Resolution - RE n. 899, of May 29, 2003

D.O.U. 02/06/2003

The Deputy of the Collegiate Board of Directors of the Brazilian Sanitary Surveillance Agency, in the use of the attribution vested in him by Administrative Order n. 238, of March 31, 2003,

WHEREAS

provided in Article 111, clause II, item “a” of paragraph 3 of the Bylaws approved by Administrative Order 593, of August 25, 2000, re-published in the Federal Official Journal of December 22, 2000

that the matter was submitted to the examination of the Collegiate Board of Directors, which approved the matter in a meeting held on March 6, 2003, decides:

Article 1 - To determine the publication of the "Guide for validation of analytical and bioanalytical methods”, attached.

Article 2 – Resolution RE n. 475, of March 19, 2002, is hereby revoked.

Article 3 - This Resolution enters into force on the date of its publication.

DAVI RUMEL

ANNEX

GUIDE FOR VALIDATION OF ANALYTICAL AND BIOANALYTICAL METHODS

1. General considerations

1.1. The information contained in this Annex presents the characteristics to be considered during the validation of analytical procedures. The objective of a validation is to demonstrate that the method is appropriate for the intended purpose, that is, the qualitative, semi-quantitative and/or quantitative determination of drugs and other substances in pharmaceutical products.

1.2. This information is applicable to:

1.2.1. analytical techniques that employ methods of gas chromatography (GC), high performance liquid chromatography (HPLC);

1.2.2. non-chromatographic methods, as long as they provide acceptable selectivity (e.g. titlemetry, UV-VIS spectrophotometry);

1.2.3. immunological or microbiological tests, as long as the degree of variability usually associated to these techniques is observed.

1.3. The validation must guarantee, through experimental studies, that the method meets the

requirements of the analytical applications, ensuring the reliability of the results. For this, it must present suitable specificity, linearity, range, precision, sensitivity, quantification limit and accuracy.

1.4. Reference standard substances made official by the Brazilian Pharmacopoeia or, for lack of it, by any other code authorized by the current legislation must be used. In the absence of these substances, working standards will be admitted provided their identity and levels are proved.

1.5. For the purpose of this guide, analytical run is the successive measurements of the same analyte, carried out under the same conditions: method, analyst, instrumentation, place, conditions of use and at short time interval between the measurements.

1.6. In the case of analytical methodology described in official pharmacopoeia or official forms, duly recognized by ANVISA, the methodology will be considered validated.

1.7. In the case of analytical methodology not described in official pharmacopoeia or official forms, duly recognized by ANVISA, the methodology will be considered validated, provided the following parameters are evaluated, as specified in Tables 1 and 2.

1.7.1. Specificity and Selectivity

1.7.2. Linearity

1.7.3. Range

1.7.4. Precision

1.7.5. Detection limit (sensitivity)

1.7.6. Quantification limit

1.7.7. Accuracy

1.7.8. Robustness

1.8. In the case of the transference of methodologies from headquarters to subsidiary in Brazil and/or of the national companies to the center of pharmaceutical equivalence studies, the methodology will be considered validated, as long as the parameters of precision, specificity and linearity are evaluated. A copy of all the original documentation referring to methodology validation must be attached, as proof that the methodology was originally validated and must contain, at least, all the parameters listed in item 1.7.

1.9. To ensure the analytical quality of the results, all the equipment used in the validation must be properly calibrated and the analysts must be qualified and adequately trained.

