

## Chapter 2

# Understanding Bioanalysis Regulations

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**Abstract** Bioanalytical data submitted to global health authorities in support of drug approval applications is expected to meet certain regulatory standards and requirements. Bioanalytical assay validation and the conduct of study sample analysis is addressed with a multitude of regulatory guidance documents. This chapter provides an overview of the regulations from a historical perspective to current day developments. Originally written around bioanalysis to support pharmacokinetic studies, bioanalysis regulatory language is now being applied to endogenous biomarker assays and evaluating immunogenicity of large molecule therapeutics. Quality management and documentation are also discussed in this chapter along with the process of how modern bioanalysis regulations are continuing to develop.

**Keywords** Bioanalysis • Regulatory • Regulated • LC-MS/MS • LBA • Guidance • PK • Immunogenicity • Biomarker

Charged with ensuring public health, health authorities enact regulations, guidance, and guidelines when they see deficiencies within the content of new drug filings or during inspections of facilities performing the work for such filings. The newly introduced regulations have ranged from minor to dramatic with many having a significant impact on the pharmaceutical industry practices. In many cases, new regulations address deficiencies observed in practices at individual companies but nonetheless are broadly extrapolated and applied; thus, many pay for the errors of a few. Over the past decade, there has been a steady expansion of not only the breadth

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of topics being regulated, but also the number of countries developing their own requirements. Due to this complex and sometimes conflicting set of rules, the pharmaceutical industry has responded with regional organizations and multiregional consortia providing feedback in an attempt to harmonize practices. This chapter provides a historical perspective on the issue, enabling the reader to understand the context from which today's complex bioanalytical regulations arose.

A historical timeline depicting the evolution of bioanalytical regulation and guidance is presented in Table 2.1. Good Laboratory Practices (GLPs) are the foundation upon which health authorities have built today's bioanalytical regulations. The first GLPs were enacted in 1972 in New Zealand and Denmark [1, 2]. In 1975, data irregularities found by the US Food and Drug Administration (FDA) in records at two laboratories resulted in congressional hearings. The hearings resulted in more inspection staff being added to the FDA, and a philosophical shift from trusting in the data submitted, to requiring demonstration that the data was reliable, with confirmation through agency audits. Subsequent to this, activities continued within the Agency culminating on December 22, 1978, when the GLPs became law within Section 21 of the Code of Federal Regulations Part 58 (21 CFR Part 58) [3]. Of note is that the US FDA GLPs only address animal safety studies. While widely followed for clinical bioanalytical studies, they are not required.

Since 1978, other countries and regions have implemented their own versions of the GLPs. In 1978, the Organisation for Economic Co-operation and Development's (OECD) Working Group released its "Principles of Good Laboratory Practice" [4] based on the 1976 draft FDA proposal on GLPs. The Principles were recommended by the OECD for use by member countries in 1981. Subsequently, a series of guidance documents for industry and health authorities were released, covering in detail additional aspects of GLPs, including the roles of the Sponsor and Study Director, the use of computerized systems, multisite studies and auditing, as well as updates to the primary GLP document [5].

The 34 OECD member countries follow the organization's GLPs and many nonmember countries also frequently follow them or similar local regulations. But, in an odd twist, while the FDA is a member country and has adopted many aspects of the OECD GLPs, unlike most of the OECD member nations, the FDA does not certify laboratories and facilities for GLP compliance. The FDA's inspection program is based on directed (inspectors sent to assess clinical (bioequivalence) and nonclinical [GLP] content within a filing) and nondirected (inspectors sent to GLP facilities that are frequently listed within filings) audits. While the outcome of these inspections may or may not result in the issuance of a Form 483 noting deficiencies in practice, the FDA does not issue a certification. This is increasingly becoming an issue for US sponsors since the UK's Medicines and Healthcare Products Regulatory Agency (MHRA) and the European Union's (EU) European Medicines Agency (EMA) have begun questioning the acceptability of clinical and nonclinical data from laboratories that have not had recent inspections [6].

In December 1990, industry and regulatory authorities representing the American Association of Pharmaceutical Scientists (AAPS), Federation International Pharmaceutique, Health Protection Branch (today known as Health Canada),

**Table 2.1** Timeline of significant regulations impacting bioanalysis

1972	Denmark and New Zealand introduce GLPs
1978	USA: FDA GLPs Implemented, following 1977 identification of fraudulent records by FDA inspectors
1992	Canada: Health Canada—Guidance for Industry: conduct and analysis of bioavailability and bioequivalence studies—Part A: Oral dosage formulations used for systemic effects
1997	USA: FDA 21CFR Part 11 Electronic records; Electronic signatures
1997	Japan: Ministerial ordinance concerning the standards for the conduct of nonclinical studies on the safety of drugs (Ministry of Health and Welfare Ordinance No. 21, dated March 26, 1997)
1998	International: OECD Principles of good laboratory practice and compliance monitoring
2001	USA: FDA Guidance for industry: bioanalytical method validation
2003	USA: FDA Bioavailability and bioequivalence studies for orally administered drug products—general considerations
2003	Brazil: Resolution No. 899, guide for validation of analytical and bioanalytical methods
2005	Brazil: revision to 2003 ANVISA regulation
2005	India: Ministry of Health and Family, Guidelines for Bioavailability and Bioequivalence Studies
2005	International: ICH Q2(R1): validation of analytical procedures: text and methodology
2005	China: CFDA Technical guideline for human bioavailability and bioequivalence studies on chemical drug products
2009	International: WHO Good Clinical Laboratory Practice (GCLP)
2010	European Union: EMA guideline on the investigation of bioequivalence
2011	China: CFDA (2011) Guidance on management of laboratory for drug clinical trial biological sample analysis (interim)
2011	European Union: EMA Guideline on Bioanalytical Method Validation
2012	Brazil: ANVISA Resolution RDC 27, minimum requirements for bioanalytical method validation used in studies with the purpose of registration and post-registration of medicines
2012	Canada: HPFB conduct and analysis of comparative BA studies
2012	European Union: EMA Reflection paper for laboratories that perform the analysis or evaluation of clinical trial samples (GCLP)

