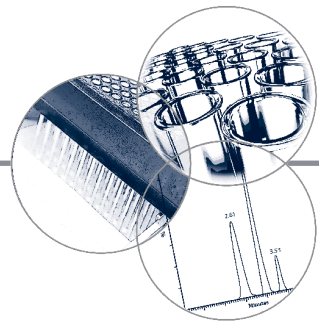

The European Bioanalysis Forum community's evaluation, interpretation and implementation of the European Medicines Agency guideline on Bioanalytical Method Validation

The European Medicines Agency's (EMA) 2011 guideline on bioanalytical method validation (BMV) was evaluated and subsequently intensely discussed by the European Bioanalysis Forum (EBF) during a 2-day workshop (EBF Workshop on the implementation of the EMA guideline on BMV, Château de Limelette, Limelette, Belgium, 15–16 March 2012). The goal of the evaluation and discussions was to come to a uniform interpretation of the guideline and thus to help facilitate a smooth implementation at our laboratories. Up front preparations for the workshop by dedicated teams concentrated on challenges on implementation: ambiguities, technical or operational challenges and issues in general. In addition, common understandings were identified as well as main differences to the 2011 US FDA guideline. The guideline was perceived as being well written with a clear structure, separating method validation from sample analysis and treating all relevant aspects one-by-one in a logical order. It is the first BMV guideline clearly addressing the specifics for ligand binding assays and it shows a good match with current scientific thinking. The EBF community considers the EMA BMV guideline an excellent basis for countries that are in the process of developing or updating their own BMV guideline.

The development of a guideline on bioanalytical method validation (BMV) by the European Medicines Agency (EMA) [101] has repeatedly been a point of attention with the European Bioanalysis Forum (EBF) [102] and was on the agenda of many of its meetings. Within the EBF formal discussions started in January 2009, shortly after the publication of the EMA concept paper on the need for a guideline on the validation of bioanalytical methods and application thereof in (pre) clinical studies [1], the group provided consolidated comments from the bioanalytical scientists of 24 pharmaceutical companies. Recognizing the challenges a second major BMV guideline could impose on industry, the EBF steering committee voiced a wish for harmonization in an editorial in *Bioanalysis* in the same year [2] and organized a session to have further discussions on the EMA concept paper with the regulators and broader bioanalytical community during the 2nd Open Symposium in December 2009 [3]. Shortly after that meeting, the draft guideline was officially published for comments [4].

As with the concept paper, EBF again collected comments among its member companies and prepared for consolidated feedback to the EMA in May whilst organizing a 2-day workshop together with the European Federation of Pharmaceutical Scientists [5,103,104]. As the EMA draft guideline was the first BMV guidance document specifically addressing ligand-binding assays (LBAs) to determine macromolecule levels, EBF also hosted a session on the EMA draft guideline during the 2010 National Biotech Conference in San Francisco [105]. In parallel, Graeme Smith published a comparison between the EMA 2009 draft BMV guideline and the US FDA 2001 Guidance, the latter being the only standard since it was released [6]. One main concern among the bioanalytical community was that guidelines in different regions proposed divergent or even conflicting procedures to be followed. This concern fuelled the industry desire to also concentrate on and work towards global harmonization [7–11,106].

The EMA released the final version of its BMV guideline [12] and an overview of the comments received [13] on 21 July 2011 with a date of implementation of 1st February 2012. The next challenge for the bioanalytical laboratories was how to implement this guideline. Our first step on addressing this essential aspect was to collect input from our member companies on this question. The outcome was presented and discussed

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during the 4th Open Symposium in November 2011 in Barcelona [14,107].

In contrast with the implementation of the FDA Guidance, which was the first of its kind and gradually adopted in the years after its release, the EBF decided to support the harmonized implementation of the EMA guidance in the individual laboratories of its members. Following up on this decision and on the discussions held in Barcelona, an internal workshop was organized where EBF members from 40 member companies extensively discussed the interpretation of the guideline aimed towards its implementation. Prior to the workshop, ten small teams, of on average six people, discussed a preselected number of sections of the guideline. The discussions focused on the following points:

- Challenges on implementation: common understandings; ambiguities; technical or operational challenges; issues;
- Differences to FDA 2001 Guidance and subsequent Crystal City White Papers [15–17].

In order to reach a concise set of major conclusions and recommendations, the different chapters were discussed within the EBF community during the workshop that took place on 15–16 March 2012 in Limelette, Belgium. The current paper outlines the outcome of these discussions.

In parallel to the activities by the EBF, the Global CRO Council held two meetings in 2011 discussing the guideline. The outcome of those discussions was published in early 2012 for *Bioanalysis* [18].

General observations

The EMA 2011 Guideline on the validation of bioanalytical methods was perceived as being well written and having a clear structure [12]. Method validation and sample analysis are clearly separated and all aspects considered relevant for either method validation or sample analysis are dealt with one-by-one in a logical order. It is the first BMV guideline clearly addressing the specifics for LBAs for the assessment of macromolecules and, in general, it shows a good match with current scientific thinking in the bioanalytical community. It also defines the implementation of quality systems such as GLP for preclinical and GCP for clinical studies. Furthermore, it has a good fit with the bioanalytical section of the EMA guideline on bioequivalence (BE) studies [19] and took into account the developing concepts within EMA on GCP for bioanalytical laboratories [20].