1.10. The tests are classified in 4 categories, according to Table 1,

Table 1. Classification of tests, according to their purpose:

Category	Test purpose
I	Quantitative tests for determination of active principle in pharmaceutical products or raw-materials.
II	Quantitative tests or limit assay for determination of impurities and degradation products in pharmaceutical products or raw-materials.
III	Performance tests (e.g. dissolution, release of active principle)
IV	Identification tests

1.11. For each category a set of tests, listed in Table 2, will be demanded:

Parameter		Category I	Category II		Category III	Category IV
			Quantitative	Limit assay		
Specificity		Yes	Yes	Yes	*	Yes
Linearity		Yes	Yes	No	*	No
Interval		Yes	Yes	*	*	No
Precision	Repeatability	Yes	Yes	No	Yes	No
	Intermediate	**	**	No	**	No
Detection limit		No	No	Yes	*	No
Quantification limit		No	Yes	No	*	No
Accuracy		Yes	Yes	*	*	No
Robustness		Yes	Yes	Yes	No	No

* may be necessary, depending on the nature of the specific test.

** if there is proof of reproducibility, the proof of Intermediate Precision is not necessary.

1.12. the analytical methodology must be revalidated in the following circumstances:

1.12.1. changes in the synthesis of the active substance;

1.12.2. changes in the composition of the finished product;

1.12.3. changes in the analytical procedure.

Certain other changes may also require validation, depending on the nature of the changes.

2. Methodology

2,1. Specificity and Selectivity

It is the capacity of the method to accurately measure a compound in the presence of other components such as impurities, degradation products and components of the matrix.

2.1.1. For qualitative analysis (identification test) it is necessary to demonstrate the capacity of selection of the method between compounds with related structures that may be present. This must be confirmed by obtaining positive results (preferably in relation to known reference material) in samples containing the drug, comparatively with negative results obtained with samples that do not contain the drug, but structurally similar compounds.

2.1.2. For quantitative analysis (content) and analysis of impurities, specificity can be determined by the comparison of the results obtained from samples (drug or drug product) contaminated with appropriate amounts of impurities or excipients and samples not contaminated, to demonstrate that the result of the test is not affected by these materials. In the absence of impurity or standard of the degradation product, the specificity of the method can be determined by comparing the results of analysis of samples containing impurities or degradation products with the results of a second, well characterized procedure (e.g. pharmacopeial methodology or other validated procedure). These comparisons must include samples stored under stressful conditions (e.g. light, heat, humidity, acid/base hydrolysis and oxidation).

2.1.3. In chromatographic methods, the necessary precautions must be taken to guarantee the purity of the chromatographic peaks. The use of peak purity tests (e.g. with the aid of photodiode arrangements or mass spectrometry) is interesting to demonstrate that the chromatographic peak is attributed to a single component.

2.2. Linearity

It is the capacity of an analytical methodology to demonstrate that the results obtained are directly proportional to the concentration of the analyte in the sample, within a specified interval.

2.2.1. It is recommended that linearity be determined by the analysis of at least 5 different concentrations. These concentrations must follow the intervals in Table 3.

2.2.2. If there is apparent linear relation after visual examination of the graph, the results of the tests must be handled with appropriate statistical methods for determination of the correlation coefficient, intersection with the Y axis, angular coefficient, residual addition of the minimum squares of the linear regression and relative standard deviation. If there is no linear relation, undertake mathematical transformation.

2.2.3. The acceptable minimum criterion of the correlation coefficient (r) must be = 0.99.

2.2.4. The curves obtained must be presented (from the experiment and from the result of the mathematical treatment).

2.3. Interval

The specified interval is the range between the limits of upper/lower quantification of an analytical method. Normally it is derived from the linearity study and depends on the intended application of the method (Table 3). It is established by the confirmation that the method presents adequate accuracy, precision and linearity when applied to samples containing amounts of substances within the specified interval.

Table 3. Percentage limits of the analyte content that must be contained in the linearity interval for some analytical methods.

Assay	Scope
Quantitative determination of the analyte in raw materials or pharmaceutical forms	80% to 120% of theoretical concentration of the test

Determination of impurities	From expected impurity level to 120% of maximum expected limit. When significant toxicological effects or unexpected pharmacological effects occur, the quantification and detection limits must be adequate in relation to the amount of impurities to be controlled.
Uniformity of content	70% to 130% of theoretical concentration of the test
Dissolution assay	Of approx. 20% over value specified for the interval. If specification for dissolution involves more than one time, the scope of the method must include –20% over lower value and +20% over higher value.