(continued)

**Table 2.1** (continued)

2013	Japan: MHLW Guideline on bioanalytical method validation in pharmaceutical development
2014	Japan: MHLW—Guideline on bioanalytical method (ligand-binding assay) validation in pharmaceutical development
2013	USA: FDA DRAFT Guidance for Industry: bioanalytical method validation
2015	European Union: EMA provides guide for reviewers that highlights the absence of US FDA laboratory certification process for GLP studies
2015	Canada: Health Canada requires stability testing to use 3 separate samples derived from separate containers, as opposed to 3 samples from a single storage container

US FDA, and the Association of Official Analytical Chemists met together at a conference entitled “Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies.” This was the first of what later came to be known as the Crystal City conferences, based on the location of this first meeting. The meetings objectives, which were met, included: (1) develop a consensus on required tests within a bioanalytical method validation and performance criteria; (2) how to apply those criteria to study sample analysis in bioavailability, bioequivalence, and pharmacokinetics studies; and [3] capture within a conference report all major consensus views for use in developing more specific regulatory guidance. The 3-day conference focused primarily on chromatographic methods (HPLC and GC with detectors that were available at the time, as LC-MS/MS was still very much in its infancy). The immunochemistry community was not forgotten at the meeting, but due to its lesser prevalence then as compared to today’s post-biotechnology revolution, only a half-page of the conference report focused on immunochemistry techniques. Microbiological assays and kit assays were also discussed but given much less air time. The five-page conference report provided the consensus of the workshop and was viewed as the first step to not only harmonized practices across the industry, but also became a set of expectations against which both Canadian and US regulators began to assess drug concentration assays [7].

Moving much more quickly than its US counterpart, the Canadian Health Protection Branch released in 1992 its Guidance for Industry on the *Conduct and Analysis of Bioavailability and Bioequivalence Studies—Part A: Oral Dosage Formulations Used for Systemic Effects* [8].

That document used the concepts of the Crystal City meeting to describe the first regulatory language on bioanalysis and referred directly to the conference report and the GLPs:

Bioavailability determinations rely on the adequacy of analytical methods for parent drugs and, when appropriate, their metabolites. This section describes the attributes of such methods and the validation procedures required in reports to establish and maintain selectivity, range, precision, and accuracy.

These concepts and the testing supporting them were delineated and incorporated into the Guidance in sufficient detail for their successful implementation by industry. The Guidance did not address which technology was covered, but given its limitation to orally administered drugs, it could be assumed that immunoassays for biologic drugs were not covered. However, as part of the required practices for bioavailability and bioequivalence study sample analysis, the fundamental concepts of today's incurred sample reanalysis (ISR) were defined when samples were analyzed in singlicate: "The purpose of the re-assaying is to establish that the degree of precision obtained with incurred samples is similar to that obtained for spiked standard or Quality Control (QC) samples." This requirement was dropped in 2003 [9], prior to the FDA-AAPS Crystal City IV discussion and the subsequent FDA expectation that ISR be routinely employed in a number of studies [10].

Another outcome of this regulation was setting the highest standard of performance for bioequivalence and bioavailability studies, studies central to labeling claims, which became more broadly applied to all nonclinical GLP studies and all clinical studies. In recent years, this broader application of very comprehensive and tough standards has been discussed frequently within scientific conferences and published on by both the industry and regulatory communities. While the concept of a tiered approach (lesser standards for some assays or studies and higher standards for others) exists, there has not been a consensus on which studies fall into each category. In this regard, some regulators have put forth the concept that studies affecting the prescribing label for a new drug should follow the highest standards. Thus, studies such as food effect, age–gender, and drug–drug interaction would be considered for the higher standard.

In 1997, the US FDA issued 21 CFR Part 11 governing the use of computerized systems, electronic records and electronic signatures [11]. This regulation, aimed at improving the use of computer systems, required that organizations operating under predicate rules (e.g., GLPs) ensured that the computer systems and resulting generated records also met the requirements of the predicate rules. For laboratories operating under the GLPs, structured computer system life cycle practices were adopted, electronic record maintenance and archive practices were improved, and audit trails were implemented to track the creation and editing of records in a GLP-compliant manner. The latter meant that during record generation, the "who did it", "when", and "what was created" needed to be tracked. In addition, when changes were made, the previous versions of the record needed to be maintained and the reason for the change documented and tracked. Even though there was no clinical counterpart to the GLPs, most bioanalytical labs used the same computer systems for clinical studies and achieved equivalent quality standards.

In December 1998, the US FDA released its draft "Guidance for Industry Bioanalytical Methods Validation for Human Studies" [12] for comment. Similar to the Canadian Guidance, the US FDA draft Guidance was focused on assay (1) accuracy, (2) precision, (3) sensitivity, (4) specificity, (5) linearity, and (6) reproducibility, but its simple Table of Contents belied the document's complexity over its Canadian predecessor. While titled for human clinical studies, it required that "The analytical laboratory conducting BA and BE studies should closely

adhere to FDA's Good Laboratory Practices (GLPs) (21 CFR Part 58) and to sound principles of quality assurance throughout the testing process."

The industry response and discussions with the Agency resulted in the second Crystal City Workshop "Bioanalytical Methods Validation—A Revisit with a Decade of Progress." It was held in January 2000 and focused on chromatographic methods. Three months later, an AAPS workshop on macromolecule measurement was held. At both events, industry and health authority representatives actively discussed the science and regulations of bioanalysis. A combined report on both workshops was issued later in the year [13]. Having heard the scientific and practical concerns of the industry, the US FDA went on to finalize and release its final version of the "Guidance for Industry: Bioanalytical Method Validation" in May 2001. It became known as the BMV [14].

