) is a subjective determination (Bérubé et al. 2011; Garofolo et al. 2011). Therefore, for analyte or methods susceptible to hemolysis, the significance of hemolysis becomes a function of the extent of hemolysis (i.e., percentage of hemolyzed samples) or which samples are hemolyzed (i.e., around Cmax range).

Therefore, as described above, stability of analytes is of concern. Consequently, depending on the analyte of interest, it is recommended that the clinical and analytical sites coordinate precautionary measures (e.g., need for stabilizers, control temperature, protect samples from light) during sample collection, and post collection, processing, storage, and shipment conditions.

16.6.4 LBA Issues

In addition to reference standards, selection of reagents including ligand agents, binding proteins, conjugated antibodies, and radioligands are critical in the development and validation of LBAs (Kelley and DeSilva 2007). The reagents should allow for suitable specificity and selectivity, and stable binding characteristics. Similar to the reference standards, reagents in LBAs are also macromolecules, hence assay sensitivity and robustness can be adversely affected due to instability. Therefore, appropriate storage and handling are paramount in maintaining the integrity of the reagents.

LBAs generally use well plates (e.g., 96-well plates) for analysis of study samples. Each analytical batch may include several well plates. In such cases, the FDA's recent draft guidance (2013) recommends that sufficient replicate QCs are used in each plate to monitor accuracy, and acceptance criteria for the batch as well for individual plates are established. Some reagents including, conjugated antibodies and radioligands, and multi-well plates have lot-lot variations. Therefore, for long-term studies or studies with large sample size, sufficient quantity for a given lot is necessary. Also, if multiple lots of reagents or well plates are used, assessment of lot-to-lot variability and comparability may be necessary.

16.6.5 Endogenous Assays

Assays for macromolecules, commonly LBAs, are often used for quantification of macromolecule therapeutics that are recombinant or modified variants of endogenous proteins or peptides. Therefore, use of routine blank matrices will not guarantee accuracy of measurement of the therapeutic macromolecule, due to the presence of its endogenous counterpart. Therefore, the FDA's recent draft guidance (2013) recommends special considerations are made for matrix selection and

conduct for such assays. One option is to use stripped matrix (e.g., charcoal, immunoaffinity) or alternate matrices (e.g., protein buffers) for calibrators, and unaltered matrix intended for the study for QCs. When using altered or alternate matrices, confirmation of the absence of measurable levels of the endogenous analyte is essential, preferably using an independent but sensitive and validated method (i.e., LC-MS/MS). Also, an attempt should be made to determine absence of matrix effects in altered or alternate matrices (DeSilva et al. 2003). For QCs in unaltered matrix, one can use the recovery experiment to assure accuracy (FDA 2013), wherein the recovery of spiked material is estimated from the unaltered matrix with quantifiable endogenous material, provided the endogenous and spiked analytes behave in an additive manner (DeSilva et al. 2003).

16.6.6 Diagnostic Kits

Kits (usually LBAs) routinely used for clinical diagnostics, are sometimes used in bioanalysis. Therefore, the FDA's recent draft guidance (2013) recommends demonstrating the reliability of such kits for quantitative determination. Some of the issues with diagnostic kits are briefly discussed below.

Manufacturers' validation data should not be relied on for diagnostic kits. Instead, the kits should be validated in-house, and a complete validation may be necessary if the kit is modified (see Sect. 16.3). Some kits may include sparse calibration standards (e.g., single- or two-point calibration curves). Therefore, it is a good practice to establish a calibration response curve with the required set of calibration standards (as described in Sect. 16.3.4) during validation and study sample analysis (FDA 2013). Also, the nominal concentrations of kit QCs are sometimes not provided, instead expressed as ranges. In such cases, in-house QCs with known nominal concentrations are recommended for use, independent of the kit-supplied QCs (FDA 2013). Proper justification and appropriate cross-validation experiments are required when standards and QCs supplied with the kits are prepared in a matrix different from the subject samples (see Sects. 16.3.4.7 and 16.6.5).

16.6.7 Automation/High Throughput Assays

Automation or high throughput analysis may require modification of validation procedures and in some cases, validation of additional factors. This may require increasing sample size of intra-batch validation, and/or additional validation batches. High throughput analysis may also require proper maintenance of instruments between batch analysis to prevent residual contamination.