The clear and logical structure can be depicted by a side-by-side comparison between 'small molecules/chromatographic assays' and 'large molecules/LBAs' table of contents as presented in Table 1. On some aspects in the guideline, EMA provides clear expectations regarding actions and/or how to evaluate the data, for other aspects it is more open. And although some may welcome a more descriptive and directive guideline, the general feeling and appreciation was that it is good that there is room for interpretation and, thus, allows the bioanalytical scientists to fine-tune validations to fit within local and corporate constrains, molecule specifics and project needs.

The EBF wants to highlight that the EMA guideline is actually focusing on bioavailability and BE studies and, unfortunately, makes no reference to the principles offered by the tiered approach, which we feel are an important alternative for documenting scientific quality for compounds for which full validation is either not needed or not possible. For example, in an earlier stage of drug development or for metabolites those are not yet fully characterized [16,21].

Differences to the FDA guidance & EMA

Observed differences between the EMA guideline and the FDA 2001 Guidance are depicted in Table 2 . The contrasts are small and are generally more the result of 10-years progress in science and a better understanding of the specific needs and challenges for bioanalysis, than illustrative of a divergent regulatory thinking in the USA compared with the EU.

Executive Summary & sections 1, 2 & 3

Common understandings

Focus of the guideline is on validation of bioanalytical methods and criteria on the application of these validated methods in the routine analysis of study samples from predominantly PK and TK studies. The guideline specifically focuses on chromatographic and ligand-binding analytical methods for the assessment of small and large molecules, respectively.

Ambiguities

The guideline does not clearly indicate for which other bioanalytical methods it is applicable, for example, radio-labeled or microdose studies. For validations in support of preclinical studies, it is clearly implied that GLP should be used as the quality standard. It is, however, unclear if compliance to GCP should also be indicated

Table 1. Section-by-section outline of the European Medicines Agency guideline on bioanalytical method validation.

General sections pertaining both small- and large-molecule analysis	
1 Introduction	
2 Scope	
3 Legal basis	
8 Report 8.1 Validation report 8.2 Analytical report	
Comparable sections pertaining either small- or large-molecule analysis	
Small molecules (chromatography)	***Large molecules (ligand-binding assays)***
4.1 Full validation of an analytical method	7.1.1 Full validation
Reference standards	7.1.1.1 Reference standards
4.1.1 Selectivity	7.1.1.3 Selectivity 7.1.1.2 Specificity
4.1.2 Carry-over	7.1.1.4 Carry-over effect
4.1.3 LLOQ	
4.1.4 Calibration curve	7.1.1.7 Calibration curve
4.1.5 Accuracy 4.1.6 Precision	7.1.1.8 Precision and accuracy
4.1.7 Dilution integrity	7.1.1.9 Dilutional linearity
4.1.8 Matrix effect	7.1.1.5 Matrix selection 7.1.1.6 Minimum required dilution
4.1.9 Stability	7.1.1.11 Stability of the samples
4.2 Partial validation 4.3 Cross validation	7.2 Partial validation and cross validation
5 Analysis of study samples	7.3 Analysis of study samples
5.1. Analytical run	7.3.1 Analytical run
5.2. Acceptance criteria of an analytical run	7.3.2 Acceptance criteria
5.3. Calibration range 5.4. Reanalysis of study samples 5.5. Integration	
6 Incurred samples reanalysis	7.3.3 Incurred samples reanalysis
	7.1.1.10 Parallelism 7.1.1.12 Reagents 7.1.1.13 Commercial kits

for validations in support of clinical studies. Following up, EBF consulted together with the European Quality Assurance Confederation and ended up with the following interpretation of the GLP requirements: *"The wording of this section has raised questions within industry. Industry believes the intent of the section is as detailed below. Bioanalytical methods used in nonclinical safety studies (GLP studies) should be validated to ensure they are fit-for-purpose. The BMV work should be conducted to a high standard commensurate with the principles of GLP. As the method validation work is not classified as a nonclinical safety study, there is no requirement to claim compliance with the Principles of GLP. This is aligned with the position of national GLP Monitoring Authorities. If it is a test facility's policy to claim GLP compliance for bioanalytical method validation, this is acceptable. This comment is not necessarily a request to amend the Guideline but a reassurance to industry that their interpretation is correct."*

Conclusions & recommendations

For method validation for pre-clinical and clinical as well as for clinical sample analysis: use the GLP system as the standard quality system for the laboratory. For sample analysis from GLP studies (pre-clinical): use GLP as the quality standard and claim compliance to GLP. Still, little clarity remains with respect to the claim of compliance to GCP for clinical studies, but referring adherence to the EMA 'GCLP' reflection paper may be good advice for these types of situations [20].

Table 2. High-level comparison of the European Medicines Agency 2011 bioanalytical method validation guideline to the US FDA 2001 Guidance and subsequent Crystal City White Papers.

