MINISTRY OF HEALTH
NATIONAL HEALTH SURVEILLANCE AGENCY

RESOLUTION - RDC # 27 OF 17 MAY 2012

Provides the minimum requirements for the validation of bioanalytical methods used in studies for the registration and post-registration of medicines.

The Executive Board of the Brazilian National Health Surveillance Agency (ANVISA), in exercise of the attributions vested under section 4 in Article 11 of the ANVISA statute approved by Decree No. 3029 of 16 April 1999, and in view of the provisions of section 2 and in §§ 1 and 3 of Article. 54 in the regulatory statute approved in accordance with Annex I of ANVISA Ordinance No. 354, of 11 August 2006, republished in the Brazilian Official Gazette (D.O.U*) of 21 August 2006, at a meeting held on 10 May 2012, adopts the following Executive Board Resolution and I, Director and Substitute Chief Commissioner, determine its publication:

CHAPTER 1
INITIAL PROVISIONS

Section 1

Objective

Article 1. The objective of this resolution is to establish the minimum requirements for the validation of bioanalytical methods employed in studies for the registration and post-registration of medicines in Brazil.

Section 2

Definitions

Article 2. The following definitions are adopted for the purposes of this Resolution:

1. Blank sample: Sample matrix processed without added analyte and internal standard;
2. Quality control (QC) sample: Sample matrix with added analyte concentration, specifically used to monitor and validate the performance of a bioanalytical method;
3. High concentration quality control sample (HQC): Analyte added to the sample matrix at a concentration between 75 (seventy-five) and 85% (eighty-five percent) of the highest calibration curve concentration;
4. Low concentration quality control sample (LQC): Analyte added to the sample matrix at a concentration up to 3 (three) times the lower limit of quantitation (LLOQ);
5. Dilution quality control sample (DQC): Sample matrix with analyte added at a concentration above the highest calibration curve concentration (ULOQ), analyzed by a pre-defined procedure and dilution ratio pre-specified by the bioanalytical laboratory;
6. Medium concentration quality control sample (MQC): Analyte added to the sample matrix at a concentration near the middle of the lower and upper limits of quantification;
7. Study sample: Biological sample that is the object of analysis;
8. Hemolyzed sample: Biological sample containing lysed red blood cells at a level pre-defined and specified by the bioanalytical laboratory;
9. Lipid sample: Sample with high lipid content, for example, from postprandial collection;
10. Processed sample: Sample ready for instrumental analysis;
11. Zero sample: Sample matrix processed with only internal standard (IS) added;
12. Samples from different sources: Biological samples originating from different individuals or different lots of mixed samples;
13. Analyte: Specific chemical compound to be measured in a matrix;
14. Sample analysis run: Analyzing a set of samples processed under the same conditions, quantified using the same calibration curve and validated by the same QC samples;

* D.O.U (Diário Oficial da União): Federal Official Gazette of Brazil
15. Calibration curve: Relationship between the instrument response and known analyte concentration;
16. Matrix effect: Effect on the response of the analyte or IS (Internal standard) caused by biological matrix components;
17. Residual effect (carryover): Effect generated by the onset or increase of analyte signal or caused by Internal Standard (IS) contamination from previous samples;
18. Stability study: Study to determine that the concentration of an analyte remains within limits in a given matrix, under specific conditions;
19. Accuracy: Agreement between the results of a test and a reference value;
20. Lower limit of quantitation (LLOQ): Lowest concentration of analyte in the calibration curve prepared in matrix;
21. Upper limit of quantification (ULOQ): Highest analyte concentration in the calibration curve prepared in matrix;
22. Matrix: Medium in which the analytes are quantified in the study;
23. Biological matrix: The biological environment in which the analytes are quantified in the study;
24. Surrogate matrix: Matrix that replaces the biological matrix of the study samples;
25. Bioanalytical methods: Analytical methods for the quantitative determination of analytes in biological matrices;
26. Chromatographic methods: Methods which employ chromatography to separate the analyte from other components of the sample and quantify it;
27. Calibration standard: Matrix to which was added a known amount of analyte used to construct the calibration curve;
28. Internal standard (IS): Chemical substance from a standard solution of fixed concentration, added in the same amount to the calibration standards, QCs and samples of samples under study;
29. Precision: Proximity of the results obtained by repeated measurements of multiple aliquots of a single source sample;
30. Selectivity: The ability of the method to distinguish and quantify the analyte and IS in the presence of other sample components;
31. Primary solution: Solution prepared from a chemical reference standard;
32. Working solution: Solution prepared by diluting the primary solution;
33. Chemical reference standard: Reference substance characterized by means of appropriate tests, properly validated and documented, having high purity and uniformity;
34. Pharmacopoeia chemical reference standard: Substance established and distributed by pharmacopoeias or official authorized institutions, possessing a high degree of purity and uniformity;
35. Validation: Confirmation testing to provide objective evidence that the particular requirements for a specific intended use are fulfilled;
36. Partial validation: Conducting part of the validation tests due to change in the validated bioanalytical method, to demonstrate that the performance and reliability of the method have been maintained, and
37. Full Validation: Completion of all validation tests of a bioanalytical method validation.