2.4. Precision

Precision is the assessment of the closeness of the results obtained in a series of measurements of a multiple sampling of the same sample. This is considered at three levels.

2.4.1. Repeatability (intra-run precision): match between the results within a short period of time with the same analyst and the same instrumentation.

The repeatability of the method is verified through a minimum of 9 (nine) determinations contemplating the linear interval of the method, that is, 3 (three) concentrations, low, medium and high, with 3 (three) replications each, or a minimum of 6 determinations at 100% concentration of the test;

2.4.2. Intermediate precision (inter-run precision): match between the results from the same laboratory, but obtained in different days, with different analysts and/or different equipment.

For the determination of intermediate precision, a minimum of 2 different days with different analysts is recommended.

2.4.3. Reproducibility (inter-laboratory precision): match between the results from different laboratories as in collaborative studies, generally applied to the standardization of analytical methodology, for example, for inclusion of methodology in pharmacopoeias. This data need not be presented for the concession of registration.

The precision of an analytical method can be expressed as the standard deviation or relative standard deviation (variation coefficient) of a series of measurements.

The precision can be expressed as relative standard deviation (RSD) or coefficient of variation (CV%), according to the formula,

$$RSD = \frac{SD}{ACD} \times 100$$

where SD is the standard deviation and ACD is the average concentration determined.

The maximum acceptable value must be defined according to the methodology employed, the concentration of the analyte in the sample, the type of matrix and the purpose of the method, values over 5% not accepted.

2.5. Detection limit

Detection limit is the lowest amount of the analyte present in a sample that can be detected, however not necessarily quantified, under the established experiment conditions.

2.5.1. The detection limit is established by means of analysis of solutions of known and decreasing concentrations of the analyte, to the lowest detectable level;

2.5.2. In the case of non-instrumental methods (CCD, titling, comparison of color), this determination can be done visually, where the detection limit is the lowest value of concentration capable of producing the expected effect (change of color, clouding, etc).

2.5.3. In the case of instrumental methods (HPLC, GC, atomic absorption), the detection limit can be estimated based on the ratio of 3 times the noise of the base line. It can be determined by the equation,

$$DL = \frac{SD_a \times 3}{CI}$$

where: SDa is the standard deviation of the intercept with the Y axis of at least 3 calibration curves containing concentrations of the drug close to the presumed quantification limit. The standard deviation can also be obtained from the analysis of the calibration curve obtained from a suitable number of blank samples; CI is the slope of the calibration curve.

2.6. Quantification limit

It is the lowest amount of the analyte in a sample that can be determined with acceptable precision and accuracy under the established experiment conditions.

The quantification limit is a parameter determined, mainly, for quantitative impurity assays, degradation products in drugs and drug products, and is expressed as concentration of the analyte (e.g. percentage w/w or w/V, parts per million) in the sample.

2.6.1. The quantification limit is established by means of analysis of solutions containing decreasing concentrations of the drug to the lowest determinable level with acceptable precision and accuracy. It can be represented by the equation,

$$DL = \frac{SD_a \times 10}{CI}$$

where: SDa is the standard deviation of the intercept with the Y axis of at least 3 calibration curves containing concentrations of the drug close to the presumed quantification limit. The standard deviation can also be obtained from the analysis of the analysis of a suitable number of blank samples; CI is the slope of the calibration curve.

2.6.2. It can also be determined through noise. In this case, the noise of the base line is determined and the concentration that produces a signal-noise over 10:1 is considered the quantification limit.