Section	European Medicines Agency 2011	US FDA 2001 and subsequent Crystal City meetings reports
Executive summary, 1, 2, 3 and definitions	Covers validation and sample analysis	Additionally covers method development
	Validations and sample analysis conducted 'following the principles of' or 'in conformity' with GLP/GCP	Analytical laboratories conducting preclinical studies for regulatory submissions should adhere to GLP
	Includes calculations to be used for accuracy and precision	Gives description only
4.1–4.1.3	Requires full validation for each species and matrix (no partial validation)	Supports the partial validation concept
	Recovery not needed for European Medicines Agency	Recovery is a validation aspect
	Does not require CoA for IS	Does not strictly require CoA for reference standards
	Strongly recommend isotope-labeled IS for MS detection	NA
	Carry-over should be <20% for analyte and <5% for IS	No target in guidance, but some inspectors see 20/5% as acceptance criterion
	Added an acceptance criterion for LLOQ in BE studies (5% of C_{max})	NA
4.1.4 Calibration curve	A relationship that can simply and adequately describe the response of the instrument with regard to the concentration of analyte should be applied.	More descriptive/directive: 'simplest model'
4.1.5 Accuracy	Within-run accuracy should be determined by analyzing in a single run a minimum of five samples per level at a minimum of four concentration levels, which are covering the calibration curve range.	Minimum of three concentrations
	Reported method validation data and the determination of accuracy and precision should include all results obtained except those cases where errors are obvious and documented.	Calculations of accuracy and precision excluding values that are statistically determined as outliers can also be reported
6 and 7.3.3 ISR	ISR explicitly described	ISR not a topic in the guideline, but main topic in Crystal City IV White Paper[†]
7. Ligand binding assays	Section specifically on LBA included	Guideline written for LC–MS based assays
7.1.1.1. Reference standards	Reference and dosing batch should be of the same origin	NA
7.1.1.2. Specificity	Details how to test drug related molecules or concomitantly administered drugs by spiking QC samples	Not specified
7.1.1.3. Selectivity	Test at least 10 different matrix sources including lipemic and hemolyzed samples. Include sources of relevant disease populations	NA in guideline. However, it is discussed in detail in Smolec *et al.*[‡]
7.1.1.5. Matrix selection	Surrogate matrices may be used if justified.	NA
7.1.1.6. Minimum required dilution	MRD should be assessed	NA
8.1 and 8.2 Reports	Table of calibrator results (validation) of accepted runs	Table of calibrator results (validation) of all runs
	Data on selectivity, LLOQ, carry-over and dilution integrity (validation)	NA
	Unexpected results with full justification and action taken (validation)	NA
	Sample reassay info (analysis) 'exclude re-assay due to analytical reasons such as run failure'	Requires a reassay SOP as appendix
	Chromatograms (validation): not specified	Chromatograms (validation): representative
	BE studies: all chromatograms from runs, which include 20% of subjects (including standards and QCs) other studies – representative chromatograms only	Chromatograms (analysis) to be submitted: 5–20% for an ANDA and representative for a NDA

[†]*Data from* [17].
[‡]*Data from* [28].
ANDA: Abbreviated New Drug Application; BE: Bioequivalence; CoA: Certificates of Analysis; IS: Internal standard; ISR: Incurred sample reanalysis; LBA: Ligand binding assays; MRD: Minimum required dilution; NA: Not addressed; NDA: New Drug Application.

4.1 Full validation of an analytical method

Common understandings

A full validation is needed for any new method validated before the first-time use in a study aimed for a regulatory submission. Furthermore, a full validation is needed for each additional matrix, species or analyte, with the exception that some stability work may not need to be repeated. Certificates of Analysis (CoA) are required for all reference standards including metabolites, but not for the internal standard (IS).

Ambiguities

The guideline text is contradictory on the application of partial validations. In 4.1 the following is stated: *"Generally a full validation should be performed for each species and matrix concerned."* While in 4.2 it reads: *"Changes for which a partial validation may be needed include … another matrix or species…"*

Technical or operational challenges

The need to have and supply a CoA is well understood, but may be quite difficult to achieve for certain metabolites (e.g., glucuronides), especially in the earlier stages of drug development.

Issues

It is unclear whether or not partial validation is still possible and acceptable, for example, in the case of another species. The safe approach, and potentially the most cost effective in the long run, is to perform a full validation when there is a species change.

Conclusions & recommendations

Always run a full validation for each species, matrix and when you add an analyte. This may not strictly be needed following the partial validation principles, but EBF experience is that it is more efficient to do full validations in the long run.

Obtain a CoA for all analytes and use a stable isotope labeled IS whenever possible.

■ 4.1.1 Selectivity

Common understandings

Selectivity of the method towards endogenous substances needs to be demonstrated in blank matrix of six different individuals and (potential) degradation of labile compounds should be addressed when sample collection/sample preparation is defined.

Ambiguities

Challenges in this section concern what are considered 'rare matrices' and what defines a metabolite being labile. Investigation of back conversion if no reference standard is available can be prone to error.

Technical or operational challenges

Back conversion of metabolites to the parent compound will hamper a correct determination of drug plasma levels. This can occur in samples upon aging, but also, for example, during the sample work-up procedure or even in the source of the mass spectrometer. Assessing and quantifying back conversion in the earlier stages of drug development cannot always be done given the lack of authentic metabolite standards at that stage.

Issues

Selectivity should be tested using independent individual sources of blank matrix. For small rodents this can result in practical or ethical challenges, hence the question if pools can still be used for rodents.

