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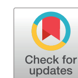
Review

Bioanalytical method validation: new FDA guidance vs. EMA guideline. Better or worse?



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ABSTRACT

Bioanalysis concerns the identification and quantification of analytes in various biological matrices. Validation of any analytical method helps to achieve reliable results that are necessary for proper decisions on drug dosing and patient safety. In the case of bioanalytical methods, validation additionally covers steps of pharmacokinetic and toxicological studies – such as sample collection, handling, shipment, storage, and preparation.

We drew our attention to the difference of both the newest FDA Guidance and the EMA Guideline on bioanalytical method validation. We aimed to point out advantages of both documents from the laboratory perspective.

The FDA and the EMA documents are similar, but not identical. The EMA describes the practical conduct of experiments more precisely, while the FDA presents reporting recommendations more comprehensively. There are also differences in recommended validation parameters. We hope that the International Council for Harmonisation will combine advantages of both documents to avoid confusing differences in terminology as well as the unnecessary effort of being compliant with two or more guidelines.

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1. Introduction

Bioanalysis concerns the identification and quantification of analytes in various biological matrices (blood, plasma, serum, saliva, urine, feces, skin, hair, organ tissue). Validation of any analytical method helps to achieve reliable results. In the case of bioanalytical methods, validation additionally covers steps of pharmacokinetic and toxicological studies - sample collection, handling, shipment, storage, and preparation - that are important for drug discovery and development as well as for regulatory submission [1,2]. Reliable measurements are necessary for proper decisions on drug dosing and patient safety. Thus, assuring the high quality of bioanalytical methods is of critical importance.

The U.S. Food and Drug Administration (FDA) has been a leader in guiding regulated bioanalysis since the early 1980s [3]. In 2001 the FDA issued the first extensive Guidance covering validation recommendations and conduct of sample analysis of small as well as large molecules [4,5]. Since then the field of bioanalysis has grown, e.g. liquid chromatography - mass-spectrometry (LC–MS) became a leader of bioanalytical techniques and new technologies like dried blood spots were introduced. Moreover, in the evolving era of biologics, ligand binding assays (LBAs) have gained significant importance. They support every step of drug development, including pharmacokinetic analyses, assessing immunogenity and searching for biomarkers [6].

A draft version of the FDA Guidance [7] was released for consultations in 2013, two years after the European Medicine Agency's (EMA) Guideline [8]. This draft contained new additions compared to the first edition [5], including incurred sample reanalysis (ISR). Both the FDA and the EMA have emphasized the importance of method reliability on the basis of its accurate, precise, selective, stable and sensitive evaluation. However, there were many differences between the FDA 2001 [5] and 2013 Guidances [7] and the EMA 2011 Guideline [8], which made it difficult to validate a method that was compliant to recommendations of both agencies.

Abbreviations: EMA, European Medicines Agency; FDA, U.S. Food and Drug Administration; ICH, International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; IS, internal standard; ISR, incurred sample reanalysis; IUPAC, International Union of Pure and Applied Chemistry; LBAs, ligand binding assays; LC–MS, liquid chromatography - mass-spectrometry; LIMS, laboratory information management system; LLOQ, lower limit of quantification; QC, quality control; SOPs, Standard Operating Procedures; ULOQ, upper limit of quantification.

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The recently published final FDA Guidance [9] is a good step to create uniform rules for bioanalytical method validation. The more similar the documents are in the U.S. and Europe, the better it is for the global bioanalysis environment, both in the industry and in the academia.

The recent FDA Guidance [9] emphasized in its background the importance of the well-suited validation parameters: lower and upper limit of quantification (respectively LLOQ and ULOQ) or selectivity and also the sampling or storage that could affect the reliability of the bioanalytical methods. In this paper, we drew our attention to the differences between of both the newest FDA Guidance [9] and the EMA Guideline [8] on bioanalytical method validation. In light of the expected global guideline ICH M10 on Bioanalytical Method Validation [10], we try to point out advantages of both documents from the laboratory perspective. We also suggest some alternatives and improvements for the future of bioanalysis.

2. Methodology

We compared the recent FDA [9] and the current EMA recommendations [8] by focusing on additions introduced by the FDA. The EMA's Guideline was treated as a reference because it is well established in the global bioanalytical community. The differences between the methodology applied to small and large molecules were not emphasized, similarly to the FDA approach in the new Guidance [9]. The most important differences are pointed out in the main text, whereas a more detailed comparison is collected in the Supplementary Data.