2.7. Accuracy

The accuracy of an analytical method is the closeness of the results obtained through the method under study in relation to the true value.
Several methodologies for determination of accuracy are available:

2.7.1. Drug

2.7.1.1. applying the analytical methodology proposed in the analysis of a substance of known purity (reference standard);

2.7.1.2. comparison of the results obtained with those resulting from a second well characterized methodology, whose accuracy has been established;

2.7.2. Dosage Form

2.7.2.1. in the analysis of a sample, in the which a known amount of the drug was added to a mixture of the components of the drug product (contaminated placebo);

2.7.2.2. in the cases where samples of all the components of the drug product are unavailable, analysis though the method of standard addition may be accepted, in which known amounts of the analyte (reference standard) are added to the drug product.

2.7.3. Impurities

2.7.3.1. analysis through the method of standard addition, in which known amounts of impurities and/or degradation products are added to the drug product or the drug;

2.7.3.2. in the case of unavailability of samples of certain impurities and/or degradation products, comparison of the results obtained with a second well characterized method may be accepted (pharmacopeial methodology or another validated analytical procedure).

The accuracy is calculated as percentage of recovery of the known amount of the analyte added to the sample, or as the percentage difference between the averages and the accepted true value, with the addition of the reliability intervals.

The accuracy of the method must be determined after the establishment of the linearity, the linear interval and its specificity, being verified from, at least, 9 (nine) determinations contemplating the linear interval of the procedure, that is, 3 (three) concentrations, low, average and high, with 3 (three) replications each. The accuracy is expressed by the relation between the average concentration determined experimentally and the corresponding theoretical concentration:

$$\text{Accuracy} = \frac{\text{average experimental concentration}}{\text{theoretical concentration}} \times 100$$

2.8. Robustness

The robustness of an analytical method is the measurement of its capacity to resist small and deliberate variations of the analytical parameters. It indicates its reliability during normal use.

The robustness assessment must be considered during the phase of method development. If susceptibility to variations is verified in the analytical conditions, they must be properly controlled or precautions must be included in the procedure.

Table 4 lists the main parameters that can result in variation in the response of the method.

Table 4. Factors that must be considered in the determination of the robustness of the analytical method.

Preparation of samples	·Stability of analytical solutions ·Extraction time
Spectrophotometry	·pH variation of the solution ·Temperature ·Different solvent manufacturers
Liquid Chromatography	·pH variation of the mobile phase ·Variation in the composition of the mobile phase ·Different batches or column manufacturers ·Temperature ·Flow of mobile phase
Gas chromatography	·Different batches or column manufacturers ·Temperature ·Speed of residual gas

BIOANALYTICAL METHODS

1. Definitions

Sample – general term that includes: controls, blank, processed and unknown samples.

Blank sample – sample of a biological matrix to which no analyte was added, used to evaluate the specificity of the bioanalytical method.

Quality Control Sample (QC) – sample of added biological matrix of the analyte, used to monitor the performance of a bioanalytical method and to assess the integrity and validity of the results of the analyzed unknown samples in an individual run.

Processed sample – final extract (prior to the instrumental analysis) of a sample that has been submitted to several manipulations (e.g.: dilution, extraction, concentration).

Unknown sample – biological sample that is the object of analysis.

Analyte – specific chemical compound to be measured, may be the untransformed drug, biomolecule or its derivative, metabolite or degradation product in a biological matrix.

Analytical run (or batch) – complete set of samples under study, with an appropriate number of standards and QCs for its validation and that undergoes complete analysis in the same conditions.

Specificity – ability of the bioanalytical method to measure and distinguish the analyte from components that may be present in the sample, such as metabolites, impurities, degradation compounds or components of the matrix.

Stability – parameter aimed at determining if an analyte remains chemically unchanged in a given matrix under specific conditions, at certain time intervals.

Accuracy – represents the degree of match between the individual results found and a value accepted as reference.

Quantification range– corresponds to a concentration range, including the HLQ and the LLQ, that

can reliable and can be quantified through replication with accuracy and precision, by means of the concentration-response ratio.

Detection Limit (DL) – lowest concentration of an analyte that the bioanalytical procedure can distinguish reliably from the background.

