Guidelines and Recommendations from Scientific Societies

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A protocol for testing the stability of biochemical analytes. Technical document

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Abstract: Stability of a measurand in a specimen is a function of the property variation over time in specific storage conditions, which can be expressed as a stability equation, and is usually simplified to stability limits (SLs). Stability studies show differences or even inconsistent results due to the lack of standardized experimental designs and heterogeneity of the chosen specifications. Although guidelines for the validation of sample collection tubes have been published recently, the measurand stability evaluation is not addressed. This document provides an easy guideline for the development of a stability test protocol based on a two-step process. A preliminary test is proposed to evaluate the stability under laboratory habitual conditions. The loss of stability is assessed by comparing

measurement values of two samples obtained from the same patient and analyzed at different time points. One of them is analyzed under optimal conditions (basal sample). The other is stored under specific stability conditions for a time set by the laboratory (test sample). Differences are expressed using percentage deviation (PD%) to facilitate comparison with specifications. When the preliminary test demonstrates instability, a comprehensive test is proposed in order to define the stability equation and to specify SLs. Several samples are collected from a set of patients. The basal sample is analyzed under optimal conditions, whereas analysis of test samples is delayed at time intervals. For each patient PD% is calculated as the difference between measurements for every test sample and its basal one and represented in a coordinate graph versus time.

Keywords: guideline; preanalytical phase; sample stability.

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Introduction

The stability of a biochemical analyte is defined as the capability of a sample/specimen material to retain its properties over time. Hence, loss of stability is calculated as a function of property variation and time in specific storage conditions (type of container, temperature and light exposure, among other factors). A stability limit (SL) is defined as the period of time in which a property variation does not exceed a maximum permissible instability (MPI) [1]. MPI is established *a priori* in the laboratory on the basis of regulations and clinical use. SLs will depend on the MPI established and local laboratory practice.

A myriad of experimental stability tests have been performed for the analytes most frequently measured in clinical laboratories. Yet, differing or even inconsistent results have been obtained due to the lack of standard experimental designs and wide variability in MPI specifications,

as shown in the systematic literature reviews published [1–3]. In addition, the Clinical and Laboratory Standards Institute (CLSI) guidelines for the management of laboratory specimens only provide general SLs based on specific studies [4–6].

The stability of an analyte depends, among other factors, on the characteristics of the container. SLs are a basic aspect of routine laboratory practice, as defined in ISO 15189 [7]. Yet, assessing SLs is not mandatory for manufacturers of *in vitro* diagnostic products. In relation to sample containers, the EU 98/79/CE directive [8] requires manufacturers to test the stability of container components, but not of container contents. Although guidelines for the validation of sample collection tubes have been published recently [9, 10], they do not address the issue of loss of analyte stability in specimens.

In 2006, the Board of Laboratory Quality of the Spanish Society of Laboratory Medicine approached this problem and developed a stability testing protocol [11]. This new version of the protocol is aimed at providing a simplified formula based on a preliminary stability test and a comprehensive stability test that help define stability test formulas.

Purpose and scope

The purpose of this document is to provide guidelines for the development of a stability test protocol. This protocol is focused on the specimens most frequently used in clinical laboratories (blood and urine), which are generally collected using vacuum collection tubes. This protocol may be applicable to other types of specimens, provided that the characteristics of each type of specimen are considered.

This document can serve as a guide to manufacturers of diagnostic *in vitro* products, helping them evaluate the characteristics of their sample collection devices. At the same time, this protocol may be useful for medical laboratories interested in verifying manufacturer's data.

Definitions

Stability: Capability of an analyte to retain its properties over time.

Loss of stability: Significant change in the properties of a biological analyte over time.

Maximum permissible instability (MPI): Maximum permissible deviation of a value obtained after a specific

storage time under given conditions from a reference value obtained under optimal storage conditions. It is expressed as a percentage of baseline value.

Stability limit (SL): The storage time in which the loss of stability of an analyte exceeds the MPI.

Factors affecting stability

Apart from time – which modifies all properties –, there are other variables that affect the stability of analyte properties, as follows:

- *Cellular metabolism.* Contact with cells causes an exchange of materials that does not necessarily involve cell destruction [12–14]. Contact with cells primarily affects electrolytes and substrates of intermediary metabolism and, to a lesser extent, complex molecules. The presence of cellular material after centrifugation and separation from cells is more frequent in plasma than in serum, and it depends on the separation and centrifugation method employed.

Cells of blood origin and microorganism can be found in urine samples [15, 16].

