The quality of diagnostic samples

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While analytical quality standards seem well established, there has been a paucity in the development of such standards for the preanalytical phase.

Technical recommendations regarding sampling, transport, and identification have been developed in national (NCCLS, DIN) and international (IFCC, ISO) consensus organizations.

More recently, recommendations of the Working Group on Preanalytical Variables of the German Society for Clinical Chemistry and the German Society for Laboratory Medicine have been published, including the definition of optimal sample size, the use of anticoagulants, and the stability of analytes regarding transport and storage.

The present short review summarizes a smaller part of these recommendations [1].

Introduction

In recent years, an increasing awareness of preanalytical variables has broadened the quality definitions in clinical chemistry and laboratory medicine.

Therefore, the German Society for Clinical Chemistry initiated a working group on the preanalytical phase in 1996, which since 1998 has run as a joint working group of this society and the German Society for Laboratory Medicine.

Together with corresponding members and representatives of the respective industry this working group has edited four recommendations on medical quality criteria in the preanalytical phase [2-5]. In 2001, a summary was published entitled "The Quality of Diagnostic Samples [1] of which the following pages give a short view.

The working group presently consists of the following members:

F. da Fonseca-Wollheim, W. G. Guder (chairman), W. Heil, Y. M. Schmitt, G. Töpfer, H. Wisser, and B. Zawta with G. Banfi, Italy, K. Bauer, Austria, A. Deom and P. Hagemann, Switzerland, C. G. Fraser, Scotland, J. Henny, France, P. Hyltoft Petersen, Denmark, A. Kallner, Sweden, E. A. Leppänen, Finland, S. Narayanan, USA, M. A. Peca Amaral Gomes, Portugal, and M. Neumaier and W. Probst, Germany as corresponding members.

Serum, plasma, or whole blood? Which anticoagulants to use?

To obtain valid medical laboratory results, it is imperative that the in vivo state of the quantity in the body fluid under investigation be presented unchanged to the analytical process. This is not always possible when measuring extracellular and cellular components of blood.

Thrombocytes and coagulation factors are activated when the blood vessels are punctured. These processes continue, when the sample containers used do not contain anticoagulant. Historically, serum obtained using this method was the preferred assay material for determining extracellular concentrations of quantities in blood.

Coagulation-related changes of some of the quantities can be largely avoided by using anticoagulants added to the sample containers.

The types and concentrations of anticoagulants used in venous blood samples were defined in the international standard [6] published in 1996 and are now used as the basis for standardized plasma samples throughout the world.

The goal of this recommendation is to compile the findings described in the literature and those obtained by the members of the Working Group (including its corresponding members) on the use of anticoagulants.

These findings will be used as the basis for determining the acceptability of various sample types in diagnostic procedures. The overview presented here was developed in meetings of the Working Group after discussion with representatives from the diagnostics industry and corresponding experts in other countries [2].

Definitions

Whole blood

A venous, arterial, or capillary blood sample in which the concentration and characteristics of cellular and extracellular quantities remain relatively unchanged when compared with the *in vivo* state. This is achieved by means of *in vitro* anticoagulation.

Serum

The undiluted, extracellular portion of blood after adequate coagulation is complete.

Plasma

The virtually cell-free supernatant of blood containing anticoagulant obtained after centrifugation.

Anticoagulants

Anticoagulants are additives that inhibit the clotting of blood and/or plasma, thereby ensuring that the quantity to be measured is changed as little as possible before the analytical process. Anticoagulation is achieved by either the binding of calcium ions (EDTA, citrate) or by the inhibition of thrombin activity (heparinates, hirudin).

TABLE I: Definitions of whole blood, serum, plasma, and anticoagulants.

Use of anticoagulants

It is important that the blood is mixed with the following concentrations of solid or liquid anticoagulant immediately after sample collection:

EDTA

A salt of ethylene diamine tetraacetic acid. Dipotassium (K_2), tripotassium (K_3), and disodium (Na2) are used as cations. Concentrations: 1.2 to 2.0 mg/mL blood (4.1 to 6.8 mmol/L blood) based on anhydrous EDTA.

Citrate

Trisodium citrate with 0.100 to 0.136 mol/L citric acid. Buffered citrate with pH 5.5 to 5.6: 84 mmol/L trisodium citrate with 21 mmol/L citric acid. 0.109 mol/L (3.2 %) was recommended to achieve standardization. WHO and NCCLS recommend 3.2 % since differences have been noticed between 3.2 % and 3.8 % when reporting INR using responsive reagents [7]. The International Society for Thrombosis and Haemostasis (ISTH) has recommended the use of Hepes-buffered citrate for all investigations of hemostastic functions.