CHAPTER 2

GENERAL PROVISIONS

Article 3. For amenable molecules, analysis by chromatographic methods should be the first choice.

Sole Paragraph. If a chromatographic method is not used, a technical justification should be presented.

Article 4. IS (internal standard?) should be used wherever applicable.
§ 1 IS (internal standard?) labeled with stable isotope should preferably be used for methods utilizing liquid chromatography coupled to mass spectrometry.
§ 2 Use of IS can be waived if technically justified.

Article 5. The tests can be adjusted or modified if alternative techniques are used, provided that the fundamentals of this Resolution have been met.

Article 6. Before implementing a bioanalytical method, full validation shall be performed.
§ 1 Validation must include testing of precision, accuracy, calibration curve, residual effect, matrix effect, selectivity and stability, according to the specifications of each test.
§ 2 The long-term stability study can be concluded after analyzing the study samples, provided it started before the analysis of the first sample.
Article 7. Metabolites, concomitant drugs and degradation products formed during the sample preparation, and nicotine, caffeine and other possible interferences should be evaluated whenever necessary.

Article 8. The possibility of conversion of a metabolite of the parent compound during analysis procedures must be evaluated and monitored when necessary.

Article 9. Whenever changes occur in a validated bioanalytical method, either partial or full validation should be performed, according to the importance of the modification.

Sole paragraph. When the impact of a change is unknown, a full validation should be performed.

Article 10. Pharmacopoeia chemical reference materials should preferably be used.

Sole paragraph. Characterized chemical reference materials may be permitted provided when the following information is available:

1. Documentation issued by the manufacturer with the following information:
   a) Nomenclature (Brazilian Common Denomination (DCB) name or the International Nonproprietary Name (INN);
   b) CAS number;
   c) Chemical name;
   d) Synonyms;
   e) Molecular and structural formula;
   f) Molecular weight;
   g) Physical form;
   h) Physicochemical properties;
   i) Impurity profile; and
   j) Care in handling and storage; and
2. An analytical report issued by the manufacturer stating:
   a) Identity;
   b) Content; and
   c) Expiration or retest date.

CHAPTER 3
VALIDATION TESTING

Section 1

Selectivity

Article 11. Samples from biological matrix obtained from at least six (6) different sources must be analyzed.

§ 1 When the biological matrix is plasma, samples from four (4) normal, one (1) lipid and one (1) hemolytic lot should be evaluated.

§ 2 When the biological matrix is whole blood, samples from six (6) lots: five (5) normal and one (1) lipemic, should be evaluated.

§ 3 When other biological matrices are used, their characteristics must be evaluated and tested.

Article 12. Test results should be compared with those obtained for processed LLOQ samples.

§ 1 Response of interfering peaks near the analyte retention time should be less than 20% (twenty percent) of the response of analyte in the LLOQ samples.

§ 2 Response of interfering peaks near the IS retention time should be less than 5% (five percent) of the IS response.

Article 13. If samples from one or more samples exhibit interference above the limits established in Article 12, new samples from at least another six (6) different sources must be tested.

Sole paragraph. If one or more samples from the second group have interference above the limits established in Article 12, the method should be changed to eliminate the interference.
Section 2

Residual Effect

Article 14. Must be performed using at least three (3) injections of the same blank sample, one before and two immediately after the injection of one or more processed ULOQ sample(s).
§ 1 Results should be compared with those obtained from processed LLOQ samples.
§ 2 Responses of interfering peaks at the analyte retention time should be less than 20% (twenty percent) of the analyte response in the processed LLOQ samples.
§ 3 Responses of interfering peaks at the IS retention time should be less than 5% (five percent) of the IS response.

Article 15. If the residual effect is unavoidable, specific procedures to manage the effect must be adopted when running the method to ensure that the precision and accuracy of the method are not affected.