In 2003, Brazil's ANVISA released its bioanalytical regulations, which were updated in 2005 and more recently in 2012 [15]. Different still from the USA and Canadian Guidances, which are regulatory recommendations for best practices, ANVISA's regulations are law and leave no wiggle room for variations in science or compliance. Aimed primarily at BE studies, these regulations included specifics, such as using the simplest regression model and use of the " $T = 0$ " measurement for assessing stability (i.e., using the actual concentration determined at the initiation of a stability study (time zero) as the comparator for subsequent stability time point measurements), that have generated challenges for industry practitioners. For HPLC or LC-MS/MS assays, use of any model other than linear required demonstrated statistical evidence that the chosen model was appropriate. Use of  $T = 0$ , rather than nominal concentrations for stability measurements in QC samples, meant that data demonstrating stability against nominal concentrations accepted by other countries could fail when compared to the  $T = 0$  concentration data. This often required the reporting of stability data in two ways at a minimum for each method, and in some cases it required repeating stability measurements to obtain acceptable data for both approaches [10]. In later updates to its regulations, ANVISA harmonized its practices to assessing stability against nominal concentration and other updates have kept pace with changes in other regions.

Through two additional meetings with industry, Crystal City III (2005) and IV (2006), the FDA was able to refine a number of its expectations for bioanalysis and put forward the concept of incurred sample reproducibility as a means of assessing method robustness and analyst execution. The conference reports for these meetings, while not FDA Guidance, did become the basis for their expectations for bioanalytical method validation and sample analysis [16, 17]. Based on FDA observations of instances of poor data reproducibility in the field, the FDA's expectation for incurred sample reanalysis (ISR) was included in the Crystal City III consensus report. The requirement for repeating a portion of the samples to demonstrate assay quality was highly controversial with industry and Crystal City IV focused specifically on the issue, refining the concept beyond method quality to include a quality of execution assessment. ISR is now embedded within routine bioanalysis.

Further expansion of regional regulations came with the EMA issuing a draft and then finalized “Guideline on Bioanalytical Method Validation” in 2011. This Guideline provided a comprehensive set of requirements for chromatographic and ligand-binding immunoassays based on the then current state of the art for both technologies [18]. It became the de facto global standard for bioanalytical regulation. The Guideline included a few items that further harmonized practices, but for the most part industry was already in compliance. A larger, but less noticeable change came with a 2012 EMA reflection paper of Good Clinical Laboratory Practices (GCLP), which blended aspects of Good Clinical Practice (patient confidentiality and safety) with documentation and evidentiary aspects of the GLPs, thus requiring all bioanalytical clinical and nonclinical work to have identical compliance standards [19]. While most of the industry already analyzed clinical samples in accordance with the principles of GLP or in a GLP-like manner, this reflection paper put forward the equivalent standard and eliminated any uncertainty in practice.

Regional expansion continued in 2012 when Health Canada indicated that it would operate under the EMA’s BMV Guideline [20]. In 2013 and 2014, Japan’s Ministry of Health, Labour and Welfare (MHLW) issued its regulations for bioanalysis using chromatographic and ligand-binding assays, respectively [21, 22]. These guidances were consistent with the EMA Guideline, but were less strict and provided more flexibility to incorporate science into bioanalytical practice. They also differed from the FDA and EMA guidance/guideline creation process in that MHLW’s working group included industry representatives as part of the writing team, rather than relying on industry comments to draft documents. In 2013, the FDA issued a draft revision to its BMV guideline [23] that resulted in both positive and negative responses from industry, and another AAPS-FDA Workshop, Crystal City V. The workshop report included only minor changes to drug concentration measurement practices. While that workshop report did provide an initial framework on the FDA’s inclusion of biomarkers by chromatographic and ligand-binding assay (LBA) methods within the BMV, no specific recommendations were made. An in-depth discussion of the unique issues related to measuring biomarkers was continued with the FDA at Crystal City VI in 2015. At the time of the writing of this chapter, its conference report is pending. Similarly, the final revision to the FDA’s revised BMV guidance, as well as an English version of a guidance from China’s CFDA, are also pending.

## 2.1 Industry Collaboration

For more than 25 years, the pharmaceutical industry’s bioanalytical community has communicated, collaborated, and worked with various health authorities to implement science-based regulations. In addition to the AAPS Crystal City meetings, others organizations (EBF, APA, CFABS) have held conferences, some with health authority representatives, and produced conference reports/white papers that

reflect consensus positions held by conference attendees. However, for most of that time, the content of these conferences tended to be region specific. That changed in 2010, when bioanalytical scientists from Canada, the EU, and USA joined together to form the Global Bioanalysis Consortium (GBC). Representing a number of regional organizations (Applied Pharmaceutical Analysis, AAPS, Canadian LC-MS/MS Group, and European Bioanalysis Forum), this group invited other regional organizations to join or to form groups and join. Thus, organizations in India (APA-India) and Brazil (AC-Bio) joined, and organizations formed and joined from China and Japan (China Bioanalysis Forum and Japan Bioanalysis Forum). As a worldwide organization consisting of representatives from various scientific associations, the mission of the GBC was “to harmonize and merge existing or emerging bioanalytical guidance to create one, unified consensus document that can be presented to the regulatory bodies/health authorities in various countries” [24]. This mission was fulfilled through the formation of global working groups charged with developing scientific best practices for bioanalytical chromatographic and immunoassay techniques. The fruits of their work resulted in a number of consensus white papers. These white papers are a valuable resource in understanding the science behind regulatory expectations [25].

## **2.2 Bioanalytical Method Validation and Sample Analysis in Support of Pharmacokinetic Studies**

As mentioned previously, early discussions on the content of BMVs for drug development emphasized methods used for the measurement of drugs and metabolites in biological fluids as important components of pivotal bioavailability and bioequivalence studies [8]. Over time, however, the conduct of BMV has become a standard expectation for a much broader range of drug development studies. Indeed, the 2012 EMA guidance on BMV, which is amongst the more comprehensive and contemporary guidance at the time of this printing, indicates it is applicable to “animal toxicokinetic studies and all phases of clinical trials” [18]. Hence, in practice companies often initiate the first bioanalytical method validation on a drug candidate in preparation for the first GLP toxicology study, which is typically well in advance of any regulatory submission or clinical trial. Subsequent to these first in human (FIH) enabling toxicology studies, BMV in human matrix is undertaken in preparation for the FIH study. Generally, a “full” BMV is performed on the parent drug and any major metabolites [26, 27] in each species and matrix used during the conduct of regulated (GLP or GCP/GCLP) studies. Supplemental or “partial” validations are conducted when changes are made to a previously validated method. Typical alterations accommodated through a partial validation include changes in the following: anticoagulant type, storage conditions, assay range, equipment, matrix (e.g., serum vs plasma), reagents (e.g., critical ligand binding assay reagents), laboratory conducting assay, population (e.g., healthy