3. Terminology

The terminology is quite consistent in both documents. The glossary proposed by the FDA is more comprehensive, whereas the EMA explains most of the terms in the main text. The FDA is less restrictive in the application of terms and uses some of them interchangeably, e.g. accuracy/trueness. On the other hand, the FDA complicated the straightforward EMA's terminology of calibration curve standards: a zero calibrator and non-zero calibrators replaced EMA's zero sample and calibration standards. The confusion comes also when both agencies use different terms for the same meaning and when terms are used solely by one of the agencies and not the other (Table 1). The FDA still includes *recovery*, *reproducibility* and *system suitability* evaluation but have not introduced *matrix-factor* and *back-conversion* terms used by the EMA. For more details and author's personal feedback on the terminology see Supplementary Table A1.

The differences between terms used in bioanalysis and other areas of analytical chemistry, e.g. defined by the International Union of Pure and Applied Chemistry (IUPAC), are beyond the comparison of both guidelines but important to be aware of [11]. This issue was discussed by Huang et al. [12] and Kruve et al. [13,14]. Also adopting harmonized terminology for sample storage conditions - like room temperature, refrigerator, freezer and ultrafreezer - could facilitate communication between clinical sites, laboratories, sponsors and assessors [15].

Table 1
Key differences in terminology.

FDA	EMA
bench-top stability	short-term stability
zero calibrator	zero sample
non-zero calibrators	calibration standards
repeat analysis	reanalysis of study samples
Sensitivity	LLOQ
diagnostic kits	commercial kits
–	matrix factor
–	back-conversion
–	drug-naive matrix
system suitability	–
recovery	–
reproducibility	–

Table 2
Topics to be described in Standard Operating Procedures or in the study plan.

Topic	FDA	EMA
Bioanalytical method	+	+
Criteria for acceptance or rejection of an analytical run and the separate batches in the run	+	+
Reasons for reanalysis of study samples and criteria to select the value to be reported	+	+
Chromatogram integration and re-integration	+	+
Validation criteria, e.g. linearity assessment, cross-validation	+	
Internal standard response variability (for chromatographic methods)	+	
System suitability test	+	
ISR	+	
Instrumentation	+	

4. Validation parameters and experiments

Both agencies agree on the scope of validation, but some variations exist in methodology and acceptance criteria. We highlighted the differences most important from the laboratory perspective (see also Supplementary Tables A2 and A3).

Among the general differences, recommendations referring to the Standard Operating Procedures (SOPs) are described more precisely by the FDA (Table 2). We have found extending list of required SOPs as well grounded. The EMA, in turn, appears more flexible for whether information should be set in SOPs or study plans and enables GLP-certified laboratories to build their own fit-for-purpose quality system. Unlike the FDA, the EMA provides consistent presentation of numerical values through exact formulas, e.g. for calculation of accuracy and precision. The FDA Guidance only included the formula for ISR.

4.1. Pre-study validation

The recommendations on the calibration curve (also called standard curve by the FDA) differ slightly. Replicates of calibration standards and their acceptance criteria are raised only by the EMA. In the case of a calibration standard exclusion, the FDA prefers not to change the model, whereas the EMA suggests reevaluation of the ňewčalibration curve. Also different samples are suggested to calculate noise at the LLOQ: blank sample (EMA) and zero sample (FDA). In the latter case, the result might be distorted because of cross-talk between an analyte and an isotope-labeled internal standard (IS) [16].

The definition of low quality control (QC) is not harmonized and concentrations of medium and high QCs are not specified by the FDA at all. Moreover, the acceptance criteria for accuracy are referred in the EMA to the mean concentrations, whereas it is not clear if the FDA's criteria are related to the mean or to each sample. Inconsistencies of accuracy and precision are also specific for LBAs. Firstly, the FDA recommends at least three replicates per QC level in all accuracy and precision runs, whereas the EMA indicates that the number should reflect the study sample analysis procedure. Secondly, the FDA suggests freshly prepared QC samples (for at least one analysis), whereas the EMA advises frozen ones - treated in the same way as the study samples.

The sample stability assessment in conditions reflecting sample handling and analysis is recommended by both agencies. They

advise evaluation of the stock solutions stability, but only EMA recommends working solutions stability and the bracketing approach. The FDA assumes that long-term stability study at −20 °C also covers lower temperatures. But the EMA accepts the bracketing approach only for small molecules, whereas the stability of large molecules has to be studied at each storage temperature.

Both agencies have similar recommendations referring to cross-validation. But while the FDA suggests using shared matrix QCs and non-pooled subject samples, the EMA allows for testing the same set of QC samples or study samples.

Due to the growing number of LC–MS assays, both the FDA and the EMA recommend studying the matrix effect, but only the EMA specifies how to do it. Moreover, the EMA only recommends using a stable isotope-labeled IS that is an easy and effective method to overcome the matrix effect [17,18].