Conclusions & recommendations

Use six individual sources of matrix for selectivity experiment and if there is, or are indications of, a labile metabolite in the assay: test for back conversion.

■ 4.1.2 Carry-over

Common understandings

A maximum of 20% of LLOQ response should be a target for the first blank, but this criterion is not an absolute blocker. If carry-over is unavoidable, specific measures such as additional blanks can be employed to keep things under control. The number and positions of blanks needed in such a case is determined during validation and the findings are applied during routine application of the method.

Conclusions & recommendations

Try to reduce carry-over to <20% of LLOQ if possible. If carry-over is higher than 20%, define appropriate measures for study sample analysis.

■ 4.1.3 Lower limit of quantification

Common understandings

No changes to the general LLOQ concept: LLOQ is the lowest concentration of analyte that can reliably be quantified and is considered to be the lowest acceptable calibration standard. The demand: *"For BE studies the LLOQ should not be higher than*

5% of the C_{max}" was regarded realistic and should, in general, not be an issue to achieve.

Conclusions & recommendations

The LLOQ in BE studies should be less or equal to 5% of C_{max}.

■ 4.1.4 Calibration curve

Common understandings

Weighted regression analysis can be used and regarded as a 'standard' procedure to assess the calibration curve parameters and all accepted runs have to be presented (curve parameters and back-calculated values). The report should show the number of and reason of failed runs but no detailed statistics are required.

Ambiguities

How to treat rare matrices? Or to what extent should surrogate matrices be allowed and which level of scientific documentation would be required.

Technical or operational challenges

The EMA position that the calibration range should match with the concentration range of the samples is impossible to meet for early toxicology and first-in-man dose escalation studies and dilution of over-range samples may need to be done to an extent that goes beyond the agencies expectations. Case-by-case it should be judged which approach to follow, but in general adaptation of the calibration range and (partial) revalidating the method does not make much sense before a final dose range is known, for example, at the start of Phase III.

Conclusions & recommendations

The following aspects are recommended: allow documented use of surrogate matrix in case of ethical or scientific need, change of anticoagulant counter ion is not a change of matrix and does not call for partial validation, weighted regression can be used as 'standard' procedure and all accepted runs have to be presented (curve parameters and back calculated values). The report should show the number and reason of failed runs.

■ 4.1.5 Accuracy

Common understandings

Four QC samples: LLOQ, low, medium and high during validation is common practice, but for the medium level QC, in contrast to what the guideline indicates *"…around 50% of the calibration curve range (medium QC)… "*, there was a common understanding among the members that the 50% should be interpreted on a geometric scale rather than arithmetic. For each of the QC levels, accuracy is to be presented 'within-run' and 'between-run', which should be read as accuracy for each batch/run and the overall accuracy.

Ambiguities

Are 'different days', different calendar days or would different batches suffice? This is an important distinction for high-throughput methods. Second, how should we to treat obvious errors and outliers? We recommend reporting of the data with and without outliers and to provide justification for outliers using preset criteria, albeit that it is unclear if EMA would accept this for validation studies.

Technical or operational challenges

Independent stock solutions should be prepared for calibrators and QCs *"unless the nominal concentration(s) of the stock solutions have been established."* But how to deal with preparing independent stock solutions from scarce or expensive reference compounds? (e.g., non-proprietary compounds in presealed vials with CoA including concentration measurements).

Issues

Obvious errors and outliers: reporting the data with and without outliers can be performed as long as a justification for the outliers has been defined using preset criteria.

Conclusions & recommendations

50% of the calibration range for the medium QC can be read as the geometric mean rather than arithmetic. With respect to the spiking solutions for calibration standards and QCs the amount of solvent added to blank matrix should be limited to prevent impact on stability (i.e., preventing enzymatic instability) and impact on differences in extraction recovery.

■ 4.1.6 Precision

Common understandings

As for accuracy, precision is to be presented for each QC level 'within-run' and 'between-run'. This should be read as precision for each batch/run and the overall precision.

■ 4.1.7 Dilution integrity

Common understandings

The impact of dilution of (over range) samples on the observed concentration should be assessed

during validation. Additional dilution factors may need to be investigated during sample analysis.

Ambiguities

It is unclear whether an investigation of dilution integrity validates the used dilution factor or the highest tested concentration. For example, for a method with a 1–1000 ng/ml calibration range, would a tenfold dilution of a 8500 ng/ml sample increase the range of the method to 8500 or 10,000 ng/ml (with proper dilution)?

Conclusions & recommendations

In the case example, how would you deal with extrapolations and would you still accept data within ±15% from this 8500 ng/ml? As a consequence, the EBF proposed to choose the concentration to validate the dilution factor of above the curve sample to be within 80–85% of the ULOQ after dilution rather than the highest tested concentration. Following this proposal, the highest concentration and the dilution factor will be very similar, and the proposal removes the ambiguity created on extrapolations.

■ 4.1.8 Matrix effects

Common understandings

A total of six lots of unique sources of blank matrix are required and the investigation should be done at a QC high and QC low level. Matrix factors (MF) should be calculated for each of the investigated matrices and the CV of the IS normalized matrix MFs should be not more than 15%. Assessment of the matrix effect should include plasma derived from problem excipient containing formulations and hemolyzed and hyperlipidemic plasma.