A mixture of one part citrate with nine parts blood is recommended for coagulation tests. One part citrate is mixed with four parts blood to determine the erythrocyte sedimentation rate [6].

Heparinates

12 to 30 international units/mL of unfractionated sodium, lithium, or ammonium salt of heparin with a molecular mass of 3 to 30 kD is recommended to obtain heparinized plasma [6].

Calcium-titrated heparin at a concentration of 40 to 60 IU/mL blood (dry heparinization) or 8 to 12 IU/mL blood (liquid heparinization) is recommended for the determination of ionized calcium [8].

Hirudin

Hirudin is an antithrombin extracted from leeches or prepared by a genetic engineering process. Hirudin binds thrombin to form a 1:1 hirudin-thrombin complex. It is used at a concentration of 10 mg/L [9].

The color codes of anticoagulants described in ISO/CD 6710 are: EDTA = lavender/red; citrate 9:1 = light blue/green; citrate 4:1 = black/mauve; heparinate = green/orange; no additives (for serum) = red/white [6].

TABLE II: Recommended use of anticoagulants.

Plasma or serum?

The definitions of whole blood, serum, plasma, and anticoagulants are stated in **Table I**. **Table II** lists the recommended use of anticoagulants.

Advantages of using plasma

The following aspects support the preferential use of plasma versus serum in laboratory medicine:

- Time saving: Unlike serum, in which coagulation is not complete until after 30 minutes, plasma samples can be centrifuged directly after sample collection.
- Higher yield: 15 to 20 % more plasma than serum can be obtained from the same volume of blood.
- Prevention of coagulation-induced changes: Independent of coagulation-dependent changes in blood composition postcentrifugal interferences by coagulation in primary and secondary sample tubes are prevented which otherwise may interfere with the analytical process (like blockage of sample needles).
- Prevention of coagulation-induced interferences: The coagulation process changes the concentrations of numerous quantities in the extracellular fluid beyond the maximum allowable limit. This is induced by the following mechanisms:
  - Increase in platelet components in serum as compared with plasma (e.g. potassium, phosphate, magnesium, aspartate aminotranferase, lactate dehydrogenase, serotonin, neuron-specific enolase, zinc). Release of amide-NH_3 from fibrinogen as induced by factor XIII.
  - A decrease in the concentration of quantities in serum as a result of cellular metabolism and the coagulation process (total protein, platelets, glucose).
  - Activation of the cell lysis of erythrocytes and leukocytes in non-coagulated blood (cell-free hemoglobin, cytokines, receptors).

Due to coagulation-induced changes, some determinations yield valid results only when plasma is used (e.g. neuron-specific enolase, serotonin, ammonium).

Disadvantages of plasma over serum

The addition of anticoagulants can interfere with some analytical methods or change the concentration of the quantities measured:

- Contamination with cations: NH_4^+, Li^+, Na^+, K^+.
- Assay interference caused by metals binding with EDTA and citrate (e.g., inhibition of alkaline phosphatase activity by zinc binding, inhibition of metallo-proteinases, inhibition of metal-dependent cell activation in function tests, binding of calcium (ionized) to heparin [8]).
- Interference by fibrinogen in heterogeneous immunoassays.
- Inhibition of metabolic or catalytic reactions by heparin, e.g., Taq polymerase in the polymerase chain reaction (PCR) [10].
- Interference in the distribution of ions between the intracellular and extracellular space (e.g., Cl^-, NH_4^+) by EDTA, citrate [11].
- Serum electrophoresis can be performed only after pretreatment.

Table III provides information on the utility of anticoagulants for the measurement of analytes in whole blood, plasma, or serum.

Table III (Click to see the table as pdf.)

Recommendations

Table III indicates materials that are recommended for a specific test.

The table also contains information on the utility of other sample materials as long as the measured results by that method do not exceed the maximum allowable deviation of measurement based on medical needs as defined from the biological deviation [12, 13].

A maximum deviation of 10 % is considered acceptable if the quantity is not included in the current list. In this case, other samples can also be used for the analysis.

Sample collection and transport time

The following sequence is recommended to avoid contamination when filling tubes: blood culture, serum (avoid serum as first tube when electrolytes are to be measured [14]), citrate, heparinate, EDTA, tubes containing additional stabilizers (e.g. glycolytic inhibitors).

Only the recommended quantity of anticoagulant (see Table II) should be added, wherever required, to avoid errors in results. Tilt the tube repeatedly (do not shake, avoid foaming) directly after filling in order to thoroughly mix the sample with anticoagulant.

To separate serum from whole blood drawn from non-anticoagulated patients, leave the containers at room temperature for at least 30 minutes. This period is shorter when coagulation is activated. The length of time that the sample is left at room temperature should not exceed the period of time indicated in the table regarding stability in whole blood.