volunteers versus patients), and concomitant medications. Tests conducted during partial validation focus on demonstrating the validity of aspects of the method likely to be impacted by the change in assay conditions. Hence, a partial validation can vary from as little as a single analytical run to almost a complete revalidation, depending on the nature of the change. Cross validation is a supplemental validation conducted to compare results when two distinct methods are used either within a study or across studies during the development of a compound. Cross validation should also be considered when sample analyses within a study are analyzed at more than one site/laboratory. The goal of a “cross” validation is to demonstrate intermethod or interlaboratory consistency and reliability.

The most comprehensive and widely referenced regulatory guidance on BMV are those from the EMA (effective in 2012, [18]), Japan’s MHLW (effective in 2013 and 2015 for LC-MS/MS [21] and LBA [22] methods, respectively), Brazil’s ANVISA (effective in 2012, [15]) and the U.S. FDA (effective 2001, [14]). As mentioned previously, the FDA is in the process of updating its 2001 Guidance to reflect many of the outcomes of the Crystal City meetings which have taken place since its issuance. Since there are subtle differences in validation requirements among guidances, scientists performing contemporary BMV for international submissions must consider all of them to develop an inclusive validation and analysis package. This being said, all guidance documents require demonstration of the following fundamental method attributes within certain criteria (unless otherwise justified in the method and validation documentation):

- Selectivity—the ability of the method to measure the analyte in the presence of other sample components.
- Accuracy—the closeness of test results from the method to the true (or nominal) value (concentration).
- Precision—the variability between individual test results determined in repeated measurements.
- Sensitivity or Lower Limit of Quantitation (LLOQ)—the lowest concentration of analyte which can be quantitated reliably.
- Calibration Curve—the relationship between analyte concentration and instrument response (also referred to as Standard Curve).
- Stability—the stability of the analyte in the matrices, containers and conditions used for sample collection, storage and analysis. Note: Health Canada has recently released specific requirements for the use of at least 3 replicate tubes exposed to each stability condition (each time point, condition, etc.) [28].
- Dilution Integrity (when applicable)—the ability to generate accurate and precise results when diluting a high concentration sample into the calibration range of the method.
- Carryover (when applicable)—when the potential for carryover exists, it should be investigated by measuring the analyte response in blank samples placed immediately following a high concentration sample.
- Matrix Effect—the alteration of analyte response due to the presence of other matrix components.

- **Reproducibility**—the precision of the method under the same operating conditions over a short period of time (several runs or days). This is typically determined using matrix spiked QCs. Also see “Incurred Sample Reproducibility” below.
- **Reference Standards**—analytical standards of known identity and purity should be used during method validation and sample analysis. A certificate of analysis or other documentation supporting the identity and purity of the standard should be available for each batch used.

For specifics on the acceptance criteria required for each of these validation elements, the reader is directed to the guidance documents themselves.

In addition to providing specifics on the conduct of method validation, the aforementioned BMV guidance also describe run acceptance criteria and quality expectations for the conduct of routine sample analysis. Key expectations are described for the following:

- **Run Acceptance**—Criteria on the use (e.g., number and concentration) and performance of blank samples, calibration curve standards and quality control samples included within the run.
- **Reanalysis of Study Samples**—Instances when it is appropriate, process and documentation needed for reanalysis of study samples. This type of reanalysis can be broken down into two categories: (1) Reanalysis for analytical reasons, where there is an assignable cause such as instrument failure, documented dilution error, poor chromatography, incongruence of replicate results in LBAs, failure of run acceptance criteria, concentration above the Upper Limit of Quantitation (ULOQ), etc.; (2) Reanalysis for pharmacokinetic (PK) reasons (incongruous result) such as having measureable drug in pre-dose, placebo, or control samples, or where a sample switch seems likely. Note, some guidances prohibit and most strongly discourage reanalysis for PK reasons during the conduct of BE studies; in any situation these reanalyses should be guided by a pertinent SOP that clearly defines circumstances in which sample reanalysis is permissible, the sample reanalysis procedure, and the manner in which a final reported result is determined from all replicate measurements that have been made. When reanalyzing samples, the laboratory is expected to provide a clear justification for why a reanalysis was performed, and the manner in which a final reportable result was generated [18].
- **Incurred Sample Reanalysis (ISR)**—ISR is broadly defined as the selection, analysis, and reporting of a subset of study samples; reanalyzed to establish the reproducibility of the method using incurred samples. ISR is generally performed once per species used in toxicokinetic assessments and as part of pivotal clinical studies. The ISR process is now viewed as a required and critical element of bioanalytical method assessment. The main purpose of ISR is to ensure reliability in reported sample concentrations. Because ISR investigation requires access to incurred study samples, the actual test occurs during the sample analysis phase and therefore reflects both the validity of the assay and its

execution. ISR may also provide information related to the stability of the analyte(s) under sample storage conditions for the time period between initial and ISR testing. Together with data generated by analysis of reference material spiked QC samples, ISR data demonstrates precision and accuracy characteristics for a given bioanalytical procedure. During ISR analysis, the original and repeat sample analysis results obtained by the same method but in different runs are compared. The requested number of samples to be tested in ISR analysis varies among regulatory guidelines.

ISR should be established at least once for each method in nonclinical species. Requirements for ISR investigations in human studies vary between regulatory guidelines but are generally expected for BE and all pivotal PK/PD studies, notably those whose results will be incorporated within the prescribing label.

Examples of clinical evaluations where ISR is recommended are:

- First-in-human studies
- First-in-patient studies
- Drug–drug interaction studies
- Studies in hepatic or renally impaired populations
- BE and biocomparability studies
- Pivotal Phase III studies
- First time use of a method in a new laboratory.