Ambiguities

Assessing the matrix effect due to an excipient by spiking excipient into blank matrix implies that it is assumed that no biotransformation *in vivo* of the excipient takes place. The better alternative is to dose the test species with blank excipient to generate matrix, which is difficult to achieve and potentially unethical. Another important aspect is how to obtain or prepare standard and acceptable hemolyzed and hyperlipidemic test matrix. For example, should hemolyzed plasma samples be prepared from frozen blood or by spiking a few percentage blood into plasma?

Technical or operational challenges

Operational challenges are encountered for assessing the matrix effect due to excipients, as excipient placebo matrix may not be available. Furthermore matrix effect investigation for special populations cannot easily routinely be carried out given the lack of representative blank matrix prior to running clinical trials in the specific population.

Conclusions & recommendations

Determination of IS normalized MF and CV acceptance criteria of ≤15% should be generally accepted. Spiked excipients in matrix should only be investigated during primary validation if excipient is known to cause analyte response variability. With respect to hemolyzed and hyperlipidemic test matrix, EBF agreed to continue the discussion and generate more experience prior to recommending.

■ 4.1.9 Stability

Common understandings

Stability evaluation of all preparation processes and storage conditions as well as 'on instrument' stability should take place. In multi-analyte cases, the stability in matrix containing all analytes is to be assessed. Assessment of back conversion of metabolites cannot be limited to metabolites that need to be quantified according to metabolites in safety testing [22,23].

Ambiguities

Blood-stability investigation is not without practical challenges; for example, must it be fresh blood or would frozen suffice and how much time may have passed from collection for blood still to be considered fresh. Furthermore, there is no differentiation between re-injected result reproducibility and stability with respect to a fresh calibration curve

Technical or operational challenges

An unanswered question is, if some analytes in multi-analyte investigation fail; should all batch data be discarded? Conducting all stability investigations for a multi-analyte situation is costly and time-consuming and seems rather overdone when there is ample stability information on each of the individual analytes. Furthermore, there is to date no solid scientific evidence known within the EBF community that indicates that the stability of one analyte in the matrix is positively or negatively influenced by the presence of the other analyte.

Preparation and initial storage conditions of samples is not always in the CRO's control, whereas it is relevant to describe storage periods and conditions in the study reports.

Conclusions & recommendations

At least 72 h of autosampler stability of processed sample should be assessed using fresh calibration standards and QCs. The stability of the IS does need not be evaluated in multi-analyte long-term storage investigation and an assessment of preventative analyte instability in matrix should only be undertaken on a case-by-case basis.

■ 4.2 Partial validation

Common understandings

No differences from the FDA Guidance and Crystal City conference papers and no different than the general understanding of partial validation [15–17].

Ambiguities

The inconsistency between sections 4.1 and 4.2 regarding partial validations has already been pointed out. Another aspect of partial validation concerns a matrix change. It is unclear from the guideline if a change in anticoagulant counter ion is considered a change in matrix by the agency. The EBF has thoroughly investigated the impact of a change in counter ion and concludes that there is no ground for a (partial) validation when such a change occurs [24–26].

Issues

Sections 4.1 and 4.2 may be seen to be in conflict. Thus, good scientific justification is required.

Conclusions & recommendations

If a partial validation is conducted than at least precision and accuracy must be assessed with additional validation aspects justified by reason of change to method or application.

4.3 Cross-validation

Common understandings

No differences from the FDA Guidance and Crystal City conference papers and the general understanding and application of cross-validations.

5 Analysis of study samples

Ambiguities

Not well understood is how the statement: *"Before start of the analysis of the study samples the performance of the bioanalytical method should have been verified"* should be interpreted. Does this mean that just a statement needs to be provided on validation of the method before start of analysis or does this mean that an additional verification (pre-study performance testing) is needed?

Technical or operational challenges

Albeit there was a discussion on a number of details, however, there were no technical or operational challenges identified for application of the guideline to routine sample analysis.

Conclusions & recommendations

The EBF community recommends verifying the performance of 'sleeping' bioanalytical methods prior to the start of study sample analysis. It is the company's decision after which duration of inactivity and to what intensity they would want to conduct such performance verification. A practical mode could be to combine performance testing with staff training on a method.

5.1 Analytical run

Common understandings

Common practice on how an analytical run should be built and processed is aligned with the description in the guideline, along with the requirement to predefine the acceptance criteria.

5.2 Acceptance criteria for a run

Common understandings

The acceptance criteria for calibrators: back-calculated values should not deviate more than 15, and 20% at LLOQ from the spiked levels and this should be the case for at least 75% of total standards or six calibrators at minimum, for QCs: 4–6–15, are in agreement with current practices.

If the lowest or the highest calibration standard is lost in processing or failed to meet the acceptance criteria, the next lowest or highest standard will be the LLOQ or ULOQ, respectively.

5.3 Calibration range

Common understandings

The calibration range should fit with the (expected) levels of the study samples. In cases where a substantial number of the samples show levels above the ULOQ, the range should be extended. When the study samples all fall into a limited range, much shorter than the calibration range, it is advised to add an additional QC specifically for that sample range.