16.6.8 Human Errors

Human errors can also contribute to lack of accuracy of study data. It has been shown that lack of homogeneity of study samples can result in errors in quantitation (Tan et al. 2009; Yadav and Shrivastav 2011; Nováková 2013). Also, inconsistent addition of IS to study samples may affect analyte/IS ratios and therefore its reported concentration (Tan et al. 2009). Switching samples during bioanalysis has been reported (Yadav and Shrivastav 2011). Therefore, adequate training of analysts and establishing ruggedness of methods are required.

16.7 Incurred Sample Reanalysis

Reproducibility issues in study samples from dosed subjects (i.e., incurred samples) are observed although the samples are analyzed using validated bioanalytical assays. Many of the assay issues discussed in the earlier section can contribute to reproducibility issues. Briefly, reproducibility issues may arise due to matrix effects, insufficiently validated method (e.g., improper extraction conditions, inadequate enzyme inhibitor, inadequate stability validation) and/or poor execution (inconsistent mixing, processing error) (Matuszewski et al. 2003; Jemal and Xia 2006; Wang et al. 2007; Besnard et al. 2008; Tan et al. 2009; Jemal et al. 2010; Silvestro et al. 2010; Meng et al. 2011; Yadav and Shrivastav 2011; Tan et al. 2012). Reanalysis of a subset of study samples to check for reproducibility in incurred samples is often referred to as incurred sample reanalyses (ISR). ISR is used to confirm that an analytical method performs as intended in clinical and nonclinical studies. Therefore, ISR serves as a confirmatory tool to ensure that all factors contributing to assay performance are in control during study sample analysis, and thereby assures the reliability of study data. ISR over the years has become an integral part of bioanalysis.

The concept of reanalysis of a subset of study samples was adopted by Health Canada in 1992 although the strategy for interpretation of the data was not clear, and the requirement was abandoned in 2003. The reanalysis of a subset of study samples as part of ISR and the need for implementation of ISR was first discussed at the 2006 AAPS/FDA Workshop (Viswanathan et al. 2007). The consensus for procedures for ISR conduct was reached at the 2008 ISR Workshop (Fast et al. 2009). Many of the ISR recommendations proposed at the 2008 ISR Workshop (Fast et al. 2009) have been adopted by the industry over the years, and recently by regulatory agencies including, the EMA (2011) and Health Canada (2012). In addition, the FDA has proposed similar ISR recommendations in their recently issued draft BMV guidance (2013). The consensus at the 2008 ISR Workshop was that ISR conduct is important for nonclinical and clinical studies where PK assessment is the primary end point, especially for all BE studies (Fast et al. 2009). Also, the consensus at the workshop was to use a sample size of 5–10 %

of the total study samples depending on size of the study (e.g., minimum of 5 % for a large study) for ISR. However, the recent FDA draft guidance (2013) recommends ISR sample size of 7 % of the total study samples. Sample selection for ISR includes selection of samples from individual subjects, with fewer samples (at the Cmax and elimination range of the PK profile) from more subjects. ISR is acceptable when at least 67 % of the reanalyzed concentrations are within 20 % (30 % for LBAs) of their original concentrations when normalized to their means of the original and reanalyzed concentrations (Fast et al. 2009; FDA 2013). It is a good practice to select samples from across the duration of sample analysis. If samples identified for reanalysis were diluted during the original analysis, then the same dilution factor used for the original result needs to be employed for ISR. Also, it is important that the number of replicates and the acquisition method used for ISR are the same as those used during the original analysis.

Acknowledgments The author thanks Drs. Brian P. Booth, Ethan M. Stier, Robert Lionberger, and Yongsheng Yang for their critical review of the manuscript.

Disclaimer The chapter reflects the views of the author and should not be construed to represent FDA's views or policies. No official endorsement by the FDA is intended or should be inferred.