Results of an ISR evaluation may be reported as an addendum to the assay validation report or as part of the sample analysis report for a study. Investigations into possible causes of ISR failures should be carefully documented to allow for detailed study reconstruction.

Again the reader is referred to the individual guidance documents for specifics on each of the above topics related to routine sample analysis for pharmacokinetic assessments.

## **2.3 Validation and Application of Assays for Immunogenicity Assessments**

The majority of protein therapeutics are known for their potential to induce a drug-specific immune response that is commonly evaluated by the measurement of antidrug antibodies (ADA). Treatment-induced ADA response is expected to vary in immunoglobulin isotype (e.g., IgG, IgM, IgE), affinity and epitope specificity of drug binding and may include drug neutralizing antibodies (NAb). NAb can be defined as an ADA that is capable of completely or partially blocking drug interaction with its target. Induction of drug-specific ADA often results in various degrees of impact on drug pharmacokinetics (PK) including shorter drug half-life due to faster clearance of large drug–ADA immune complexes. A direct impact of drug-specific ADA on the performance of a PK assay is also possible as the ADAs

may interfere by blocking the drug analyte binding with assay reagents. For example, ADA with neutralizing specificity may block drug-target binding, therefore impacting PK assay performance if target or a molecule with similar specificity of binding is used as one of the assay reagents. A careful investigation may be required in order to determine the true nature of ADA impact on the observed PK profile. Detection and evaluation of ADA development, particularly in clinical studies, is a critical element of biotherapeutic development. Initial groundwork toward the harmonization of ADA-detecting assays was made by industry groups [29] with the focus on developing recommendations for the design of ADA-detecting assays. Similarly, industry groups pioneered steps and methodologies required for the development of assays set up to detect the presence of ADAs with drug neutralizing activity (NAb) [30]. Particular focus was directed toward developing risk-based strategies for ADA evaluation and characterization [31]. Later, industry positions on ADA and NAb assay development were presented [32]. The EMA position was finalized and incorporated in the EMA Guideline on Immunogenicity Assessment [33], followed by a draft FDA Guidance for the Industry [34]. At the time this chapter was written, the FDA guideline remains a draft document. The above two regulatory guidance focus on the approaches and various critical aspects of importance during ADA and NAb detecting method development and validation. In addition, FDA has issued a regulatory guideline designed to provide recommendations for immunogenicity risk factor identification and mitigation [31]. This 2014 FDA guideline provides a critical connection between product immunogenicity risk assessment and design of appropriate immunogenicity testing strategies. Most recently, the EMA has issued a draft update of its guideline [35].

Typically, a tiered approach to immunogenicity evaluation is applied and includes an initial screening test followed by an ADA specificity confirmatory test. In addition, tests may be conducted to determine final reportable ADA titer value, characterize ADA isotype, and to determine whether the ADA has a drug neutralizing specificity (NAb). Drug-specific immune response is expected to be polyclonal, i.e., to contain a mixture of various isotype immunoglobulins with different epitope specificity and binding affinity to the drug. The initial ADA assay is expected to detect ADAs with all possible specificities, affinities, and isotypes. Therefore, it is not possible to generate a true reference material that would be able to reflect performance of the variety of analytes expected to be found in the sample. Positive Control (PC) samples are routinely used to monitor ADA assay performance, but do not allow for a quantitative reporting of ADA assay results. Consequently, ADA assays most typically report semiquantitative results, (e.g., titer values) [36]. The same can be said about neutralizing antibody (NAb) assays. While ADA assessments usually employ ligand binding assays (LBA), NAb assays can be cell or noncell based, depending on the drug's mode of action (MOA). NAb assays are most applicable during clinical investigations and are rarely conducted in support of nonclinical studies, unless a translation between nonclinical and clinical safety signals can be drawn.

Details regarding ADA assay development and validation can be found in several regulatory guidelines and industry white papers [29, 34–36]. In the initial screening test, samples are identified as putatively positive versus negative based on comparison with the screening assay cut point value. The cut point value is developed during ADA assay validation by statistically analyzing the distribution of signals generated from the analysis of individual samples obtained from drug naïve subjects representative of the study population. Typically, the 95th percentile of the distribution is compared to the performance of the assay negative control to determine an assay-specific cut point correction factor. The particular distribution cutoff is chosen to ensure that all putative positive samples are detected by establishing a 5% false positive rate in the initial screening assay investigation. Putative ADA positive samples are then confirmed for the specificity of binding to the drug by, for example, analysis of the unlabeled drug's ability to inhibit signal in the ADA assay. Here again, ADA—drug specificity of binding is confirmed through comparison to a predefined, assay-specific confirmatory cut point. As for the screening assay, the confirmatory assay cut point is defined through statistical analysis of signal inhibition values generated by drug naïve, study population specific samples. A sample is defined as ADA positive and drug-specific if the corresponding screening signal is equal to or greater than the screening assay cut point value and the percentage inhibition value generated in the confirmatory assay is equal to or greater than the confirmatory assay cut point value.

During sample testing, positive and negative control samples are routinely applied to determine the appropriateness of assay performance based on predetermined acceptance criteria. Assay Negative Control (NC) typically consists of a pooled drug naïve, study-relevant, species matrix sample. Assay PC is typically a matrix sample spiked with either a monoclonal or polyclonal, drug-specific, non-human origin antibody raised by hyperimmunizing various laboratory species. Several of the assay characteristics are determined based on the PC material performance, including assay sensitivity and drug tolerance. The regulatory expectation is that a clinical ADA assay should be able to detect 250 ng/mL of PC material (500 ng/mL for nonclinical assays). Assay drug tolerance is a parameter that reflects the ability of unlabeled drug to block ADA binding to the assay reagents (e.g., biotin-labeled drug) and, therefore, inhibit ADA assay signal. Residual drug found in the sample can subsequently be expected to reduce ADA assay sensitivity. The ADA assay ability to tolerate a certain amount of drug in the sample can be assessed based on the performance of PC material, although this does not directly reflect sample ADA performance. Numerous variations of ADA protocols intended to improve assay tolerance have been reported [37, 38].