5.4 Re-analysis of study samples

Common understandings

Possible reasons for reanalysis and reported data should be predefined and discussed in study report. In the case of predose or PK re-analysis, all relevant information should be available.

Re-injection for instrument failure is basically always authorized but not if calibrators or QCs failed for no apparent reason.

5.5 Integration

Common understandings

Section matches with the common understandings and is free of ambiguity.

6 & 7.3.3 Incurred sample reanalysis

Common understandings

A SOP for incurred sample reanalysis (ISR) is considered mandatory and its conduct should be identified in the study plan. Studies for which ISR should be conducted include first-in-man, multiple ascending dose, BE, hepatic and renal impaired patients and proof-of-concept for clinical and at least once per species for preclinical. ISR should also be conducted in the first study directly following a major method change. Sample selection should be based upon: maximizing the number of subjects and one sample around C_{max} and one from the terminal phase. The number of samples subjected to ISR should be 10% for studies of less than 1000 samples plus 5% of the number of samples above 1000 for studies more than 1000 samples. There is no minimum number for (very) small studies, but 20 samples is considered to be a good lower limit for ISR.

The acceptance criteria are: two-thirds should fall within 20% from mean for small molecules/chromatographic assays and 30% from mean for large molecules/LBAs. Non-numeric values are excluded from the evaluation. The criteria are straightforward and realistic from a process control point of view, they, however, do not take into account cases in which a limited number of samples show rather large differences between the original and the repeat. From a method control perspective, the EBF members are of the opinion that in these latter kinds of cases an investigation is warranted.

ISR is best conducted early on in the study and it considered best practice to conduct ISR in the batch directly following the original measurement. ISR failure puts bioanalysis on hold, triggers an investigation and implies that an investigation report is issued.

Ambiguities

ISR is being used both as method control and process control and this may not only cause some confusion, but also warrant a different approach to investigate ISR failures. It also impacts on how to deal with cases in which a few values deviate substantially. In addition, timing of performing ISR is critical. While many bioanalysts see the value of doing ISR in each subsequent analysis batch, the normal laboratory routine and more specifically the challenges produced by commonly used Laboratory Information Management Systems make it a less prefered approach.

Despite the guidance clearly stating the studies that need to be considered for ISR, CROs are experiencing clients requesting ISR for most studies. In this respect, EBF is happy to see the guideline being in alignment with the earlier thinking of the group and limit ISR to a selection of studies rather that performing it in virtually all studies [27].

Issues

On the '10% question', there are no issues with respect to conducting ISR other than the added workload, but the community strongly wonders what the continued added value is of doing ISR in hundreds of samples in larger clinical trials while essential information on method performance can be picked up using a less extensive number of samples.

Conclusions & recommendations

To follow current established methodologies for ISR and consider incurred sample reproducibility inclusion in drug–drug interaction studies, in which a substantial impact on metabolism may be anticipated. In situations of formally acceptable ISR, in which a limited number of samples demonstrate rather large differences between original and repeat, we advise to conduct an investigation.

7 Ligand binding assays

Common understandings

Because of the complex nature of macromolecules and consequently the differences in analysis compared with small molecules, the EMA included a chapter on LBAs. Macromolecules are usually analyzed using LBA, although chromatography-based techniques are becoming more readily available over the past few years. Whereas section 7 indicates the requirements specifically pertaining LBA methods, other sections (i.e., sections 4 and 5) are also applicable for macromolecules as well. Section 7.1 includes 13 subsections discussing the requirements for full validation of a LBA method. The subsequent section 7.2 on partial validation and cross-validation

refers to sections 4.2 and 4.3 where this more general topic is discussed. Finally, section 7.3 outlines the analysis of study samples such as the analytical run, acceptance criteria and ISR.

■ 7.1.1 Full validation

Common understandings

This section with its subsections deals with the general requirements for the full validation of LBA for the assessment of PK properties of macromolecules.

7.1.1.1 Reference standards

Common understandings

Reference standards to be used should be in the purest form available while it is recommended that the drug used for dosing and reference standard are of the same batch. In case of batch changes, an analytical characterization as well as bioanalytical evaluation should be performed to ensure that performance characteristics are not changed.

Technical or operational challenges

To ensure optimal comparability between the drug determined in the nonclinical or clinical sample and the reference standard used for preparation of the calibration standard and QCs, the guideline strongly recommends that both should be of the same origin. This might be challenging because the batch used for dosing may not yet be available during validation or may be difficult to obtain. In the case of change of batch, the actual lot should be compared with the previous one by means of calibration standards and QC samples.

Conclusions & recommendations

Preferentially, the reference standard and dosing batch should be of the same origin. However, in some cases this may not be possible. In those cases it is recommended to document this in the report.

7.1.1.2 Specificity

Common understandings

The guideline stipulates that to assess the specificity of the assay, drug-related molecules or drugs expected to be concomitantly administered should be tested with QC samples by adding these in increasing concentrations to the naïve serum matrix. Both lower and higher concentrations of the related molecule(s) should be tested. Accuracy at both the LLOQ and ULOQ levels should not exceed 25% of the nominal values.