The FDA does not provide detailed procedure on how to test the carry-over and only advises to address and eliminate the problem. The FDA suggests that the carry-over should not be greater than 20% of the LLOQ. The EMA describes the test and expands the acceptance criteria for the IS (equal or less than 5% of the LLOQ). Additionally, the EMA recommends the evaluation of back-conversion of metabolites into a parent compound which might be of great importance for LC–MS assays.

In the case of LBAs there are differences in the assessment of the dilution effects. A sample dilution has a significant impact on the observed concentrations because a pre-assay sample in LBAs is prepared in very limited volumes. Thus, there is a high risk of cross-reactivity and nonspecific interactions between analytes and other molecules [19]. The FDA neither mentions the minimum required dilution nor describes conditions for studying parallelism. In contrast, the EMA suggests testing high QC samples close to the expected maximum concentration in study samples, diluted at least three times with a precision not exceeding 30%. Moreover, the FDA highlights parallelism only in relation to the endogenous compounds whereas the EMA does not. Minor differences appear also in the dilution linearity test. The FDA indicates five replicates per dilution whereas the EMA does not mention their number. It is not clear whether the number of samples should be either the same as in the dilution integrity test for small molecules or reflect the number in the accuracy and precision section for LBAs. The acceptance criteria are, in turn, more specifically defined by the EMA.

Some discrepancies are also marked when assessing selectivity and specificity for LBAs. In both cases the FDA recommends testing a blank matrix (including haemolyzed and lipemic matrix), LLOQ and high QC samples. The EMA advises using samples at or near LLOQ including a haemolyzed and a lipemic matrix, the high QC samples are suggested, but blank matrix is not mentioned at all. In the case of specificity, the distinctions between the agencies also concern sample concentrations, type of matrix, and concentration of interfering molecules.

Both agencies advise revalidation of commercial kits, but the FDA only gives special recommendations, e.g. mentioned the proceeding with sparse standards or QC samples expressed in ranges.

4.2. In-study validation

In the FDA Guidance, testing for lack of analyte in blank and zero samples is not supported by acceptance criteria in case of IS. The approaches to a calibration range that is found to be too wide are similar. The FDA recommends adding new QC samples at concentrations reflecting those of the study samples. The EMA favors narrowing the calibration curve range and adapting concentrations of QC samples.

A noticeable addition in the FDA Guideline is monitoring of QC samples drift and evaluation of its impact on the accuracy. Moreover, the FDA includes QC outliers in the assessment of accuracy and precision from accepted runs. This requirement seems to be unnecessary and might falsify reported results of method accuracy and precision.

The IS variability may affect results of the chromatographic method. Thus, the FDA suggests its monitoring, whereas the EMA allows reanalysis in case the variability is beyond the criteria established in a dedicated SOP.

In LBAs the EMA recommends using at least 2 replicates per study sample whereas the FDA does not mention this issue at all. It is surprising considering the high variability in LBAs and that both the calibration curve and the QCs are advised by the FDA at least in duplicate.

The FDA recommends additional procedures like the system suitability test, which was also included in the previous version of the Guidance [5]. Yet, records of system suitability are not needed in reports except for audits. Moreover, if unique high concentrations of metabolite are found in study samples, development of a fully validated assay for the metabolite determination should be considered. This rule may increase the time and costs of the analysis, disproportionately to the risk of invalid results.

5. Documentation & reporting

One of the main advantages of the FDA Guidance is the detailed and clear presentation of documentation and reporting. The table in the appendix suggests documentation at the analytical site, validation report, and analytical study report. The FDA also defines the presentation of QC results from passed and failed runs, including outliers. Other additions include: blank matrix (description, receipt dates, and storage - which are in line with GLP requirements [20]), 100% of e-chromatograms, laboratory information management system (LIMS), and correspondence records. Critical reagents are associated with both small and large molecules analysis, while the EMA uses this term only for large molecules. The FDA encourages QC graphs trend analysis which is similar in approach to recent papers on bioanalytical data visualization [21,22]. Example of an overall summary table for method validation report is helpful for both laboratory staff and assessors.

6. What is missing & future perspective

The bioanalytical method validation is a well-established area of regulatory science [1], especially for small molecules. But there are still open questions or areas which need further studies. Therefore, in this section we provide personal feedback, suggesting alternatives and improvements to serve the global harmonization process.

LC–MS is a golden standard in bioanalysis. Thus, it is very surprising that the FDA does not indicate how to evaluate the matrix effect. The absolute and relative matrix effects introduced by Matuszewski et al. [23] seem to be replaced by matrix factors [8]. The calculation of the latter ones includes also neat solutions and appears be more conservative [24]. Yet, the new concepts may be interesting alternatives for regulatory decision makers [25,26].