Other parameters evaluated during ADA assay development and validation include:

- Identification of appropriate assay reagents, selection of the assay PC
- Identification of appropriate NC for the assay
- Selection of assay analytical platform and format

- Establishment of assay conditions, including assay buffer, reagent conjugation conditions, minimum required dilution (MRD), assay incubation times
- Identification of confirmatory assay conditions including drug concentration spiked
- Determination of both Screening and Confirmatory cut point values during ADA assay validation
- Determination of assay precision, robustness and sensitivity using assay PC material performance
- Establishment of acceptance criteria based on PC and NC material performance.

Study incurred samples are commonly tested at multiple dilutions to produce a final reportable titer value. Titer value can be determined as the maximum sample dilution that produces signal at or above the assay cut point value. Similar approaches to report NAb assay data are also applied.

## 2.4 Chromatographic Versus Ligand-Binding Assays

Compared to LC-MS/MS assays, typical LBA methods do not include a significant sample pretreatment step. Neat matrix samples are usually diluted in order to fall into the assay range of quantitation and if a higher than the minimum-required dilution (MRD) is required, assay diluent buffer containing related matrix is used. Matrix components can play a significant role in the final assay performance based on their ability to interact with the analyte, various assay reagents and components (e.g., plates or beads). The concentration and binding properties of these non-specific and generally low-affinity interferences frequently vary from patient to patient and are highly dependent on the nature of assay matrix, disease, and sample collection strategy. The aspects of selectivity and specificity are, therefore, critical to LBA methods. Matrix component interference can be particularly noticeable at the lower and upper limits of the detection curve. As a result, it is critical to understand assay performance at the LLOQ/ULOQ levels [18].

Biotherapeutics may be applied at high doses while LBA methods typically have a narrow dynamic range of quantitation. Therefore, samples can be diluted 100,000 fold and higher. A careful evaluation of the dilutional linearity of LBA assays is critical to ensure accurate reporting of sample concentrations. Dilutional linearity is expected to be performed during assay validation and should demonstrate an assay's ability to detect accurately the analyte concentrations similar to expected  $C_{max}$  levels. This test is particularly important as LBAs commonly produce non-linear sigmoidal response curves which are analyzed using four- and five-parameter logistics or similar fitting algorithms.

A parallelism analysis, in principle, is a test that is similar to dilutional linearity but is designed to address questions related to possible analyte modifications while in circulation in vivo, including partial degradation or other forms of covalent or non-covalent modifications.

Relatively high well-to-well variability observed for older generation, plate-based LBA protocols resulted in the requirement to conduct duplicate measurements per sample. Similarly, acceptable ranges for assay precision (20%) and accuracy (25% for LLOQ/20% for other QCs) have also been extended compared to a typical LC-MS/MS method. A requirement for duplicate analysis has been recently challenged and may evolve with the advent of newer and more precise LBA platforms. The nonlinear nature of a typical LBA concentration–response curve often calls for inclusion of standards, known as anchor points, which extend outside of the quantifiable range. Inclusion of anchor points may improve mathematical fitting of the calibration curve and, therefore, improve QC and standard performance. At least eight calibrators with at least a 75% passing rate are expected to be included in the assay range of quantitation in order to support robust fitting of the data.

## 2.5 Biomarkers

Biomarker measurements are typically performed to reflect a drug's pharmacodynamic (PD) effects. While the requirements for PK and ADA analysis are well described in the existing regulatory guidance, regulatory expectations for biomarker analysis are much less mature. In fact, the FDA's 2013 draft guidance is the only BMV guidance that mentions biomarkers. As previously discussed, even the most recent Crystal City VI meeting offered only a preliminary and high-level framework for biomarker validation requirements. This cautious pursuit of biomarker bioanalysis regulations by both regulatory and industry scientists is clear recognition of the unique challenges biomarker analysis presents, and indicates a need for the science and practice related to biomarkers to mature before being codified within regulations. Among the unique aspects of biomarkers are the endogenous nature of the analyte and the difficulty in producing a reference standard that is identical to the endogenous compound (e.g., proteins). Other factors are the complex matrix in which many biomarkers reside (e.g., membranes, cells, tissues) and the rapid introduction of new and innovative approaches for measuring biomarkers. These challenges make it difficult to settle on a stable set of quality standards that can be broadly applied.

## 2.6 Other Laboratory Tests

The above regulations apply to the work typically conducted in bioanalytical laboratories as part of PK, immunogenicity, and PD assessments during drug development. It is important to recognize that there are a large number of laboratory tests performed during drug development and after drug approval that fall outside the bioanalytical realm. These tests may be conducted for a variety of reasons that

include, but are not limited to: patient diagnosis, drug safety testing, clinical pathology, or even therapeutic drug monitoring. These tests are beyond the scope of traditional bioanalysis and hence this book, and fall under their own set of regulatory requirements. A few of these from the USA are described briefly below to add context for the reader. It should be noted that similar practices and rules exist in other regions.

- Clinical Laboratory Improvement Amendments (CLIA): a quality/compliance program directed by the Division of Laboratory Service within the Centers for Medicare and Medicaid Service in the United States. The CLIA program regulates laboratories performing tests on patient samples to ensure they produce accurate and reliable test results. It is noteworthy that CLIA does not address the validity of an assay, that is, its ability to measure or predict the presence or absence of a clinical condition, which falls under FDA jurisdiction. CLIA laboratories must establish and maintain certain performance characteristics on their assays and must pass state administered inspections on a biennial basis [39].
- Laboratory Developed Test (LDT): A LDT is a type of in vitro diagnostic device that is designed, manufactured, and used within a single laboratory. These tests typically identify, detect, or measure the presence or absence of a clinical condition or predisposition in a patient. In 2014, the FDA released its first draft framework for the oversight of LDTs, citing the risk that flawed LDTs may pose to patients [40]. The FDA also cited a gap in current regulations with respect to their oversight of LDT validity (ensuring they are safe and effective). In the draft framework, FDA describes a three-tier, risk-based system that it will apply to regulate LDTs beginning six months after the final guidance is issued.
- Premarket Approval (PMA) and 501(k): Many commercial devices used to perform clinical tests on patient samples require regulatory approval prior to marketing and use. The type of application [PMA or 501(k)] depends on the associated risk to the patient and whether a test already exists for the endpoint. FDA review of these devices (tests) is focused on ensuring they are safe and effective for their intended use [41].