7.1.1.3 Selectivity

Common understandings

As for specificity, the same is also true for selectivity testing. A total of ten sources of matrix should be tested. These should include hyperlipidemic and hemolyzed samples. Also, sources from relevant diseases should be taken into account. Matrix effects at higher as well as lower (near LLOQ) analyte levels should be assessed.

Technical or operational challenges

At least ten sources of matrix should be included in selectivity testing. These different matrices should also include hyperlipidemic and hemolyzed samples. These nonstandard matrices are not difficult to obtain, but a clear definition on what can be considered a hyperlipidemic or hemolyzed sample is lacking. Until more experience is built within the bioanalytical arena, there should be some liberty in choosing exactly which of these kinds of samples to include in the validation.

7.1.1.4. Carry-over effect

Issues

Potential carry-over should be investigated when using robotic liquid-handling systems. However, this section does not address crosstalk in, for example, electrochemiluminescence assays. Crosstalk can lead to over quantification of low concentration samples which are placed next to samples with high analyte level and which consequently have a very intense signal in that sector of the plate. Assessment of carry-over effect should therefore not be limited only to robotic systems.

Conclusions & recommendations

As described above, the guideline stipulates that the carry-over effect should be investigated when assays are performed using robotic liquid-handling systems. The EBF recommends the extension of this investigation is extended to other platforms where carry-over (or crosstalk) might be an issue. The electrochemiluminescence assay format is a good example.

7.1.1.7 Calibration curve

Common understandings

A minimum of six calibration standards should be run at least in duplicate to generate a calibration curve. The calibration standards should be spaced approximately evenly on a logarithmic scale. A minimum of six independent runs should be evaluated and reported in a tabulated

fashion to establish the overall robustness of curve fit model to be used. The back-calculated concentrations of the calibration standards should be within 20% of the nominal value for at least 75% (25% for LLOQ and ULOQ) of the calibration standards. Anchor calibrators do not require acceptance criteria as they are out of the quantifiable range.

7.1.1.8 Precision & accuracy

Common understandings

To assess the precision and accuracy of the assay, at least six independent runs over several days should be performed. At least five QC levels (LLOQ, low, medium, high and ULOQ) should be used. These QCs should not be freshly prepared, but frozen to mimic the treatment of study samples. The within- and between-run accuracy should not exceed 20% (25% at the LLOQ and ULOQ) of the nominal value at each concentration level. The within- and between-run precision should not exceed 20%. In addition, it is recommended that the total error should not exceed 30% (40% at LLOQ and ULOQ).

7.1.1.9 Dilutional linearity

Common understandings

In order to ensure that samples containing drug levels above the ULOQ can be accurately measured, dilution linearity should be assessed. This should also be done to evaluate an eventual prozone or hook effect. The CV of back-calculated concentrations for each dilution should not exceed 20% with respect to the nominal value.

Ambiguities

Dilutional linearity should be shown in order to ensure that samples can be diluted when the analyte exceeds the ULOQ (i.e., confirm integrity of the sample upon dilution). It was discussed how these dilutions should be made in order to reflect the influence of the matrix in an appropriate way and how to proceed in case of a nonlinear dilution experiment. So far, no agreement was achieved, whether different dilutions of a sample should be analyzed on each plate. However, it is advised to specify this aspect in the relevant operational SOP.

7.1.1.10 Parallelism

Common understandings

As soon as study samples are available, parallelism between the calibration curve (spiked reference standards) and serially diluted study sample should be tested using a high concentrated study sample. The precision of the back calculated value between the dilutions should not be higher than 30%. In case nonparallelism is observed, a procedure for reporting a result should be defined a *priori*.

Ambiguities

Parallelism of incurred samples is a relatively new item in guidance documents. In this part of the EMA guideline, some ambiguities were observed. In most of cases, validation of an assay is performed prior to the start of any nonclinical and clinical study. As a consequence, incurred samples will only be available after completion of the validation and after the start of the study. Therefore, the reporting needs to be defined prior the validation in either a validation plan or an SOP. Consequently, it is also unclear where these results can best be reported: in the sample analysis report or the method validation report? In addition, it is not clear whether parallelism of incurred samples should be implemented once per matrix, as suggested for ISR.

Conclusions & recommendations

The EBF strives towards raising awareness in the scientific community on this topic and will continue to have discussions on the assessment of parallelism. Attention should be given to informed consent to ensure that these tests are covered.

7.1.1.11 Stability of the samples

Common understandings

As described in other guidance documents and White Papers, it is stipulated that short-term stability at room or sample-processing temperature as well as freeze–thaw stability should be tested. Long-term stability during storage should also be studied. The mean difference in observed concentrations should not exceed 20% CV of the nominal value.

Ambiguities

Stability testing of samples should be performed on low and high level QC samples.

As clinical samples are usually frozen, it is assumed that the spiked assay control sample should be frozen and not freshly prepared. This is not clearly described in the guideline.

7.1.1.12 Reagents

Common understandings

The section on reagents focuses on critical reagents (i.e., antibodies). Since these reagents

have a direct impact on the results, their quality should be assured. The effect on analytical performance must be assessed upon batch change of a critical reagent.

Ambiguities

What makes a reagent a critical reagent is a point of attention and should be thoroughly investigated during method development. For some reagents, such as binding proteins, it is evident; however, for many other reagents it may be more difficult to clearly identify them as either critical or noncritical. Another point of attention is the verification of an old versus new batch of reagents. The effort needed to verify the influence and comparability of different lots of reagents is due to sound science and will be discussed further within the EBF.