Contrary to the EMA, the FDA does not recommend investigation of unmatched results in ISR. This area needs further research and knowledge sharing among the bioanalytical community. The work by Tan et al. [27] may be a good starting point. Another open question is the sample size for this test. It is appreciated that the sample size calculation methods are now harmonized between the FDA and the EMA. Still, there were many signals that the number of samples tested is too large [28–30]. Recently, it was suggested that the sample size does not match acceptance criteria [21].

In both stability recommendations, the reference and after-storage samples are treated as independent sets of data. Thus, in

the case of small molecules test acceptance criteria are met if reference samples are +14.9% and after storage are -14.9% of nominal concentration. In our opinion, the difference of 29.8% in the above example raises a serious question on the validity of the results. This problem would be solved by using a ratio of means or confidence intervals in both datasets [31,32].

The FDA treats the evaluation of co-medications influence more precisely than the EMA, which refers more generally to other analytes. The FDA suggests studying their influence not only for selectivity (specificity) but also for stability. However, they may also impact accuracy and precision, e.g. for higher analyte concentrations when the extraction solvent is saturated. Thus, more experiments may be necessary [33]. The dealing with co-medications in bioanalysis requires more guidance, beyond a single recommendation from the European Bioanalysis Forum [34].

Both agencies present principles that are not suitable for endogenous analytes or biomarkers, regardless of whether LC–MS or LBA is used. The quantitative determination of endogenous compounds is complicated because of the lack of analyte-free matrix or commercially available samples with accurately known analyte concentrations [35–38]. The FDA only presents some general recommendations on use of an analyte-free matrix or QCs preparation. The lack of recommendations [39,40] or very general tips [41] in other regulatory guidelines indicate a global need for refined requirements. Furthermore, the validation of endogenous analysis should be specified in details. As suggested by Tsikas [42], the guidelines should indicate boundaries of the calibration range. This is a specific topic, so a supplement or an appendix to the guideline focused particularly on this subject could be a solution.

Both the FDA and the EMA define the small molecules acceptance criteria for LLOQ and ULOQ at 20% and 15%, respectively. These two points on a calibration curve are determined with higher uncertainty than others. It is caused by possible variation of slope between sequences. This is why one may observe wider confidence interval for calibration curve at LLOQ and ULOQ than in the middle of the concentration range in the case of $y = ax + b$ model. In the case of $y = ax$ model the widest confidence interval for calibration curve is observed at ULOQ (like at Figure 3 in [43]). Thus, acceptance criteria for LLOQ and ULOQ should be equal - just like for LBAs, even though different calibration models are used in both instances.

The FDA opens the door for the dried blood spots as a novel bioanalytical technique in an additional issues section [9]. Dried blood spots use exceeds pharmacokinetic and toxicokinetic studies [44] and includes also therapeutic drug monitoring [45] as well as forensic toxicology [46]. The information on specific validation tests lacks a detailed description and acceptance criteria. Correlating results obtained with dried blood spots to traditional sampling may be problematic in the case of variable drug plasma to blood partition ratio [47]. Also setting the standards for dealing with hematocrit-related issues would be encouraged [48].

The interesting issue of bridging data from multiple bioanalytical technologies is also raised. Comparison of methods using a set of a minimum of 20 incurred samples is advised. This arbitrarily selected number is not explained and is much smaller than the number of samples recommended for ISR. It is a bit strange because the results obtained by the two techniques are expected to be more different than the results of a sample reanalyzed using the same method. Moreover, there are no acceptance criteria for bridging data indicated.

What are the future perspectives of bioanalysis beyond introducing novel techniques? It seems that the current validation methodology ensures reliable concentration measurements, but international proficiency testing may further enhance the measurement quality [49]. Recently, European Bioanalysis Forum proposed to replace current technology-based acceptance criteria with decision-based ones [50]. This proposal is intriguing and may challenge the fundamentals of bioanalysis. There are no doubts that bioanalysis is a global issue [1,51,52]. Thus, it is good news that the recommendations of both agencies are closer than ever. We hope that the International Council for Harmonisation (ICH) will combine the advantages of the FDA and the EMA documents soon. Avoiding confusing differences in terminology as well as the unnecessary effort of being compliant with two or more guidelines is much-anticipated.

7. Conclusions

The FDA and the EMA recommendations on bioanalytical method validation are similar, but not identical. For example, there are differences in suggested validation parameters. We have found the FDA Guidance structure more clear and the tables presented in its appendix very helpful. The EMA describes the practical conduct of experiments more precisely. The FDA presents reporting more comprehensively. We hope that the ICH will combine advantages of the FDA and the EMA documents to avoid confusion in terminology as well as the unnecessary effort of being compliant with two or more guidelines.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2018.12.030.

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