Section 3

Matrix Effect

Article 16. Processed biological matrices with subsequently added analyte and IS and solutions prepared at the same LQC and HQC concentrations should be analyzed.
§ 1 When the biological matrix is plasma, eight (8) samples from different lots, four (4) normal, two (2) lipemic and two (2) hemolyzed, should be analyzed.
§ 2 When the biological matrix is whole blood, six (6) samples from different sources, 4 (four) normal and 2 (two) lipemic, should be analyzed.
§ 3 When other biological matrices are used, six (6) samples from different sources must be analyzed.
§ 4 In patient studies in patients where any change in the characteristics of the biological matrix is exhibited, this specific matrix must be employed in this assay.

Article 17. For each sample, Matrix Factor Normalized by IS (MFN) should be obtained using the following formula:

\[
MFN = \frac{\text{Analyte Response in Matrix}}{\text{IS Response in Matrix}} \times \frac{\text{Analyte Response in Solution}}{\text{IS Response in Solution}}
\]

Section 18. The coefficient of variation (CV) of NMFs for all samples must be less than 15% (fifteen percent).

Sole paragraph. If the CV of NMFs of all plasma samples exceeds 15% (fifteen percent) due to discrepant results for the hemolyzed matrix, a new CV without NMFs of the hemolyzed samples can be calculated and the hemolyzed samples may not be analyzed.

Article 19. Samples with a higher degree of hemolysis than those passing this test may not be analyzed.
Article 20. If the bioanalytical method does not permit the assessment of the matrix effect as described above, an alternative procedure should be implemented.

Section 4

Calibration Curve

Article 21. At least three (3) calibration curves should be constructed and evaluated, including a blank, a zero sample and at least six (6) samples of different concentrations of standard analyte with IS added.

Sole paragraph. Standard and IS must be initially added to the calibration curve samples and then subjected to the same preparation procedure which will be used for the study samples.

Article 22. The calibration curve must be constructed using the same matrix proposed for the study.
Article 23. Scientific justification for the concentration range covered by the calibration curve must be presented.
Article 24. An equation representing the relationship between instrument response and known analyte concentrations must be provided.
§ 1 Responses of blank and zero samples should not be used in the construction of the equation.
§ 2 It is preferred that the simplest mathematical model should be adopted, usually the linear model.
§ 3 When a nonlinear model is proposed, it must be mathematically demonstrated that the linear model is not adequate.

§ 4 For nonlinear models, at least eight (8) samples at different concentrations should be included in the calibration curve.

§ 5 If the error variance is not constant across the quantification range of the analytical method, weighting that presents the lowest value for the sum of the relative errors of the nominal value of calibration standards versus their values obtained by the equation of the curve should be employed.

§ 6 The equation for the curve should not include calibration standards that do not meet approval criteria.

§ 7 When a calibration standard is not acceptable, the calibration curve should be recalculated without this standard.

§ 8 When a calibration standard meets the criteria for approval, this standard should not be excluded from the curve equation.

Article 25. Calibration standards are approved if they meet the following criteria:
1. Deviation less than or equal to 20% (twenty percent) relative to nominal concentration for the LLOQ standards, and
2. Deviation less than or equal to 15% (fifteen percent) relative to nominal concentration for the other calibration standards.

Article 26. The calibration curve must meet the following criteria to be approved:
1. At least 75% (seventy-five percent) of the calibration standards must meet approval criteria, and
2. At least six (6) calibration standards of different concentrations, including the LLOQ and ULOQ, are approved in accordance with the above criteria.

Section 5

Precision

Article 27. Precision shall be determined in the same run (intra-run precision) and in at least 3 (three) different runs (inter-run precision).

§ 1 At least five (5) replicates of a minimum of five (5) QC concentrations (LLOQ, LQC, MQC, HQC and DQC) must be analyzed in each run.

§ 2 Precision evaluations should cover inter-run evaluations on different days.

Article 28. Precision must be expressed as relative standard deviation (RSD) or coefficient of variation (CV%), not accepting values greater than 15% (fifteen percent), except for the LLOQ, for which values less than or equal to 20% (twenty percent) according to the following formula:

\[ CV\% = \left( \frac{\text{Standard Deviation}}{\text{Mean Experimental Concentration}} \right) \times 100 \]

§ 1 Intra- and inter-run CV should be calculated based on all values obtained.

§ 2 If the CV does not meet the stated limits, the test should be repeated.

Section 6

Accuracy

Article 29. Accuracy must be determined in the same analytical run (intra-run accuracy) and in at least three (3) different runs (inter-run accuracy).