Centrifugation

Blood cell constituents can be rapidly separated from plasma/serum by centrifugation. Relative centrifugal force (rcf) and rotations per minute (rpm) can be calculated by using the rotating radius r (the distance between the axis of rotation and the base of the container in mm). The following equation applies:

$$rcf = 1.118 \times r \{rpm/1{,}000\}^2$$

It is recommended that blood containers be centrifuged in 90°-swing-out rotors so that the sediment surface forms a right angle to the container wall, thus preventing contact between the sampling needle in the analyzer and the cell surface or separating gels.

Serum

When coagulation is complete, the sample should be centrifuged for at least 10 minutes at a minimum speed of 1500 g.

Plasma

To obtain cell-free plasma, centrifuge the anticoagulated blood (citrated, EDTA or heparinized blood) for at least 15 minutes at 2,000 to 3,000 g. When separating serum or plasma, the temperature should usually not drop below 15 °C or exceed 24 °C.

Storage

Uncentrifuged samples can be stored at room temperature for a specified time given in the recommendations on stability (see **Table III**). After centrifugation, the serum or plasma from primary tubes should be analyzed within the time period as indicated in the recommendations for whole blood if the whole-blood sample is stored at room temperature without the use of separating gels or a filter separator.

If serum or plasma must be refrigerated or frozen for preservation or because of labile quantities, blood cells must first be separated. Avoid freezing whole-blood samples before or after centrifugation, also when polymer-separating gels are used.

Analyte stability in sample matrix

The aim of a clinical laboratory test is to determine the true value of a diagnostically relevant analyte in a body fluid at the sampling time by in vitro analysis. This assumes that the composition of the samples taken for this purpose does not change during the preanalytical phase (sampling, transportation, storage, sample preparation).

The members of the Working Group have made the following recommendations. In cases where results of their own investigations were not available, they have carried out a comprehensive search of the literature on the subject. The present list has been compiled as part [1] of the book "Samples: From the Patient to the Laboratory" [11].

Definition of stability

Stability is understood to be the capability of a sample material to retain the initial value of a measured quantity for a defined period of time within specified limits when stored under defined conditions.

The measure of the instability can be described as an absolute difference, as a quotient, or as a percentage deviation.

Example:
During the transportation of whole blood for three to four hours at room temperature, the concentration of potassium rises from 4.2 mmol/L to 4.6 mmol/L.

Absolute difference: 0.4 mmol/L
Quotient: 1.095
Percent deviation: +9.5 %

Since the changes in a sample can only be measured in experimental studies, the Working Group has decided on a practical form of the definition:

As the maximum permissible instability a deviation is defined which corresponds to the maximum permissible relative imprecision of the analysis. According to the current directives of the German Federal Medical Council, this is generally 1/12 of the biological reference interval [12].

The deviation should be smaller than half of the total error derived from the sum of biological and technical variability [13]. The stability during the preanalytical phase is determined by the temperature, the mechanical load, and time. Since, in addition to other factors, time plays a major role in changes, the stability is stated as the maximum permissible storage time under defined conditions.

The maximum permissible storage time is defined as that period of time at which the stability requirement of 95 % of the samples is met. This is a minimum requirement since under pathological conditions the stability of an analyte in the sample can be greatly reduced.

The storage time is stated in suitable units of time (days, hours, minutes). A distinction is made between the storage of the primary sample (blood) and the storage of the analytical sample (e.g. plasma, serum).

Since only a few systematic studies on stability are available, the observations described in the literature have been critically examined and summarized. Literature references have been reduced to a minimum by referring to collective works when the original paper is quoted therein [15].

Recommendation for quality assurance of the relevant preanalytical times

The transportation time is to be documented for each sample tested in the laboratory. This is derived from the difference between the blood sampling time (time of day in hours and minutes, in general to an accuracy of at least a quarter of an hour) and the registration time of the request and/or the arrival of the sample in the laboratory.

The preanalytical time in the laboratory is calculated from the difference between the time of analysis and the registration time of the sample. When the time at the end of the analytical phase (i.e., printing time of the result) is noted, the analysis time stated in the description of the method is to be subtracted.

For the documentation of the preanalytical transportation time, it is recommended that the sampling time and the arrival time of the sample in the laboratory be stated with the report.

If the maximum permissible storage time of the sample is exceeded, a medically relevant falsification of the results is to be assumed. It is the responsibility of the person in charge of the laboratory to mark the results obtained from such samples with an appropriate note or to refuse to carry out the test.

The latter measure is especially advisable when medical conclusions may be drawn from the result which are to the disadvantage of the patient. The clinician should be informed via a comment or a refusal.

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