Conclusions & recommendations

Critical reagents have a direct impact on the result of the assay and should therefore be carefully monitored. The definition of 'critical reagents' and their requirements should be described in a clearer way. Furthermore, the EBF recommends defining a verification method to test system suitability. A good way to keep track on critical reagents is the trending of the assay response using the QC samples, which are routinely assessed during the assay runs.

7.1.1.13 Commercial kits

Common understandings

Since commercially available assays may be developed for purposes different from PK assessment, they need to be revalidated to assure that they meet with the objectives of the study, in particular that LLOQ and QC samples must meet the accuracy and precision criteria.

Conclusions & recommendations

In order to fulfill the validation requirements using commercial kits, it may be needed to change the calibration standards and QC's provided with kit to achieve the necessary number of level including LLOQ and ULOQ. In addition, the influence of the matrix of interest needs to be taken into account.

7.3 Analysis of study samples

Common understandings

Section 7.3 delineates the guidance for analysis of study samples and is divided into sections on analytical run, acceptance criteria and ISR.

■ 7.3.1 Analytical run

Common understandings

The common understanding of the EBF members was that analytical runs may include multiple plates. Each plate should contain a set of calibration standards and QC samples. However, in order to increase capacity, in some platforms it may be acceptable that a set of calibration standards be placed on the first and the last plate and QC samples on every single plate.

Ambiguities

Reference is made to the limited capacity of platforms. Clarification is needed if platform is used to describe different assay formats or different (automated) techniques.

Conclusions & recommendations

For most LBAs, samples are at least measured in duplicate (two wells per independent samples). However, no criteria on the variation between the replicates are indicated, most companies allow a variation of 15 and 20% CV between the two measurements.

■ 7.3.2 Acceptance criteria

Common understandings

Regarding the acceptance criteria for the study sample analysis, the guidance indicates that each plate should contain at least three levels of QC samples at least in duplicate. At least 67% QC samples and 50% at each concentration level should be within 20% of the nominal value and exceptions should be justified.

Ambiguities

The guidance states that each plate should contain at least three levels of QC samples at least in duplicate.

As stated in chapter 7.1.1.8, QC samples may be reported as the mean of two replicates (measurements of the same sample in two different wells). If so, to fulfill the requirements for assay acceptance it is needed to analyze four samples (duplicates of two replicates) per level. This is not expressed clearly in the guideline and may be a source of continuous discussions on the number of wells needed per level.

8 Reports

Ambiguities

Although there appeared to be many different flavors and different interpretations among the participants of the workshop, the general view was that the section is clear and unambiguous.

The only doubt raised was whether or not SOPs should be appended to reports or whether referencing them was sufficient.

Technical or operational challenges

CoAs are not always available and, if available, they do not always contain all the relevant data as: purity and expiry/retest dates. Acquiring fully detailed CoAs is often not within the possibilities of the bioanalytical laboratory. This aspect is more common when provision is from smaller biotechnology companies and especially for metabolites.

With respect to a quality assurance (QA) review/statement in validation and analytical reports, CROs and Pharmaceutical companies (sponsors) appear to have different internal processes. Validation activities may be covered by facility/process audits rather than full audit of every validation.

■ 8.1 Validation report

Common understandings

The validation report should contain validation performance summary, source of analytical method, the assay procedure, description of reference material including batch, origin and stability, calibration standards and QC storage conditions, tables of QC results, stability data including long-term stability, recovery tests and matrix effects, selectivity, LLOQ, carry-over, dilution integrity. and representative chromatograms.

Conclusions & recommendations

Validation reports: an observed difference between CRO and the R&D pharmaceutical industry practices is the QA involvement in validation, for CROs this is standard, whereas for the pharmaceutical industry this is not always the case. Performing validation studies under GLP implies a QA involvement. EBF recommends submitting all data for all (including failed) runs with unexpected results.

■ 8.2 Analytical report

Common understandings

The sample analysis report should contain description of analysis/assay procedure, reference material batch(es), run acceptance criteria, sample storage conditions, sample conditions on receipt, table with runs and analysis dates, calibration standards and QC results, identification of the failed runs, deviations from method/SOPs and the impact that may have had, details on any reassay and ISR results.

Issues

A lack of clarity is noted for preclinical study reports, whether it should be a single bioanalytical TK contribution or two separate (phase) reports and secondly if SOPs should either be appended or referenced or neither.

Conclusions & recommendations

Analytical reports: present sample receipt dates and contents in each shipment, calibrators and QC preparation dates and storage conditions. SOPs may be referenced and do not need to be appended. Some companies do not have a separate bioanalytical phase report for preclinical as it is included in the TK report. Our recommendation is to always issue a separate bioanalytical report.

Statement/EBF recommendation

The EBF community considers the EMA BMV guideline an excellent basis for countries that are in the process of developing or updating their own BMV guideline. Harmonization of bioanalytical guidance documents and of knowledge and working principles around the globe is to the benefit of both industry and health authorities. EBF is an active member of the Global Bioanalysis Consortium and actively supporting harmonization efforts [108].

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Disclaimer

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