References

Abou-Auda HS, Najjar TA, Al-Khamis KI et al (2000) Liquid chromatographic assay of nifedipine in human plasma and its application to pharmacokinetic studies. J Pharm Biomed Anal 22 (2):241–249

Andersson A, Ehrsson H (1995) Stability of cisplatin and its monohydrated complex in blood, plasma and ultrafiltrate-implications for quantitative analysis. J Pharm Biomed Anal 13:639–644

Bérubé ER, Taillon MP, Furtado M et al (2011) Impact of sample hemolysis on drug stability in regulated bioanalysis. Bioanalysis 3(18):2097–2105. doi:10.4155/BIO.11.190

Besnard T, Renée N, Etienne-Grimaldi MC et al (2008) Optimized blood sampling with cytidine deaminase inhibitor for improved analysis of capecitabine metabolites. J Chromatogr B 870 (1):117–120. doi:10.1016/j.jchromb.2008.05.040

Bozovic A, Kulasingam V (2013) Quantitative mass spectrometry-based assay development and validation: from small molecules to proteins. Clin Biochem 46:444–455. doi:10.1016/j.clinbiochem.2012.09.024

Briscoe CJ, Hage DS (2009) Factors affecting the stability of drugs and drug metabolites in biological matrices. Bioanalysis 1(1):205–220. doi:10.4155/BIO.09.20

Chen J, Hsieh Y (2005) Stabilizing drug molecules in biological samples. Ther Drug Monit 27 (5):617–624

Christophersen AS (1986) Tetrahydrocannabinol stability in whole blood: plastic versus glass containers. J Anal Toxicol 10(4):129–131

Desilva B, Smith W, Weiner R et al (2003) Recommendations for the bioanalytical method validation of ligand-binding assays to support pharmacokinetic assessments of macromolecules. Pharm Res 20(11):1885–1900

DeSilva B, Garofolo F, Rocci M et al (2012) 2012 white paper on recent issues in bioanalysis and alignment of multiple guidelines. Bioanalysis 4(18):2213–2226. doi:10.4155/BIO.12.205

Dufresne C, Favetta P, Paradis C et al (2001) Stability of sufentanil in human plasma samples. Ther Drug Monit 23:500–552

EMA (2011) Guideline on bioanalytical method validation. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf. Accessed July 2013

Fast DM, Kelley M, Viswanathan CT et al (2009) Workshop report and follow-up—AAPS workshop on current topics in GLP bioanalysis: assay reproducibility for incurred samples—implications of Crystal City recommendations. AAPS J 11(2):238–241. doi:10.1206/s12248-009-9100-9

FDA (2001) Guidance for industry: bioanalytical method validation. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf. Accessed July 2013

FDA CFR (2013) Code of Federal Regulations Title 21 Part 320. http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?CFRPart=320&showFR=1&subpartNode=21:5.0.1.1.7.2. Accessed July 2013

FDA (2013) Draft guidance for industry: bioanalytical method validation (revised). http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM368107.pdf. Accessed Sept 2013

Findlay JWA, Das I (2006) Enzyme immunoassay and related bioanalytical methods. In: Swarbrick J (ed) Encyclopedia pharmaceutical technology. Informa HealthCare, New York, pp 1566–1579

Fura A, Harper TW, Zhang H et al (2003) Shift in pH of biological fluids during storage and processing: effect on bioanalysis. J Pharm Biomed Anal 32(3):513–522

Garofolo F, Rocci ML, Dumont I et al (2011) 2011 white paper on recent issues in bioanalysis and regulatory findings from audits and inspections. Bioanalysis 3(18):2081–2096. doi:10.4155/BIO.11.192

Guan F, Uboh C, Soma L et al (2003) Sensitive liquid chromatographic/tandem mass spectrometric method for the determination of beclomethasone dipropionate and its metabolites in equine plasma and urine. J Mass Spectrom 38(8):823–838

Health Canada (1992) Guidance to industry. Conduct and analysis of bioavailability and bioequivalence studies-part A: oral dosage formulations used for systemic effects. http://www.hc-sc.gc.ca/dhp-mps/alt_formats/pdf/prodpharma/applic-demande/guide-ld/bio/gd_cbs_ebc_ld-eng.pdf; http://faculty.ksu.edu.sa/64448/Documents/Guideline%20BA%20-%20BE%20Part%20A.pdf. 1371 Accessed Oct 2013

Health Canada (2003) Notice to industry-removal of requirement for 15% random replicate sample notice affecting guideline A and guideline B. http://www.hc-sc.gc.ca/dhp-mps/alt_formats/pdf/prodpharma/applic-demande/guide-ld/bio/gd_cbs_ebc_ld-eng.pdf. Accessed Oct 2013