Lower limit of quantification (LLQ) – lowest amount of an analyte in a sample that can be quantitatively determined with acceptable precision and accuracy.

Higher limit of quantification (HLQ) – largest amount of an analyte in a sample that can be quantitatively determined with precision and accuracy.

Linearity – corresponds to the capacity of the method to provide resulted directly proportional to the concentration of the substance under examination (analyte).

Biological matrix – distinct material of biological origin, that can be sampled and processed in a manner that can be reproduced.

Method – understandable description of all the procedures used in analyses of samples.

Calibration standard – calibration matrix to which a known amount of analyte was added. The calibration standards are used to build the calibration curve, with which the concentrations of the analyte in the QCs and the unknown samples under study are determined.

Internal standard (IS) – generally composed of structural characteristics similar to the analyte, added to the calibration standards and samples in known and constant concentrations, to facilitate the determination of the analyte.

Precision – represents the degree of repeatability between the results of individual analyses, when the procedure is applied various times to the same homogeneous sample, in identical assay conditions.

Recovery – extraction efficiency of an analytical method, expressed as the percentage of the known amount of an analyte, obtained from the comparison of the analytical results of blank samples added to standard and submitted to the extraction process, with the analytical results of standard solutions not extracted.

Reproducibility – precision between two laboratories. It also represents the precision of the method under the same operational conditions, in a short period of time.

Partial validation – modification in the validated bioanalytical method that does not require the need for a total revalidation.

Total validation – establishment of all the validation parameters of a bioanalytical method, applicable to the analysis of the samples.

2. General considerations

2.1. The information contained in this guide is applicable to bioanalytical methods, such as gas chromatography (GC, high efficiency liquid chromatography (HPLC) and these combined with mass spectrometry (MS) such as LC-MS, LC-MS-MS, GC-MS, GC-MS-MS, used for the quantitative drug determination and their metabolites in biological matrixes, such as blood, serum, plasma or urine. It is also applicable to other analytical techniques, such as microbiological and immunological methods, or for other biological matrixes, although in these cases, a high degree of variability can be observed.

2.2. The validation must guarantee, through experimental studies, that the method meets the requirements of the analytical applications, ensuring the reliability of the results. For this, it must present precision, accuracy, linearity, detection limit and limit of quantification, adequate specificity, reproducibility, stability and recovery suitable for the analysis. Thus, it is important to highlight that all the equipment and materials must be properly calibrated and the analysts must be qualified and adequately trained.

2.3. Reference chemical substances and/or biological standards made official by the Brazilian Pharmacopoeia or by any other code authorized by the current legislation must be used. In the absence of reference chemical substances and/or biological pharmacopeial standards, studies using secondary standards will be admitted provided their certification is proved.

2.4. For the relative bioavailability and bioequivalence studies the internal standard must be used whenever chromatographic methods are used. The impossibility of its use must be justified.

2.5. Total validation must be carried out before the implementation of a bioanalytical method for the quantification of a drug and/or metabolites.

2.6. Partial validations must be carried out when modifications take place in the bioanalytical method already validated. The partial validation assays can involve from a small determination, such as the determination of intra-assay accuracy and precision, to close to total validation. The typical changes that require partial validation include, among others:

2.6.1. change of methods between laboratories and analysts;

2.6.2. changes in the analytical methodology, for example, substitution of the detection system;

2.6.3. change of anticoagulant in the collection of the samples;

2.6.4. matrix change, for example, from plasma to urine;

2.6.5. change in the sample preparation procedure;

2.6.6. significant changes in the concentration range;

2.6.7. changes of instruments and/or software;

2.6.8. demonstration of selectivity of the analyte in the presence of concomitant medications;

2.6.9. demonstration of selectivity of the analyte in the presence of specific metabolites.

2.7. The robustness assessment must be considered during the phase of method development. If susceptibility to variations is found in the analytical conditions, they must be properly controlled or precautions must be included in the procedure. Variation examples are:

2.7.1. stability of the analytical solutions.