- *Contact with air, diffusion and evaporation.* When a tube is opened during an assay, the solvent evaporates partially. As a result, concentrations of most analytes increase [17] and diffusion of dissolved gases augments. This causes a rise of carbon dioxide levels and an increase in pH [5, 18, 19]. Even when the tube is capped, gas diffusion occurs across the walls of the container, as it occurs with oxygen in plastic tubes [20, 21].

Urine is collected in contact with air (except when collected by puncture), even although it is rapidly stored in vacuum containers.

- *Exposure to light.* Tubes are generally transparent. Therefore, specimens are exposed to light throughout the whole assay. The loss of stability of some analytes depends on the intensity and type of light they are exposed to [22].
- *Tube storage position and spinning.* Spinning affects the velocity of chemical reactions. It is an important aspect in long-distance transportation of specimens. Serum tubes are placed in an upright position to allow the specimen to fully clot, reduce constituent spinning, and prevent fibrin adherence when the tube is capped [4].
- *Adsorption.* A transfer of constituents from the specimen to tube components or *vice versa* may occur when

plastic tubes or tubes containing a separating gel are used. This phenomenon is more frequent in trace elements, medications and hormones [23–25].

- *Temperature.* Temperature is an important catalyst of the chemical reactions causing loss of stability [26–30].

Many of these factors are subject to significant inter-individual variability. This variability is explained by cellular concentration variations in a specimen or genetic variability in degradation mechanisms [31].

The factors interfering with analyte stability are affected by the characteristics of the container, such as tube composition, separator, lid and sample handling method (timing and duration of centrifugation). European laws and regulations require manufacturers of sample collection devices to assess the limitations of their products. Therefore, manufacturers should provide information about the stability of the analytes commonly measured in each type of container.

a preliminary test. In a preliminary test, the values obtained under optimal conditions (shortest time between sampling and analysis) are compared with those obtained after the maximum time considered acceptable in the standard laboratory practice. If bias does not exceed the MPI, there is no loss of stability at the storage time studied. A comprehensive test will be performed if deviation exceeds the MPI or a stability equation is to be developed. A comprehensive test involves testing different storage times.

The conditions and purposes of a stability test will be set by each manufacturer or laboratory according to their needs. It is recommended that the manufacturer always performs a comprehensive test to provide users with a stability equation. This formula will allow users to determine an SL that meets the quality specifications of each laboratory.

The following general aspects should be considered in the design of comprehensive tests.

Experimental design

Generalities

The stability of an analyte under standard laboratory conditions can be determined experimentally by

Stability conditions

The factors and conditions affecting the stability of each analyte should be explored. In all cases, laboratories should investigate the effects of contact with cells, storage time and temperature, among others. The storage time to be studied will be set by each laboratory, based on routine practice and applicable laws and guidelines.

Figure 1: Standard blood handling conditions in laboratories with regard to the main factors affecting stability.

In routine laboratory practice, there are “three basic conditions” affecting the stability of analytes in blood samples (Figure 1), namely:

- From collection to centrifugation. Stability of the analyte in whole blood stored in sealed tubes at room temperature exposed to light.
- Stability in serum/plasma generally stored in the primary container at room temperature, temporarily uncapped and exposed to light.
- Storage conditions. Stability in serum/plasma generally stored in a sealed refrigerated primary container in the dark. In the case of prolonged storage in a serum bank, generally frozen and separated in a secondary tube.

In routine laboratory practice, a maximum storage time is set for each of these conditions affecting stability. Maximum storage time limits are determined for pre-analytical processes prior to delivery to the laboratory, intra-laboratory pre-analytical processes + analytical processes and post-analytical processes.

Types of specimens/samples

It is recommended to use samples from patients rather than healthy subjects. This way, researchers will ensure that concentrations are near clinical decision limits. Use recent samples and avoid the presence of hemolysis, icterus or lipemia.

For the results obtained to be applicable to regular laboratory practice, the type of specimen and container used in a stability test should be the same as those used in routine measurements.

Significant differences have been obtained in measurements made in the same sample stored in different tubes. To ensure the validity of all tubes, phlebotomy should be performed in accordance with standard guidelines [32].

Analyte concentrations

The stability of an analyte may depend on its baseline concentration [21]. In general, stability tests should be performed using samples which concentrations are near clinical decision limits, in accordance with the type of laboratory and reference population. Yet, recruiting patients whose concentrations are near clinical decision limits is challenging, as it is difficult to predict concentrations, except in very controlled cases. Therefore, most

specimens will contain concentrations within normal limits.

Experimental design

Ideally, samples should be analyzed in a single run to avoid analytical bias. To analyze all samples in a single run, storage at -70°C could be an alternative. However, not all analytes can be frozen, as freezing can cause deterioration. Use of quality control samples of the same batch on each analytical run could be useful to determine between-run systematic error. Between-run systematic error is defined as the difference between measurement values for quality control samples in each analytical run.