Health Canada (2012) Notice to guidance document: conduct and analysis of comparative bioavailability studies. http://www.hc-sc.gc.ca/dhp-mps/alt_formats/pdf/prodpharma/applic-demande/guide-ld/bio/gd_cbs_ebc_ld-eng.pdf. Accessed July 2013

Izhizuka T, Fujimori I, Kato M et al (2010) Human carboxymethylenebutenolidase as a bioactivating hydrolase of olmesartan medoxomil in liver and intestine. J Biol Chem 285 (16):11892–11902. doi:10.1074/jbc.M109.072629

Jemal M, Xia YQ (2000) Bioanalytical method validation design for the simultaneous quantitation of analytes that may undergo interconversion during analysis. J Pharm Biomed Anal 22 (5):813–827

Jemal M, Xia YQ (2006) LC-MS development strategies for qualitative bioanalysis. Curr Drug Metab 7(5):491–502

Jemal M, Ouyang Z, Xia YQ (2010) Systematic LC-MS/MS bioanalytical method development that incorporates plasma phospholipids risk avoidance, usage of incurred sample and well thought-out chromatography. Biomed Chromatogr 24(1):2–19. doi:10.1002/bmc.1373

Kearney AS, Crawford LF, Mehta SC et al (1993) The interconversion kinetics, equilibrium, and solubilities of the lactone and hydroxyacid forms of the HMG-CoA reductase inhibitor, CI-981. Pharm Res 10(10):1461–1465

Kelley M, DeSilva B (2007) Key elements of bioanalytical method validation for macromolecules. AAPS J 9(2):E156–E163

Korfmacher WA (2005) Foundation review: principles and applications of LC-MS in new drug discovery. Drug Discov Today 10(20):1357–1367

Kruger R, Vogeser M, Burghardt S et al (2010) Impact of glucuronide interferences on therapeutic drug monitoring of posaconazole by tandem mass spectrometry. Clin Chem Lab Med 48 (12):1723–1731. doi:10.1515/CCLM.2010.333

Le Guellec C, Gaudet ML, Lamanetre S et al (1997) Stability of rifampin in plasma: consequences for therapeutic monitoring and pharmacokinetic studies. Ther Drug Monit 19(6):669–674

Li W, Luo S, Smith HT et al (2010) Quantitative determination of BAF312, a S1P-R modulator, in human urine by LC-MS/MS: prevention and recovery of lost analyte due to container surface adsorption. J Chromatogr B 878(5–6):583–589. doi:10.1016/j.jchromb.2009.12.031

Li W, Zhang J, Tse FLS (2011) Strategies in qualitative LC-MS/MS analysis of unstable small molecules in biological matrices. Biomed Chromatogr 25(10):258–277

Matuszewski BK, Constanzer ML, Chavez-Eng CM (2003) Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal Chem 75:3019–3030. doi:10.1021/ac020361s

Meng M, Reuschel S, Bennett P (2011) Identifying trends and developing solutions for incurred sample reanalysis failure investigations in a bioanalytical CRO. Bioanalysis 3(4):449–465. doi:10.4155/BIO.10.212

Mulvana DE (2010) Critical topics in ensuring data quality in bioanalytical LC-MS method development. Bioanalysis 2(6):1051–1072. doi:10.4155/BIO.10.60

Niederlander HA, Koster EH, Hilhorst MJ et al (2006) High throughput therapeutic drug monitoring of clozapine and metabolites in serum by on-line coupling of solid phase extraction with liquid chromatography–mass spectrometry. J Chromatogr B 834(1–2):98–107

Niessen WM (2003) Progress in liquid chromatography-mass spectrometry instrumentation and its impact on high-throughput screening. J Chromatogr A 1000(1–2):413–436

Nováková L (2013) Challenges in the development of bioanalytical liquid chromatography-mass spectrometry method with emphasis on fast analytes. J Chromatogr A 1292:25–37. doi:10.1016/j.chroma.2012.08.087

Rozet E, Marini RD, Ziemons E et al (2011) Advances in validation, risk and uncertainty assessment of bioanalytical methods. J Pharm Biomed Anal 55(4):848–858. doi:10.1016/j.jpba.2010.12.018

