Collection of Blood Specimens by Venipuncture for Plasma-Based Coagulation Assays

Necessity of a Discard Tube

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Key Words: Preanalytics; Discard tube; Activated partial thromboplastin time; APTT; Prothrombin time; PT; Specialized coagulation assay

Abstract

The Clinical and Laboratory Standards Institute (CLSI) recently abandoned its recommendation for drawing a discard tube when performing a prothrombin time (PT)/international normalized ratio (INR) or an activated partial thromboplastin time (APTT). Because there is currently no evidence that a discard tube is necessary for more specialized coagulation assays, we studied the need for a discard tube for some of these tests. Blood was obtained from 88 subjects in 2 subsequent citrate tubes. Platelet-free plasma was tested for PT, APTT, antithrombin, protein C, and factors II, V, VIII, IX, and X. Difference and bias between tubes were tested using the Wilcoxon signed rank test and Bland-Altman plots. For only APTT, antithrombin, and protein C was a small, statistically significant mean bias found (0.5 seconds; \( P = 0.001 \); \(-0.7\%; \ P = 0.002; \) and \(-0.8\%; \ P < 0.0001, \) respectively), but the bias of individual samples was not clinically relevant. This was also true for the other parameters tested. The recent CLSI recommendation that a discard tube is not necessary for PT/INR and APTT can be extended to include more specialized plasma-based coagulation assays as identified in this study.

Preanalytic variables may affect test results. In particular, coagulation assays are sensitive to variation in conditions during the collection, transport, and processing of blood specimens. During the last few years, there has been much debate whether a discard tube should be drawn for plasma-based coagulation assays when using a standard evacuated tube collection system. The rationale for a discard tube is based on historic coagulation testing using the whole blood clotting time, in which tissue thromboplastin released and activated during venipuncture could lead to erroneous results. A few studies have already shown that with today’s coagulation reagents and venipuncture procedures, using extremely sharp low-resistance needles, a discard tube is not necessary for prothrombin time (PT) and activated partial thromboplastin time (APTT).1-4 Because of these studies, the Clinical and Laboratory Standards Institute (CLSI), formerly known as National Committee for Clinical Laboratory Standards, has abandoned the recommendation for a discard tube for PT/INR and APTT testing in its latest guideline, H21-A5.5 Because there is no proof of the necessity that a discard tube should be drawn for other, more specialized coagulation assays, the CLSI was not able to report a recommendation for these assays. Therefore, the aim of this study was to determine whether drawing a discard tube for several plasma-based specialized coagulation assays is required.

Materials and Methods

Patient Recruitment and Methods

From October until December 2007, 88 consecutive patients, for whom coagulation tests were requested, were asked to participate in the study. By Dutch law, ethical approval is not needed for studies in which samples are anonymously...
used for quality enhancement or evaluation protocols of clinical chemistry assays unless the patient refuses that his or her sample be used for this purpose. To be sure that each patient had no objection, we obtained written informed consent. To be able to investigate the whole concentration range of coagulation factors and their inhibitors, subjects were recruited from several subpopulations, including 30 random control subjects (for concentrations in the normal range), 30 patients receiving coumarin therapy, 13 patients with hemophilia A or B, and 15 subjects suspected of having a bleeding disorder.

From each subject, blood was consecutively drawn into 2 labeled 5-mL plastic evacuated tubes (tube 1 and tube 2) containing 0.109 mol/L trisodium citrate (BD Vacutainer, Becton Dickinson, Plymouth, England) by venipuncture. Each tube was filled with 4.5 mL of blood and 0.5 mL of buffered citrate solution (ie, 9:1 blood/buffer ratio). If other blood tests were ordered, tubes for these were drawn after the duplicate citrated tubes for the study. The sampling procedure was performed according to the guidelines of the European Concerted Action on Thrombosis and Disabilities.6 In short, blood was drawn using a 21-gauge needle with minimal tourniquet application to prevent stasis and was immediately mixed by gently inverting the tube 5 times. The sample was centrifuged within 30 minutes at 2,500g and 20°C for 15 minutes without brake. Plasma was carefully removed and transferred to a nonactivating centrifuge tube using a plastic pipette and was centrifuged again using the same conditions. Platelet-free plasma was divided into aliquots and stored at −80°C until analysis. Just before analysis, frozen samples were rapidly thawed at 37°C for 5 minutes. For each parameter, tube 1 and tube 2 were analyzed consecutively within the same run.

Parameters were tested using the Advance Coagulation analyzer (Instrumentation Laboratory, Breda, the Netherlands), according to the instructions of the manufacturer. Reagents and factor-deficient plasma used for each parameter are listed in Table 1 and were obtained from Instrumentation Laboratory. In short; APTT and PT were measured using optical clot detection, individual coagulation factors using a 1-stage PT assay (factors II, V, and X) or a 1-stage APTT assay (factors VIII and IX), antithrombin using a factor Xa–based assay, and protein C using a chromogenic substrate–based assay in which its amidolytic activity is assessed after a snake venom activation.