## 2.7 Quality Management in the Regulated Bioanalytical Laboratory

In addition to the specific requirements outlined in various BMV guidelines, laboratories conducting regulated bioanalysis are also expected to establish a robust quality management system (QMS). For laboratories conducting regulated, non-clinical bioanalysis, the fundamental expectations of a QMS system are described within the FDA and OECD GLPs [3, 4]. Similar expectations exist for laboratories conducting clinical bioanalysis, and a contemporary guidance on GCLPs is



available in the previously mentioned EMA reflection paper on the topic [19]. The GLPs and GCLPs (collectively known as “GxP”) provide a framework for the facilities, systems, procedures and documentation that should be present to ensure the reliability, quality, and integrity of the work performed and associated results. Key components of a contemporary QMS supporting GxP bioanalysis include, but are not limited to:

- **Organization, Personnel, and Facilities**—Management of establishments conducting GxP work should ensure staff roles and responsibilities are established and documented prior to conducting regulated work. Staff engaged in regulated study support should have the appropriate education, training, and experience to conduct the assigned work and this information should be documented and updated regularly. Test facilities should be of suitable size and construction to meet the requirements of the study. This includes an adequate degree of separation between activities to prevent adverse effects on the study (e.g., contamination within or between studies). Facilities should also have an appropriate controlled access space for storing study specimens and archiving data and reports in an environment that prevents deterioration.
- **Standard Operating Procedures (SOP)**—Testing facilities should have written SOPs that are approved by management and ensure the quality and integrity of the data generated in the course of the study. Deviations from SOPs related to the study should be acknowledged by the study director (nonclinical) or sponsor representative (clinical). A controlled and documented SOP revision process is required. SOPs related to bioanalysis may cover material and reagent preparation and handling, equipment operations and maintenance, receipt and storage of study samples, protocols relevant to assay validation, sample testing, records keeping, training and QC processes. Method-specific documentation sometimes is also issued as a method-specific SOP. Laboratory staff are expected to maintain role-appropriate levels of documented training as defined in relevant SOPs.
- **Quality Assurance Program**—Test facilities should have a Quality Assurance (QA) program to assure the studies performed are in compliance with GxPs. The QA program should be carried out by individuals designated by management and not involved in the conduct of the study. The QA unit should inspect each GxP study at intervals adequate to assure the integrity of the study and should maintain proper documentation of each inspection. Different levels of QA review can be conducted, including facilities, systems, equipment, personnel training, data recording, and reporting audits. Inspections should also determine that protocols and SOPs have been made available to study personnel and are being followed. Any issues found during the course of inspection that are likely to affect study integrity should be brought to the attention of the study director (nonclinical), sponsor representative (clinical), and laboratory management in a timely manner.

- **Equipment, Materials and Reagents**—Equipment, including validated computerized systems used in the generation, storage, and retrieval of data, and for environmental control of factors relevant to the study, should be suitably located, of appropriate design and capacity, and properly maintained. Chemicals, reagents, and solutions should be labeled to indicate identity, concentration, storage requirements, and expiration date. Information on the source, preparation date, and stability should be available.
- **Sample labeling, storage and chain of custody**—Samples should be labeled in a manner to allow unequivocal identification. A mechanism to ensure that samples were maintained under appropriate storage conditions prior to analysis (shipping, storage, etc.) should be implemented and maintained. This will typically include a tracking system that provides chain of custody from receipt through destruction.
- **Patient Safety, Informed Consent, and Study Blind**—In the case of clinical bioanalysis, the safety of the trial patients or subjects take precedence over all other aspects of the study. Therefore, lines of communication must be present between the laboratory, sponsor (or their representative), and clinical investigators to ensure that any issues that impact patient/subject safety are communicated rapidly. There should also be a mechanism to ensure that the laboratory is informed of what actions to take if informed consent is withdrawn by a patient or subject. Many clinical trials are blinded in order to ensure an unbiased approach in interpretation of trial data, including information on treatment outcome and adverse effects. For blinded clinical trials, laboratories should exercise due diligence to ensure they do not inadvertently compromise the study blind while communicating with investigators. In cases where bioanalytical data could unblind the trial, particular care must be taken to ensure data is only communicated with an established point of contact. A double-blinding approach is most typical during conduct of a drug development trial [18]. In a double-blind trial design, the subjects, as well as the investigator and staff involved in administering the treatment, are not aware of the received treatment. Bioanalytical scientists may be unblinded prior to sample analysis or after review of sample concentration data, but procedures must ensure the unblinding is restricted to appropriate personnel and does not impact the trial design. A set of SOPs is expected to prevent inadvertent and inappropriate distribution of treatment information, particularly during reporting of the data, to the staff that is expected to remain blinded for the duration of the study

## 2.8 Documentation

Thorough documentation of bioanalytical activities is a central and common expectation for all regulated work. All major regulatory guidelines are generally in high agreement regarding assay documentation expectations [14, 18]. The level of

detail included in the documentation should allow for reconstruction of the study as it was conducted. Reports summarizing results of activities conducted during assay validation and sample testing are expected at the conclusion of each study.

While validation activities are not formally required to be conducted under GxPs, in practice most laboratories conduct validations using the same practices and procedures applied during regulated sample analysis (in some cases excluding the QA audit activities). The main goal of documenting assay validation data is to retain and record the performance characteristics of a successfully validated method in order to demonstrate its suitability and reliability for the intended application. During the course of bioanalytical study support, assays may require additional validation or revalidation. For example, a change in the assay reagent lot, assay matrix, or disease indication may require additional tests to ensure that the same or modified assay continues to meet predefined requirements. These and similar experiments should be carefully documented in an updated version of the assay procedure and validation report. When reporting validation data, all run information should be disclosed, including data from failed runs.