2.7.2. extraction time.

Typical variations in liquid chromatography are:

2.7.3. influence of the pH variation in the mobile phase.

2.7.4. influence of the variation of the mobile phase composition.

2.7.5. different columns (different batches and/or manufacturers).

2.7.6. temperature.

2.7.7. flow rate.

Typical variations in gas chromatography are:

2.7.8. different columns (different lots and/or manufacturers);

2.7.9. temperature;

2.7.10. flow speed.

3. Pre-study validation

3.1. Specificity

3.1.1. Samples of the biological matrix must be analyzed (blood, plasma, serum, urine, or other) obtained from six individuals, four normal samples, a lipemic sample and hemolized sample, under controlled conditions in terms of time, feeding and other important factors for the study. Each blank sample must be tested using the procedure and the chromatographic conditions proposed. The results must be compared with those obtained with aqueous solution of the analyte, in concentration close to the LLQ.

3.1.2. Any blank sample that presents significant interference in the retention time of the drug, metabolite or internal standard, must be rejected. If one or more of the analyzed samples presents such interference, new samples of six other individuals must be tested. If one or more of the samples of this group present significant interference in the retention time of the drug, the method must be modified with a view to eliminating it.

3.1.3. The interferents may be components of the biological matrix, metabolites, decomposition products and drug products used concomitantly with the study. The interference of nicotine, caffeine, products sold without prescription and metabolites must be considered whenever necessary.

3.1.4. If the method is intended for quantification of more than one drug, each one must be injected separately to determine the individual retention times and to ensure that the impurities of a drug do not interfere with the analysis of the other.

3.1.5. The response of interfering peaks in the retention time of the drug must be lower than 20% of the response of the LLQ. The responses of interfering peaks in the retention time of the drug and the internal standard must be lower, respectively, than 20% and 5% of the response in the concentration employed.

3.2. Calibration/linearity curve

3.2.1. The calibration curve represents the ratio between the response of the instrument and the known analyte concentration. A calibration curve must be built for each drug and analytical run, which will be used to calculate the concentration of the drug in the samples, using the same biological matrix proposed for the study. The calibration curve must include the analysis of the blank sample (biological matrix free from the drug standard and internal standard), of the zero sample (biological matrix plus the internal standard) and at least 6 (six) samples containing drug standard and internal standard, contemplating the expected variation limit, of the LLQ up to 120% of the highest concentration intended to be analyzed.

3.2.2. For the determination of the calibration curve, samples extracted from the appropriate matrix

must be analyzed, at least 6 (six) different concentrations. Alternative procedures must be justified, such as in the attainment of a nonlinear correlation, where a higher number of concentrations of standards will be necessary.

3.2.3. The results must be analyzed by appropriate statistical methods such as, for example, the calculation of linear regression by the method of the square minimums. The curves obtained (experimental and those resulting from the mathematical treatment), the coefficient of linear correlation, the angular coefficient and intercept must be presented.

3.2.4. Acceptance criteria of the calibration curve:

3.2.4.1. deviation less than or equal to 20% (twenty per cent) in relation to the nominal concentration for the LLQ;

3.2.4.2. deviation less than or equal to 15% (fifteen per cent) in relation to the nominal concentration for the other concentrations of the calibration curve;

3.2.4.3. at least four of the six concentrations of the calibration curve must comply with the former criteria, including the LLQ and the largest concentration of the calibration curve;

3.2.4.4. the coefficient of linear correlation must be equal to or higher than 0.98.

3.3. Precision

3.3.1. The repeatability of the method is verified by using, at least, 3 (three) concentrations (low, medium and high), contemplating the variation range of the procedure, carrying out, at least, 5 (five) determinations per concentration.

3.3.2. The precision must be determined in the same run (intra-run precision) and in different runs (inter-run precision).