§ 1 At least five (5) replicates of a minimum of five (5) QC concentrations (LLOQ, LQC, MQC, HQC and DQC) must be analyzed in each run.

§ 2 Accuracy evaluations should cover inter-run evaluations on different days.

Article 30. Accuracy is determined by the Percent Relative Error (%RE), excluding values outside the range ± 15% (fifteen percent) of the nominal value, except for the LLOQ, for which values outside the range of ± 20% (twenty percent) of nominal value are excluded, according to the following formula:

\[ \%\text{RE} = \left( \frac{\text{Mean Experimental Concentration} - \text{Nominal Value}}{\text{Nominal Value}} \right) \times 100 \]

§ 1 Intra- and inter-run accuracy should be calculated based on all values obtained.

§ 2 If the CV does not meet the stated limits, the test should be repeated.
Section 7

Analyte Stability in the Biological Matrix

Article 31. Analyte stability in the biological matrix should be demonstrated by means of the following studies:
   1. Freeze-thaw Stability;
   2. Short-term Stability;
   3. Long-term Stability; and

Article 32. The conditions evaluated for the stability studies should replicate the conditions of storage, preparation and analysis of the study samples.

Article 33. Stability studies should utilize a set of biological matrix samples spiked with the analyte, IS and the same anticoagulant to be used in the study samples.

Article 34. At least three (3) samples of LQC and HQC must be used, which must be analyzed immediately after preparation and after being subjected to the test conditions.

Sole paragraph. Only samples whose test results are within ± 15% of nominal immediately after preparation should be used.

Article 35. Sample concentrations are determined using a freshly prepared calibration curve.

Article 36. Stability is demonstrated when there is no deviation exceeding 15% (fifteen percent) of the mean concentration from the nominal value.

Sole paragraph. All concentrations obtained should be included in calculating the mean.

Subsection 1

Freeze-thaw Stability

Article 37. Samples should be frozen at the indicated storage temperature for at least twelve (12) hours, then thawed at room temperature.
   § 1 When completely thawed, the samples must be refrozen and stored at the storage temperature for at least twelve (12) hours, repeating for each cycle, and quantifying analyte in samples after the last cycle.
   § 2 The number of freeze-thaw cycles should be greater than or equal to the number of cycles that will be used for study samples.

Subsection 2

Short-term Stability

Article 38. Short-term stability samples must be processed and analyzed after remaining at room temperature, or the processing temperature established for the bioanalytical method, for a longer time and under the same conditions as the samples to be analyzed will be maintained during the study.

Subsection 3

Long-term Stability

Article 39. Samples should be processed and analyzed after storage for a period of time that exceeds the time interval between collection of the first study sample and analysis of the last study sample.

Sole paragraph. The temperature used for the stability study should reproduce the temperature at which study samples are stored for the study.

Subsection 4

Post-processing Stability

Article 40. Samples must be processed and maintained under the same conditions used for study sample analysis.
§ 1 The storage period must exceed the time interval between completion of sample preparation and the end of the longest analytical run.
§ 2 If any storage is used in addition to the autosampler, stability under these conditions must be demonstrated.

Section 8

Solution Stability of the Analyte and IS

Article 41. Stability of analyte and IS should be demonstrated in at least three (3) samples of the highest primary solution concentration and the lower working solution concentration for longer than the use or storage period.
§ 1 Solutions must be analyzed after being kept under the same conditions the solutions will be subjected to during use and storage.
§ 2 The stability of primary and working solutions must be determined after appropriate dilution, taking into consideration the dynamic range of the detector.
§ 3 Mean instrumental results from the study solutions should be compared with the mean results obtained using freshly prepared solutions of analyte and IS.
§ 4 If a stable isotope IS will be used, evaluation of solution stability is not necessary, provided that the absence of isotope exchange reactions under the study conditions has been demonstrated.

Article 42. Solutions are considered stable when there is no deviation greater than 10% (ten percent) of their responses compared to the responses in freshly prepared solutions.

Sole paragraph. All instrumental responses obtained should be included in calculating the mean.

Section 9

Methods Where Analyte-free Biological Matrix is not Available

Article 43. When analyte-free biological matrix is not available, selectivity can be tested by comparing the slopes of at least six (6) standard addition curves in six (6) independent biological matrix sources (containing a baseline level of analyte) and a standard curve in solution or surrogate matrix.
§ 1 The method is considered selective if the slopes of the curves are not significantly different.
§ 2 A predefined statistical model must be used for slope comparison.