Quality performance specifications

The MPI set by each laboratory for each analyte will be based on the quality standards of the laboratory.

In the 1st Strategic Conference of the European Federation of Laboratory Medicine (EFLM), three models were defined for the selection of quality specifications [33], namely: impact on clinical outcomes, biological variation of the analyte and state-of-the-art. Guidelines were provided for the selection of one of these formulas according to the analyte to be assayed. Although a hierarchy was not established, there was a general agreement that the two first models are better suited for certain analytes than others, as these formulas are based on the clinical use of analytes. Laboratories should use quality performance specifications based on robust studies which population was similar to that of the laboratory. As to biological variation, a tool has been developed to review the studies published so far. In addition, the Task Force on Biological Variation DataBase of the European Federation of Laboratory Medicine and Clinical Chemistry is building an updated international database based on methodologically-robust studies [34].

Preliminary stability test

Experiment design

Loss of stability is assessed by comparing measurement values for two samples simultaneously obtained from the same patient and analyzed at different time points. One of them is analyzed under optimal stability conditions (basal sample). The other sample is stored under

specific stability conditions for a time set by the laboratory (test sample).

Steps:

- (1) Determine the analytes and stability conditions to be tested. Each test involves a specific combination of type of container, specimen and temperature (or other conditions). It is recommended to assess stability in at least three separate tests, namely: analyte stability in the specimen (whole blood) at room temperature, in the sample (serum/plasma) at room temperature, and in the refrigerated sample (see Section “Stability conditions”). Each laboratory should consider whether it is necessary to consider other factors based on the analyte to be tested and specific conditions of the laboratory.
- (2) Establish an MPI for each analyte under study.
- (3) Define the duration of each test. The duration of each test will be determined by the maximum time the sample can be stored in this stability conditions in standard laboratory practice. This method will make it possible to assess the stability of an analyte in the worst conditions.
- (4) Determine the number of study subjects. It is recommended to include at least three patients to assess inter-individual variations of stability.
- (5) Determine the number of replicates needed. The study must reach a statistical power and level of statistical significance that guarantees that the bias observed involves a significant loss of stability. The number of replicates needed will be estimated based on the ratio of MPI to the analytical imprecision of the method, as determined by the coefficient of variation (CVa). Table 1 shows the number of replicates needed to reach a 90% statistical power and a 95% confidence interval (adapted from CLSI EP7 A3) [35]. The minimum number of replicates to be performed will be five in all cases.

Table 1: Number of replicates required to reach a 90% statistical power and a 95% confidence interval (adapted from CLSI EP7 A3) [35].

MPI (%) / CV (%) ratio	Number of replicates
1	22
1.2	15
1.5	10
1.8	7
2	6
>2.5	5

MPI (%), maximum permissible instability; CV (%), coefficient of variation.

- (6) Determine the number of tubes required for the whole assay according to steps 1–5. We recommend using a primary tube for each stability condition instead of using aliquots. The reason is that the transfer of aliquots may alter the results with respect to standard laboratory practice.
- (7) Consider the possibility of deep freezing the sample to perform the assay in a single run. Otherwise, avoid between-run bias by analyzing the same batch of control material in each run.
- (8) Test performance. Once all the specimens have been obtained, separate the baseline specimen from the test specimens. Centrifuge the baseline specimen rapidly after collection and analyze it or store it at -70°C . In stability tests in whole blood, centrifugation of test specimens is delayed for a specific time. In stability tests in serum/plasma, test samples are centrifuged together with the baseline sample, and analysis or deep freezing are performed after a fixed storage time.

Analytical design

The data obtained are compared to assess whether a significant loss of stability occurred.

- (1) Detection of atypical values (outliers). Revise the results obtained to identify the presence of atypical values (the test to be applied will depend on the number of replicates performed, and its use must be duly justified).
- (2) Estimation of percentage deviation (PD%). PD% is the difference between measurand concentrations in optimal conditions (baseline sample; t_0 , average of replicate measurements) and its concentrations when stored for the maximum permissible storage time (test sample; t_x , average of replicate measurements). The difference is converted into a %PD from the baseline value.

$$\text{PD\%} = \left(\frac{t'_x - t'_0}{t'_0} \right) \times 100$$

t'_x : average concentration in the test sample.

t'_0 : average concentration in the baseline sample.

- (3) Interpretation of results. A PD% < MPI for all study subjects indicates that there was not a significant loss of stability within the storage time studied. A PD% $\geq$ MPI in some of the subjects indicates that there was a significant loss of stability within the storage time studied. To define the SL for an analyte, a comprehensive assay must be performed to refine the stability equation.