3.3.3. It may be expressed as relative standard deviation (RSD) or coefficient of variation (CV%), values greater than 15% not accepted, except for the LLQ, for which values lower than or equal to 20% are admitted, according to the formula:

$$RSD = \frac{SD}{ACD} \times 100$$

where, SD is the standard deviation and ACD is the average concentration determined.

3,4. Accuracy

3.4.1. The accuracy of the method must be determined using, at least, 3 (three) concentrations (low, medium and high), contemplating the variation range of the procedure, carrying out, at least, 5 (five) determinations per concentration.

3.4.2. The accuracy must be determined in the same analytical run (intra-run accuracy) and in different runs (inter-run accuracy).

3.4.3. The deviation should not exceed 15% (fifteen per cent), except for the quantification limit for which values lower than or equal to 20% are allowed.

3.4.4. The accuracy is determined by the ratio between the average concentration determined experimentally and the corresponding theoretical concentration:

$$\text{Accuracy} = \frac{\text{average experimental concentration}}{\text{theoretical concentration}} \times 100$$

3.5. Lower limit of quantification (LLQ)

3.5.1. Established by means of the analysis of the biological matrix containing decreasing concentrations of the drug up to the lowest quantifiable level with acceptable precision and accuracy.

3.5.2. The ratio of 5:1 between the signal and the noise of the base line can also be used, specifying the method used for the determination of the LLQ.

3.5.3. The LLQ must be, at least, five times higher than any interference of the blank sample in the retention time of the drug.

3.5.4. The response peak of the drug in the LLQ must be identifiable and reproducible with 20% (twenty percent) precision of and 80 – 120 % (eighty to one hundred and twenty percent) accuracy, through the analysis of, at least, 5 (five) samples of standards.

3.6. Detection limit (DL)

Established by means of the analysis of solutions of known and decreasing concentrations of the drug, up to the lowest detectable level. It is recommended that the DL be 2 to 3 times higher than the noise of the base line.

3.7. Recovery

The recovery measures the efficiency of the extraction procedure of an analytical method within a variation limit. Recovery percentages near 100% are desirable, nevertheless lower values are accepted, provided the recovery is precise and accurate.

3.7.1. This test must be done comparing the analytical results of samples extracted from three concentrations (low, medium and high), contemplating the linearity range of the method with the results obtained with standard solutions not extracted, presenting 100% recovery.

3.7.2. The calculation of the recovery must be based on the ratio of the area of the extracted and not extracted standard, both for the analyte and the internal standard separately.

3.8. Quality control (QC)

3.8.1. QC of the quantification limit (QC-LLQ): same concentration as the LLQ.

3.8.2. QC of low concentration (QCL): less than or equal 3 x LLQ.

3,8,3. QC of middle concentration (QCM): approximately the average between QCL and QCH

3,8,4. QC of high concentration (QCH): 75 to 90% of the highest concentration of the calibration curve.

3.9. Stability study of drugs in biological Liquids:

3.9.1. Relevant specific Considerations

For the stability study the parameters for accuracy, precision, linearity, detection limit, limit of quantification, specificity, variation and robustness limits previously validated must be observed.

The drug stability in biological liquids depends on its chemical properties, the biological matrix and the packing material used. The stability determined for a type of matrix and a specific packing material can not be extended to others.

The conditions for carrying out the stability assays must reproduce the actual conditions of handling and analysis of the samples. The stability of the analyte during the collection and handling of the sample must be assessed, after long term storage (freezing) and short term storage (room temperature), after cycles of freeze and thaw and under analysis conditions. Assessment of the stability of the analyte in the standard solutions must also be included, prepared with appropriate solvent in known concentrations.

The stability determinations must use a set of samples, prepared from a recent solution reserve of the drug analyzed, added to a biological matrix free from interference.