Bioanalytical data generated during the application of an assay for study sample analysis is commonly documented either in an independent bioanalytical report or a combined bioanalytical/PK report. Assay investigations conducted during study support are expected to accompany the study report and include information on issue resolution and any required assay modifications.

Typically, the following information is expected to be included in the assay documentation package maintained at the lab:

- Source (or raw) data.
- Summary of assay performance.
- Assay procedure including information on assay components and required steps.
- Assay validation report(s).
- Study sample analysis report(s).
- Failure investigations, deviations, assay changes and modifications including all supporting information.
- Assay relevant correspondence records.
- References or copies of relevant SOPs.
- Records identifying appropriate qualification, training and experience of assay personnel.
- Instrumentation maintenance and calibration records.

Documentation can be stored in either paper or electronic format, and is expected to record events in a contemporaneous manner. Although printing electronic records and storage of the paper version with deletion of the electronic record was once acceptable, most agencies currently oppose this practice. Appropriate laboratory procedures and policies should be in place explaining documentation generation and storage, including conversion of paper documents into an electronic format. The latter needs to include a thorough QC process to ensure electronic copy completeness and equivalence to the original paper copy. Any clarifying modifications to the

documentation added later should not obstruct previously created information and should be accompanied by the reason for the change. If stored in electronic format, relevant regulatory guidance is applicable, including existence of an audit trail.

Details related to the information to be documented as part of the assay package are thoroughly described in regulatory guidelines (FDA, EMA and MHLW) and GBC Team A8 report [42].

## 2.9 Conclusions

As technologies advance alongside new and evolving drug molecules and delivery systems (e.g., sRNA, adnectins, nanobodies, millamolecules, viral vector delivery systems), the nature of a bioanalytical assay will also continue to change. As observed by regulators over the past 25 years, these types of advances drive re-evaluation and updating of the performance characteristics expected of the assays during validation and sample analysis. In this way, regulators are able to ensure the quality of the data generated, and consequently the safety and efficacy assessments of new therapies.

In the realm of LC-MS/MS, small molecule bioanalysis has been advanced with UHPLC, low flow systems, newer more sensitive instruments, high resolution accurate mass instruments, and full scan systems of improved sensitivity. UHPLC has increased the throughput of assays without the loss of chromatographic resolution. Micro- and nanoflow systems are increasing sensitivity. The increases in sensitivity introduced by triple stage quadrupole mass spectrometers have kept pace with the development of more potent molecules, and thus continue to provide valid pharmacokinetic assessments. High resolution instruments with improved specificity have ensured that fewer interferences plague bioanalytical scientists and also enable the bioanalytical support of microdosing studies that use smaller numbers of patients. The use of full scan instruments and targeted selected reaction monitoring is enabling early identification and quantitation of metabolites with the practices for validation and sample analysis being worked out based on current practices for triple stage quadrupole mass spectrometers. Combined, these advances provide a larger variety of tools for bioanalytical scientists and enable more reliable and sensitive assays.

The diversity of therapeutic modalities, particularly in the field of biotherapeutics, continues to evolve. Alternative scaffold antibody and fusion proteins, bispecific antibodies, antibody–drug conjugates, nucleotide and viral vector-based biologics are now a part of the clinical development space. Such second generation biotherapeutics present additional bioanalytical challenges including increased requirements for assay sensitivity, specificity, the ability to handle smaller sample volumes, larger dynamic range of the assay, and measure specific analyte type (e.g., free versus total monoclonal antibody moiety). A new generation of LBA methodologies has advanced to address some or all of these requirements. Many of the new LBA analytical technology platforms have moved away from a plate-based

approach and may allow testing more than a typical 20 to 30 samples per analytical run. This raises a question of whether a typical LBA assay run should contain a single set of standards and QCs or should incorporate additional QCs throughout the run set. Currently, it is generally agreed that an analytical run can be defined as a set of standards with interspersed QCs and that a number of QCs greater or equal to five percent of the total number of unknown samples should be present in a run to appropriately control assay quality [43]. Questions related to the detailed positioning of QCs within the run, and size of a run batch are still left open. Currently, in a majority of LBA methods, samples are tested in duplicate; while improved precision for some LBA-based analytical platforms may allow for a single replicate analysis.

Affinity capture (AC) LC-MS/MS technology is quickly being adopted for analysis of biotherapeutics. A good example of an AC-LC-MS/MS application is the analysis of various drug species present following the administration of an antibody–drug conjugate (ADC). AC-LC-MS/MS methods are a hybrid between LBA and LC-MS/MS platforms as they include an initial affinity capture step (e.g., by using specific antidrug antibody) followed by a digestion and peptide analysis on an LC-MS/MS platform. The LC-MS/MS step adds improved specificity of detection while the AC step provides an opportunity to improve assay selectivity and sensitivity. Importantly, the existence of an AC step brings challenges similar to those observed for a typical LBA method, including potential matrix component interference, impact of sample pretreatment (e.g., dilution) on the analyte type (e.g., free/total), and nonlinearity of response. Hybrid assays may, therefore, have inherently higher variability versus other types of LC-MS/MS methods. The overall experience for both regulatory agencies and the industry with the AC-LC-MS/MS platform is still developing. Currently, there is no guideline that clarifies regulatory expectations for AC-LC-MS/MS assays.

Because of the long duration of a typical drug development cycle, bioanalytical methods and analytical platforms applied early on may be reassessed and changed later in development. An improved understanding of assay sensitivity or the type of analyte requirements may drive a platform change, for example requiring a switch between LBA platforms or a change from LBA to an AC-LC-MS/MS platform. Regulatory agencies generally expect presentation of data that support and justify the implemented analytical platform change. Some of the technologies are sufficiently different to prevent a direct comparison of absolute analyte concentrations. Currently, consensus exists that a set of bridging data should be presented to directly compare different analytical methods.

Details on current scientific practice will be discussed within other chapters, with this chapter having established the historic precedents and progress leading to today's extensive and sometimes conflicting set of regulations. As this chapter goes to press, the International Council on Harmonization announced acceptance of a proposal to generate M10—a harmonized approach to bioanalytical method validation and sample analysis. While this effort may take some time to come to fruition, it is a positive step forward for regulators and the scientific community.

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