Figure 2: Comprehensive stability equation.

- (4) Bias test. When an assay is performed in different runs, the occurrence of bias must be controlled using quality control results. To such purpose, calculate the PD% between the controls used in each run. If the PD% for controls is $>1/3$ of intraindividual biological variation, repeat the assay [36, 37].

Supplementary material shows how to perform a preliminary stability test.

Comprehensive test

The comprehensive test is used to develop the equation of PD% change vs. time and SL under given conditions in accordance with particular MPI specifications (Figure 2).

Experiment design

Loss of stability under given conditions is assessed by collecting simultaneously several samples from a patient. The control sample is analyzed under optimal stability conditions, whereas analysis of test samples is delayed at fixed time intervals. Loss of stability is measured as the difference between measurement values for the baseline sample and the values obtained for each test sample. The results drawn are shown in a coordinate graph with PD% in the Y-axis and time in the X-axis.

Steps:

- (1) Determine the analytes and stability conditions to be tested.
- (2) Establish an MPI for each analyte.

- (3) Define the timing of sampling. In case a preliminary test has been performed, sampling times should include a point above and below the test time. Otherwise, if a comprehensive test is performed without a preliminary test, sampling times will include standard laboratory processing and storage times. We recommend that sampling is performed in all subjects at the same time point to facilitate statistical analysis.
- (4) Determine the number of study subjects. We recommend that at least 10 patients are included.
- (5) Determine the number of replicates needed. In general, analysis in duplicate is adequate.
- (6) Consider the possibility of storing the sample at -70°C to perform the assay in a single run. Otherwise, to avoid bias, quality control measurements will be performed.
- (7) Based on steps 1–6, determine the total number of tubes needed. Considering the volume and number of tubes to be filled, consider the possibility of testing stability conditions and measurands in samples collected in a single sampling session. To ensure the validity of all tubes, perform phlebotomy in accordance with standard guidelines and avoid prolonged extractions [32].
- (8) Test performance. Once all specimens have been obtained, handle the baseline specimen under optimal conditions. Perform centrifugation or deep freezing/analysis of test samples at fixed storage times.

Analytical design

Once all data have been obtained the stability equation can be defined. Apply MPI to the y-axis, which will yield an SL for the analyte under study on the x-axis.

- (1) Detection of atypical values (outliers). Revise duplicate measurements to ensure that variance with respect to common analytical imprecision is not significant.
- (2) Estimation of PD%. Estimate the PD% between each storage time and the baseline sample.

$$\text{PD}\% = \left(\frac{t'_x - t'_0}{t'_0} \right) \times 100$$

t'_x : average concentration in the test sample at storage time x .

t'_0 : average concentration in the baseline sample.

- (3) Graphic representation. Design a coordinate graph displaying PD% values (abscissa) for each storage

time (ordinates) for each patient. Each patient will be labeled to identify potential cases of inter-individual variation (when the loss of stability formula for a patient differs significantly from the formulas for other patients).

- (4) Adjustment of the stability equation. The formula for testing stability can be adjusted manually on the stability chart or by the least squares method (preferred method). The regression curve must always be forced to cross the intersection of coordinates, as at time 0, loss of stability must be 0. The best adjustment may not be lineal. Adjustment of the stability equation must have enough statistical power (Pearson's coefficient >0.7). The established slope must always be significantly different from 0.
- (5) Definition of SLs. Based on the MPI set for each analyte, the SLs for a set of specific storage conditions can be obtained from the stability equation (Figure 3). Consider the confidence interval for each storage time. Calculate and represent graphically the average PD% and confidence interval for each storage time. A high specificity ("a loss of stability cannot be discarded") or high sensitivity ("it is certain" that the MPI is exceeded) can be defined.
- (6) Bias test. If an assay has been performed in different runs, calculate the PD% between the quality control samples used in each run. If the PD% for control samples is $>1/3$ of intraindividual biological variation, consider repeating the assay [36, 37].

Supplementary material shows how to perform a comprehensive assay.

Figure 3: Comprehensive stability test. Confidence intervals for each storage time.
MPI, maximum permissible instability.

Conclusions

The SL of an analyte depends on the conditions under which a sample is collected and handled and on the sample collection device used. Other factors include the MPI set by the laboratory.

A myriad of experimental assays have been performed to assess the stability of most clinically relevant analytes. However, conflicting results have been obtained and extrapolation of results from experimental assays to other laboratories is challenging. These difficulties are partially due to the lack of standard guidelines for the performance of stability tests. The purpose of this paper is to propose a standard protocol for the performance of experimental stability tests. This protocol can be useful for clinical laboratories and manufacturers for the evaluation of sample collection devices.

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