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Introduction

Clinical laboratory investigation of hemostasis has considerably progressed during the last decade with the use of high-quality instruments and reagents. In order to transfer these improvements to the quality of patient care and to increase inter- and intra-laboratory reproducibility, more stringent requirements in the management of samples from blood collection to the validation of results are needed. The outcome of even the best clinical test in hemostasis is critically dependent upon the quality of the sample used. Therefore, the quality of blood sampling, the transport to the laboratory, sample preparation and storage are factors determining the test results.

To minimize this significant source of variation, the implementation of good biological practice rules is compulsory. Both the National Committee for Clinical Laboratory Standards (NCCLS) and the European Concerted Action on Thrombosis have established and/or reinforced such guidelines [1–5]. In addition, at least in France, the clinical pathologist is responsible for the whole preanalytical phase, including blood collection, even if it is carried out by a third party [6].

In this study, the criteria approved by the ‘Groupe d’Etude sur l’Hémostase et la Thrombose’ (GEHT) are summarized and updated [7].

Material for Venous Blood Sampling: Tube and Closure

Materials used in the manufacturing of blood collection tubes are as diverse as their
impact on coagulation tests. The use of evacuated blood collection tubes has been recommended by the European Concerted Action on Thrombosis since 1987 [4]. Tubes must be sterile and the sterility guaranteed by the manufacturer. The volume evacuated should represent at least 80% of the total tube volume in order to reduce platelet activation caused by the decrease in pH observed in partially evacuated tubes [8].

**Material and General Characteristics**

The tube components should neither activate the coagulation factors nor adsorb them [9]. The inside of glass tubes must be siliconized and excessive silicone removed, since some silicones (e.g., Silwet™ Copolymer L-720) have been shown to activate platelets. Although siliconized glass remains the standard material, evacuated ‘plasticware’ tubes may also be used keeping in mind that plastics comprise different materials with different characteristics and therefore different performances: prolongation of clotting times [10–12], heparin adsorption, interference with fibrinogen assays, modification of thromboplastin sensitivities [13] or calibration [14], and activation of contact phase of coagulation [15–17] have been reported. ‘Plasticware’ tubes may require strict storage temperature conditions. In addition, their evacuation is usually incomplete, therefore their performances should be rigorously established.

For safety reasons, tube closure must be designed to decrease the risk of aerosol formation and blood spillage when opening. In addition, the stopper itself must be free of any substance that could interfere with the anticoagulant added, the blood clotting factors and drugs such as heparin.

Labeling should be in accordance with international regulatory standards, i.e., on each tube, the batch number, the date of expiry under optimal storage conditions (temperature around 20°C, absence of light exposure), the volume drawn, the volume of additives and composition must be clearly indicated. The expiry date should indicate the last day of possible use. The phlebotomist must check this information before using any tube.

**Influence of the Collection Tube Size**

The size of the collection tube must be adapted to the volume of blood drawn and not to the ease of use. Comparison of results obtained from tubes of identical sizes but partial filling (i.e., reduced volume of anticoagulant and reduced evacuation) shows significant differences in clotting times, especially for APTT [8, 18] and platelet counts. The variations in the ratio of blood volume/surface area have not been fully investigated. It certainly has an impact on blood flow in the tube and on platelet activation that may be increased by centrifugation. It also influences the sample pH through gas exchange.

**Anticoagulants**

The type, the volume, the concentration of divalent cations, the pH of the anticoagulant, its ratio to the blood volume and the hematocrit are preanalytical variables which influence blood coagulation tests.

**Type and Concentration**

The recommended anticoagulant is trisodium citrate. It can be mixed with other additives if necessary.

The recommended concentration range is 0.105–0.109 M (usually 3.2%). The 0.129 M (3.8%) citrate, which is still available, should be abandoned because its citrate concentration modifies the internal sensitivity index of some thromboplastins [19]. In addition, one must keep in mind that the calibration of the reference thromboplastin is performed with
plasmas from blood drawn on 0.109 M citrate [20]. Citrate concentration has little or no effect on coagulation tests from normal subjects; conversely, a high citrate concentration prolongs coagulation time of anticoagulated patients (heparin or anti-vitamin K) in a reagent-dependent manner [21].