Article 44. When analyte-free biological matrix is not available, evaluation of calibration curve accuracy and the residual effect may be performed using calibration standards and QCs prepared in solution or surrogate matrix, provided they meet the requirements of Article 43.

Article 45. When analyte-free biological matrix is not available, stability evaluations should be performed in the same biological matrix used for the study.

Sole paragraph. Stability studies should be performed in accordance with one of the following:
1. Comparing the concentrations obtained from freshly prepared samples with those obtained from the same samples after storage, or
2. Compared to the nominal values, provided that the matrix was previously analyzed and the baseline level of analyte was supplemented to achieve LQC and HQC concentrations.

CHAPTER 4

METHOD VALIDATION CRITERIA

Article 46. The method used to analyze study samples must be validated, using the same anticoagulant, extraction technique and analytical conditions.

Article 47. Each analytical run of study samples must contain:
1. Calibration curve;
2. LQC, MQC and HQC samples; and
3. Samples from one or more study subjects to be analyzed.
§ 1 All samples must be processed as a single batch.
§ 2 For bioequivalence / relative bioavailability studies, all samples from the same volunteer should be analyzed in the same run, except for sample reanalysis.
§ 3 The number of LQC, MQC and HQC samples to be incorporated in each analytical run shall not be less than 5% (five percent) of the number of samples to be analyzed, and should not be less than six (6) QCs, one set of duplicates for each concentration.
§ 4 DQC samples should be included in reanalysis runs with diluted samples.
§ 5 LQC, MQC, HQC and DQC samples should be distributed among the analyzed samples in a balanced manner, always with the same number of replicates at each concentration.
§ 6 If the calibration curve covers a very wide concentration range in comparison with the concentration in all subject samples, an additional quality control must be included in the run so that at least two QCs are within the range of measured concentrations.

Article 48. Only concentrations quantified between LLOQ and ULOQ should be accepted.
§ 1 If the LLOQ or ULOQ calibration standards are not approved, the next acceptable calibration standard can be regarded as the LLOQ or ULOQ for this analytical run.
§ 2 Samples with concentrations above the ULOQ must be diluted and reanalyzed.
§ 3 Samples with concentrations below the LLOQ will be reported as < LLOQ.

Article 49. For approval of the analytical run, at least 67% (sixty-seven percent) of the total QCs and at least 50% (fifty percent) of the samples at each QC concentration should deviate less than or equal to 15% (fifteen percent) from their nominal values.

Article 50. The mean accuracy and precision of QCs of all approved runs (inter-run accuracy and precision) should be calculated for each concentration level.

Sole paragraph. If the mean inter-run accuracy or precision exceeds 15% (fifteen percent), causes should be investigated and justified, and may result in rejection of data.

Article 51. For approval of the analytical run, a calibration curve must meet the criteria provided in Articles 24, 25 and 26, 1.

Sole paragraph. At least six (6) calibration standards of different concentrations must be approved according to the criteria set forth in Article 25.

Article 52. When analyte-free biological matrix is not available for study sample analysis, calibration standards and QCs should be prepared in the same matrix used for validation.

CHAPTER 5
REANALYSIS

Article 53. Study samples must be reanalyzed when:
  1. The concentration is above the ULOQ;
  2. The concentration is between the LLOQ and adjacent calibration standard, considering the situation under Article 48, § 1;
  3. Observed analytical problems preclude or invalidate the quantification, or
  4. Analyte response is above the LLOQ in predose, placebo group or control group samples.

Article 54. For bioequivalence / relative bioavailability studies, reanalysis should not be performed for pharmacokinetic reasons.

Article 55. Reanalysis of calibration standards and QCs should not be performed.

Article 56. Reanalysis procedures must be pre-established in a standard operating procedure.

CHAPTER 6
FINAL AND TRANSITIONAL PROVISIONS

Article 57. This resolution replaces the bioanalytical methods section in the Annex to Resolution - RE 899, of May 29, 2003 and paragraph “d” in Item 2 of the Annex to Resolution - RE 1170, of 19 April 2006 on 01 December 2012.
§ 1 Companies can now revise their procedures in accordance with this Resolution without prejudice to the need to observe the date referred to in this article.
§ 2 Until 01 December 2012, companies can still file the documentation in accordance with RE 899/2003 or may choose to submit documentation as provided in this Resolution.
§ 3 In the event that you choose to submit documentation before the required deadline for this resolution, study approval will depend on suitability of the submission to meet the requirements of this Resolution.
Article 58. This Resolution shall enter into force on the date of its publication.

JAIME CESAR DE MOURA OLIVEIRA