3.9.2. Stability after freeze and thaw cycles

The drug stability must be tested after three freeze and thaw cycles using a minimum of three samples of low and high concentrations determined in the validation of the analytical method, in the following conditions: the samples must be frozen at temperature indicated for storage and kept for 24 hours, after which they are defrosted at room temperature. When completely defrosted, the samples must be frozen again at temperature indicated for storage, for 12 to 24 hours, and so on successively, until the three cycles are complete, the drugs in the samples being quantified after the third cycle. The results must be compared to those obtained from the analysis of the recently prepared samples.

3.9.3. Short term stability

For verification of this stability, at least three samples of the low and high concentrations determined in the validation of the analytical method are used. Each one must remain at the room temperature for 4 (four) to 24 (twenty and four) hours (based on the time in which the samples of the study are to be kept at room temperature) and analyzed. The results must be compared to those obtained from the analysis of the recently prepared samples.

3.9.4. Long term stability

3.9.4.1. The period of storage for the study of long term stability must exceed the interval of time comprised between the first sampling and the analysis of the last, according to the schedule presented in the protocol of the relative bioavailability/bioequivalence study.

3.9.4.2. The temperature used in the assay must reproduce the one recommended for storage of the samples, usually -20ºC.

3.9.4.3. For verification of this stability at least three samples of the low and high concentrations determined in the validation of the analytical method are used. The concentrations of all the stability samples must be compared to the average of the values previously calculated for the samples on the first day of the test.

3.9.5. Post-processing stability

If equipment is used that employs an autosampler, a study of the drug stability must be undertaken, in the sample processed in the analysis, including the internal standard, at the temperature in which the test is to be carried out and for a period of time longer than the duration of the analytical race.

At least, three samples of the low and high concentrations determined in the validation of the analytical method are used. The results must be compared to those obtained from the analysis of the recently prepared samples.

3.9.6. Stability of the standard solutions

3.9.6.1. The standard solutions stability of the drug and the internal standard must be assessed, at room temperature, after a minimum of 6 (six hours) of preparation.

3.9.6.2. In the case of such solutions to be stored under refrigeration or freezing, the stability must also be assessed, contemplating their temperature and period of storage.

3.9.6.3. The results of this test must be compared to the ones obtained using recent solutions prepared from the drug and internal standard.

3.9.7. Analysis of the results

The samples will be considered stable when there is no deviation higher than 15% of the value obtained from the recently-prepared samples, with the exception of the LLQ, for which a deviation of up to 20% is accepted. Whatever the statistical method used to assess the results of the stability studies, it must be described clearly in the standard operational procedure (SOP).

4. Criteria for application of the validated bioanalytical method

4.1. The analysis of all the samples of an analyte in biological matrix must be concluded within the period of time for which the stability has been determined.

4.2. An analytical run must contain: QC samples, calibration standards and unknown samples of one or more subjects of the study. It is preferable that all the samples of a same subject be analyzed in a single run.

4.3. It is not to estimate the concentration of the samples through extrapolation of the calibration curve below the LLQ or above of the highest standard. Instead, the curve must be redefined or the higher concentration samples must be diluted and re-analyzed.

4.4. In the routine use of the validated analytical method, its precision and accuracy must be monitored regularly to ensure continuity of the satisfactory performance. For this, QC samples must be analyzed together with the other samples, in each analytical run.

4.5. The QC samples must be incorporated at appropriate intervals, depending on the total number of samples in the run, always in the same number of the replicates of each concentration (QCL, QCM and QCH).

4.6. The number of QC samples (in multiples of three) to be incorporated in each analytical run must not be lower than 5% (five percent) of the number of unknown samples. For analytical runs consisting of up to 120 samples, at least 6 (six) QCs (a duplicate of each concentration) must be present.

4.7. The results of the QC samples will serve as basis for acceptance or rejection of the analytical run. At least 67% (four out of six) of the QC samples must be within approximately 15% of their respective nominal values, except for the LLQ, for which a deviation lower than or equal to 20% is admitted; 33% (two out of six) QC samples may be outside these limits, but not for the same concentration.