**Anticoagulant/Blood Ratio**

The recommended ratio is 1 volume of anticoagulant to 9 volumes of blood. Therefore, in an underfilled tube, the final citrate concentration is increased. The APTT test is the most sensitive to variations in the final citrate concentration. It lengthens by an average of 3% for 10% underfilling and 10% for a 20% underfilling. The PT test lengthens by an average of 5% for 20% underfilling and by 15% for 30% underfilling [22, 23].

Overfilling denotes that the evacuated tubes had not been used according to the manufacturer’s specifications. Such conditions would happen if the tube was filled either after opening it or with a syringe previously used for blood drawing.

**pH Effect**

Citrate must be buffered with citric acid to a pH of 5.1–5.3 [4] in order to maintain the pH of the plasma sample between 7.3 and 7.45. Outside this range, a spurious prolongation of abnormal PT is possible. In unbuffered citrate, the pH of a plasma sample will be rapidly outside these ranges since the pH of trisodium citrate is slightly alkaline and because the plasma has lost the buffering capacity of red cells that are removed by centrifugation.

**Hematocrit Effect**

The hematocrit directly influences the ratio of plasma to anticoagulant. Any concentration of plasma protein (e.g. fibrinogen) is influenced by this dilution effect. For hematocrit values >55% or <30%, it is recommended to modify the volume of anticoagulant. It is possible to use abacuses or the equation of McGann [24]: Anticoagulant volume (ml) = 0.00185 × final volume (ml) × [100 − hematocrit (%)] or, as recommended by the NCCLS, the one of Ingram [4]: Anticoagulant volume (ml) = blood volume (ml) × [100 − hematocrit (%)]/[595 − hematocrit (%)].

The hematocrit also modifies PT and APTT through the concentration of Ca²⁺ in the test medium after recalcification. In daily practice, the use of evacuated tubes prevents any adjustment of the anticoagulant volume to the hematocrit value.

**Additives**

**Citrate and Antiplatelet Mixture**

The citrate-theophylline-adenine-dipyridamole buffer (CTAD) mixture is made of trisodium citrate/citric acid (0.105 M); theophylline (15 mM); adenosine (3.7 mM) and dipyridamole (0.198 M) with a pH between 5.32 and 5.38. This mixture is well suited for monitoring heparin therapy either by APTT or by anti-Xa assays [25, 26] since it reduces heparin neutralization by platelet factor IV very significantly. Recently, the use of the CTAD has been proposed for flow-cytometric analysis of platelets [27–30]. On the other hand, its use is proscribed for any type of platelet function testing as it inhibits platelet function. In addition, this anticoagulant is very sensitive to light; tubes must therefore be stored in their original light-proof package until assayed [31].

**Citrate and Fibrinolysis Inhibitor**

This category of additive may be of interest for monitoring fibrinolytic therapy since, in the absence of a fibrinolysis inhibitor, blood-borne plasmin will continue to proteolyze fibrinogen in the sampling tube. Therefore,
Table I. General recommendations for hemostasis testing