Sadagopan NP, Li W, Cook JA et al (2003) Investigation of EDTA anticoagulant in plasma to improve the throughput of liquid chromatography/tandem mass spectrometric assays. Rapid Commun Mass Spectrom 17(10):1065–1070

Savoie N, Booth BP, Bradley T et al (2009) The 2nd calibration and validation group workshop on recent issues in good laboratory practice bioanalysis. Bioanalysis 1(1):19–30. doi:10.4155/BIO.09.11

Savoie N, Garofolo F, van Amsterdam P et al (2010) 2010 white paper on recent issues in regulated bioanalysis & global harmonization of bioanalytical guidance. Bioanalysis 2(12):1945–1960. doi:10.4155/BIO.10.164

Saxer C, Niina M, Nakashima A et al (2004) Simultaneous determination of levodopa and 3-O-methyldopa in human plasma by liquid chromatography with electrochemical detection. J Chromatogr B 802(2):299–305. doi:10.1016/j.jchromb.2003.12.006

Silvestro L, Gheorghe MC, Tarcomnicu I et al (2010) Development and validation of an HPLC-MS/MS method to determine clopidogrel in human plasma. Use of incurred samples to test back-conversion. J Chromatogr B 878(30):3134–3142. doi:10.1016/j.jchromb.2010.09.022

Singleton C (2012) Recent advances in bioanalytical sample preparation for LC-MS analysis. Bioanalysis 4(9):1123–1140. doi:10.4155/BIO.12.73

Smeraglia J, Baldrey SF, Watson D (2002) Matrix effects and selectivity issues in LC-MS-MS. Chromatographia 55(suppl 1):S95–S99

Tan A, Hussain S, Musuku A et al (2009) Internal standard response variations during incurred sample analysis by LC-MS/MS: case by case trouble shooting. J Chromatogr B 877(27):3201–3209. doi:10.1016/j.jchromb.2009.08.019

Tan A, Boudreau N, Lévesque A (2012) Internal standards for quantitative LC-MS bioanalysis. In: Xu QA, Madden TL (eds) LC-MS in drug bioanalysis. Springer, New York, pp 1–32

Timmerman P, Luedtke S, van Amsterdam P et al (2009) Incurred sample reproducibility: views and recommendations by the European bioanalysis forum. Bioanalysis 1(6):1049–1056. doi:10.4155/BIO.09.108

Trufelli H, Palma P, Famiglini G et al (2011) An overview of matrix effects in liquid chromatography–mass spectrometry. Mass Spectrom Rev 30(3):491–509. doi:10.1002/mas.20298

Van Eeckhaut A, Lanckmans K, Sarre S et al (2009) Validation of bioanalytical LC-MS/MS assays: evaluation of matrix effects. J Chromatogr B 877(23):2198–2207. doi:10.1016/j.jchromb.2009.01.003

Van Harten J, Lodewijks MT, Guyt-Scholten JW et al (1987) Gas chromatographic determination of nisoldipine and one of its metabolites in plasma. J Chromatogr 423:327–333

Viswanathan CT, Bansal S, Booth B et al (2007) Quantitative bioanalytical methods validation and implementation: best practices for chromatographic and ligand binding assays. Workshop/conference report. AAPS J 9(1):E30–E42

Wang S, Cyronak M, Yang E (2007) Does a stable isotopically labeled internal standard always correct analyte response? A matrix effect study on a LC/MS/MS method for the determination of carvedilol enantiomers in human plasma. J Pharm Biomed Anal 43(2):701–707. doi:10.1016/j.jpha.2006.08.010

Yadav M, Shrivastav PS (2011) Incurred sample reanalysis (ISR): a decisive tool in bioanalytical research. Bioanalysis 3(9):1007–1024. doi:10.4155/BIO.11.76

Yue B, Pattison E, Roberts WL et al (2008) Choline in whole blood and plasma: sample preparation and stability. Clin Chem 54(3):590–593. doi:10.1373/clinchem.2007.094201

Zhang J, Rodila R, Gage E et al (2010) High-throughput salting-out assisted liquid/liquid extraction with acetonitrile for the simultaneous determination of simvastatin and simvastatin acid in human plasma with liquid chromatography. Anal Chim Acta 661(2):167–172. doi:10.1016/j.aca.2009.12.023