<table>
<thead>
<tr>
<th></th>
<th>Good</th>
<th>Fair</th>
<th>Poor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube</td>
<td>siliconized glass</td>
<td>plastic, after validation</td>
<td>poorly siliconized glass</td>
</tr>
<tr>
<td></td>
<td>4.5-ml evacuated tubes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anticoagulant</td>
<td>buffered trisodium citrate 0.105–0.109 M or CTAD</td>
<td>buffered trisodium citrate 0.129 M</td>
<td>any others</td>
</tr>
<tr>
<td>pH of anticoagulated plasma</td>
<td>7.1–7.35</td>
<td>&gt;7.35</td>
<td>&lt;7.1</td>
</tr>
<tr>
<td>Filling</td>
<td>100%</td>
<td>90%</td>
<td>&lt;90%</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.30–0.55 l/l</td>
<td>must be corrected if</td>
<td>&lt;0.30 or &gt;0.55 l/l and uncorrected</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;0.30 or &gt;0.55 l/l</td>
<td></td>
</tr>
<tr>
<td>Needle diameter</td>
<td>0.7–1 mm (19–22 gauge)</td>
<td>&lt;0.7 or &gt;1 mm for an adult</td>
<td></td>
</tr>
<tr>
<td>Catheter blood sampling</td>
<td>to avoid</td>
<td>after rejecting 5–10 ml</td>
<td>using the first 5 ml</td>
</tr>
<tr>
<td>Tourniquet</td>
<td>&lt;1 min</td>
<td>&gt;1 min</td>
<td></td>
</tr>
<tr>
<td>Order of sampling</td>
<td>2nd tube</td>
<td>1st tube</td>
<td>after a tube containing heparin</td>
</tr>
<tr>
<td>Transport temperature</td>
<td>ambient temperature</td>
<td>&lt;4 or &gt;30°C</td>
<td></td>
</tr>
<tr>
<td>Time to analysis</td>
<td>&lt;2 h</td>
<td>4 h if previously centrifuged</td>
<td>&gt;4 h</td>
</tr>
<tr>
<td>Centrifugation</td>
<td>2 × 2,000 g, 15 min</td>
<td>1 × 2,000 g, 15 min refrigerated</td>
<td>&lt;1,000 g, &lt;10 min overheating during centrifugation</td>
</tr>
<tr>
<td>Freezing</td>
<td>snap freezing</td>
<td>slow</td>
<td></td>
</tr>
<tr>
<td>Preserving</td>
<td>–80°C</td>
<td>–20°C (&lt;30 days)</td>
<td>&gt;–20°C</td>
</tr>
<tr>
<td></td>
<td>–20°C &lt;8 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thawing</td>
<td>fast, water bath at 37°C</td>
<td>ambient temperature microwave</td>
<td></td>
</tr>
</tbody>
</table>

the fibrinogen level will be underestimated depending on time to analysis: the longer the delay, the lower the fibrinogen. Addition of aprotinin enables a better evaluation of fibrinogen but modifies the APTT. Because of its protein nature, aprotinin does not store well in a liquid medium. Therefore, the necessity of an extemporaneous preparation hinders the use of citrate tubes containing fibrinolysis inhibitor.

Medical Devices for Venous Sampling

Needle

The needle must be adapted to the vein diameter. A needle with a diameter greater than 1 mm (19 gauge) may be traumatic for the vein, leading to the release of vascular fragments which, in the tube, could modify hemostasis [3, 32]. Needles with a diameter
under 0.7 mm (22 gauge) prolong blood collection and the pressure gradient in the needle which could lead to hemolysis and platelet activation.

Collection sets (‘butterfly’) are routinely used, especially in patients with difficult venous access. They consist of a small-diameter winged needle prolonged by plastic tubing. This system reduces the blood flow and consequently increases the risk of platelet activation. In addition, the void volume, which could reach 500–700 μl, modifies the anticoagulant/blood ratio, especially when low volumes are sampled, as for example in children. Therefore, collection sets with void volume <10% of the draw volume must be used (i.e. <500 μl for a 4.5-ml collection tube).

**Catheter**
Although not recommended, it is sometimes unavoidable to draw blood from a catheter. Published studies agree that there is a good correlation between blood from a catheter and blood obtained by direct venous puncture for the PT test and fibrinogen level [33, 34]. Conversely, results of APTT and thrombin time tests are discordant [35], especially because of the presence of heparin in the catheter. It is therefore recommended to rinse the catheter, and several techniques have been proposed: to discard the first 5 ml [36, 37], or the catheter volume plus 2 ml [25, 37], or to discard five times the void volume [38]. For some authors not even this is enough [33] and recommendations to discard volumes as high as 10 or 20 ml of blood have been found [3, 39].

**Venous Blood Collection**
The first step is the identification of the patient and the labeling of the tubes. In addition, clinical and therapeutic data and patient characteristics should be mentioned on the test request form.

**Posture**
It is well known that body posture influences the concentration of blood constituents [40]. For example, changing from the supine to the upright position reduces the plasma volume by about 12% [41]. Therefore, blood is preferably taken with the patient in the supine position. A rest of up to 30 min has been recommended for fibrinolysis tests [42].

**Vein Selection**
The large veins of the elbow (ulnar, cephalic) are to be preferred. A small diameter vein can collapse, which may cause blood coagulation and/or underfilling of the tube. The choice of the arm is also important. Therefore, blood should preferably be taken from the arm without infusion or arteriovenous fistula in hemodialyzed patients. Contamination of laboratory samples by infusion solutions (especially those containing heparin) is the most common preanalytical error in hospital [41].

**Tourniquet**
The only use of the tourniquet is to help locating the vein. If tourniquet application lasts >1 min, the levels of factor VIII, von Willebrand factor and t-PA are increased, and fibrinolysis is activated. In addition, there is a time-dependent hemoconcentration reaching its plateau after 5–6 min, which increases the level of all blood proteins [41]. Therefore, the tourniquet must not be too tight and must be kept in place less that 1 min; as soon as blood begins to flow in the tube, it must be removed.

**Procedure**
Diurnal variations and food intake can modify some parameters. Therefore, blood should be collected between 7 and 9 a.m., 12 h
after the last meal, and from an unstressed recumbent patient [41], except if time-dependent drug effects are to be monitored (e.g. heparin). The venipuncture must be as atraumatic as possible. The necessity of well-trained and qualified phlebotomists has been recently highlighted. Prothrombin fragments 1 + 2 in plasma collected by an untrained technologist was increased by an average of 21% compared to the corresponding reference specimen. For thrombin-antithrombin complexes, the increase was 277% [43].

**Order of Blood Collection**

The order of blood collection of the various collection tubes is still the subject of controversy. The NCCLS recommend either the removal of the first 5 ml or their use for another purpose than hemostasis testing [5]. Indeed the 1st ml of blood can be contaminated by tissue factor. McPhedran et al. [44] showed that APTT was more sensitive than PT. More recently, Yawn et al. [45] suggested that PT could be performed on the first tube, and finally the main point is the qualification of the operator, as proposed by Gottfried and Adachi [46].

However, the characteristics of the first tube are of paramount importance, and blood sampling for hemostasis should be avoided after sampling in either a tube containing an activator or a tube containing any other anticoagulant such as heparin or EDTA.

Practically, the tube determined for hemostasis assays may be the first one if blood is drawn under strict conditions: a 4.5-ml hemostasis tube drawn by a well-trained operator. In any other conditions, we recommend to draw the sample for hemostasis secondly, but never following any other anticoagulant or a tube containing an activator. These recommendations should be reinforced when blood is collected from a catheter or with a blood collection set.

The tube must be correctly filled. For an optimal homogenization of blood and anticoagulant, the tube must be gently mixed by 5–6 inversions but never shaken as documented by Iversen [43].

**Sample Transport**

Before sending the blood sample to the hemostasis laboratory, it must be kept at room temperature to avoid the cold activation of factor VII and platelet disruption, especially in the monitoring of anticoagulants. When several hemostasis tubes for the same patient are sent to the laboratory, it is necessary to indicate the order of sampling. Furthermore, the identification of the operator must also be clearly noted in order to be able to trace any nonconformity.

**Time to Analysis**

Since the delay between sampling and centrifugation is of prime importance, the precise time of blood sampling must be indicated on the label. Whether the delay before centrifugation is acceptable depends on the type of the coagulation test. For the PT test, a delay of up to 4 h is acceptable. For monitoring heparin therapy, because of the variable heparin neutralization by platelet factor IV, the delay before centrifugation should not exceed 1 h for a sample collected in citrate and 4 h in CTAD. This applies also to fibrinolysis parameters.

**Transport Conditions**

Tube handling must be smooth. If not protected, in a pneumatic system, protein denaturation through foaming and platelet activation may occur.
Concluding Remarks

Blood sampling is the first step for routine and specialized hemostasis evaluation. The quality of the results depends entirely upon this easy and often neglected crucial stage. Simple precautions, special training of operators and the choice of evacuated blood collection tubes and the medical devices for blood sampling will facilitate to obtain the best analytical quality.

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