Statistical Analysis

Data were evaluated statistically using Microsoft Excel 2003 software (Microsoft, Redmond, WA) and Analyse-It, version 1.72 (Analyse-It Software, Leeds, England). Because data were not normally distributed, the differences between tubes 1 and 2 were tested using the Wilcoxon signed rank test for paired samples. To correct for multiple testing, a Bonferroni correction was used, and a P value less than .006 was considered significant.9 The agreement between the sampling methods was investigated by using Passing-Bablok method comparison, and individual bias with 95% limits of agreement between paired samples was assessed by using Bland-Altman analysis. If a statistically significant difference was found, the clinical significance of this finding was evaluated by using the maximal difference (10%) between individual samples as reported by the CLSI and the critical difference (dK), a mathematical approach that shows the 95% range of all differences between 2 subsequently obtained values in a healthy person and is defined as $2\sqrt{2} \ast \sqrt{(CV_p^2 + CV_A^2)}$, with CVp the intraindividual coefficient of variation and CVA the analytic coefficient of variation. For each coagulation parameter, the dK, CVp, and CVA as assessed in our laboratory are summarized in Table 1.

Results

The results for the coagulation assays investigated are summarized in Figure 1 and Table 2. Of the routine

### Table 1
Reagents Used, Intra-assay CVs, and Critical Difference for Each Assay

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Reagent</th>
<th>Intrasubject CV (%)</th>
<th>Intra-assay CV (%)</th>
<th>Critical Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APTT (seconds)</td>
<td>HemosIL APTT-SP</td>
<td>3.3</td>
<td>1.0</td>
<td>9.8</td>
</tr>
<tr>
<td>PT (seconds)</td>
<td>HemosIL PT RecombiPlasTin</td>
<td>2.4</td>
<td>0.9</td>
<td>7.2</td>
</tr>
<tr>
<td>Protein C (%)</td>
<td>Coamatic Protein C Kit</td>
<td>7.9</td>
<td>3.3</td>
<td>24.2</td>
</tr>
<tr>
<td>Factor II (%)</td>
<td>Factor II–deficient plasma</td>
<td>4.4</td>
<td>7.8</td>
<td>25.3</td>
</tr>
<tr>
<td>Factor V (%)</td>
<td>Factor V–deficient plasma</td>
<td>3.9</td>
<td>4.6</td>
<td>17.1</td>
</tr>
<tr>
<td>Factor VIII (%)</td>
<td>Factor VIII–deficient plasma</td>
<td>4.4</td>
<td>5.9</td>
<td>20.8</td>
</tr>
<tr>
<td>Factor IX (%)</td>
<td>Factor IX–deficient plasma</td>
<td>4.4</td>
<td>7.4</td>
<td>24.4</td>
</tr>
<tr>
<td>Factor X (%)</td>
<td>Factor X–deficient plasma</td>
<td>4.8</td>
<td>4.7</td>
<td>19.0</td>
</tr>
</tbody>
</table>

APTT, activated partial thromboplastin time; CV, coefficient of variation; PT, prothrombin time.

* All reagents were purchased from Instrumentation Laboratory (Breda, the Netherlands).

† Intra-assay CVs for APTT, PT, antithrombin, and protein C derived from Wada et al7 and for factors V and X from Costongs et al.8 Intrasubject CVs for factors II, VIII, and IX are estimates based on the reported values for factors V and X (average).

2 Intra-assay CVs were determined by repeated measurement (n = 10) of a plasma pool at our laboratory.
Figure 1: Bland-Altman plots for the investigated parameters. Solid lines indicate zero bias; dotted lines, mean bias; hatched lines, 95% limits of agreement. APTT, activated partial thromboplastin time; PT, prothrombin time.

Table 2: Results for the Differences Between Plasma-Based Coagulation Assays Measured in the First and Second Tubes Drawn (n = 88)*

<table>
<thead>
<tr>
<th></th>
<th>Passing-Bablok Method Comparison</th>
<th>Bland-Altman Bias Plots</th>
<th>95% Limits of Agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(y = ax + b)</td>
<td>(y – x)</td>
<td>Lower (95% CI)</td>
</tr>
<tr>
<td>APTT (seconds)</td>
<td>1.04 (1.01 to 1.07)</td>
<td>–1.06 (–2.24 to 0.07)</td>
<td>–1.9 (–2.3 to –1.5)</td>
</tr>
<tr>
<td>PT (seconds)</td>
<td>1.00 (0.99 to 1.00)</td>
<td>0.03 (0.00 to 0.13)</td>
<td>0.0 (0.1 to 0.1)</td>
</tr>
<tr>
<td>Antithrombin (%)</td>
<td>1.00 (0.95 to 1.05)</td>
<td>–0.43 (–5.68 to 4.59)</td>
<td>–0.7 (–1.2 to –0.2)</td>
</tr>
<tr>
<td>Protein C (%)</td>
<td>0.99 (0.98 to 1.00)</td>
<td>0.21 (–0.28 to 0.89)</td>
<td>–0.8 (–1.2 to –0.4)</td>
</tr>
<tr>
<td>Factor II (%)</td>
<td>1.03 (1.00 to 1.07)</td>
<td>–3.02 (–7.63 to 1.60)</td>
<td>–0.2 (–1.1 to 0.8)</td>
</tr>
<tr>
<td>Factor V (%)</td>
<td>1.00 (0.98 to 1.01)</td>
<td>–1.00 (–1.59 to 0.41)</td>
<td>–0.6 (–1.5 to 0.2)</td>
</tr>
<tr>
<td>Factor VIII (%)</td>
<td>1.00 (0.97 to 1.01)</td>
<td>0.00 (0.00 to 1.14)</td>
<td>–0.4 (–1.1 to 0.4)</td>
</tr>
<tr>
<td>Factor X (%)</td>
<td>1.00 (0.99 to 1.02)</td>
<td>0.00 (–0.18 to 0.12)</td>
<td>0.1 (–0.8 to 1.0)</td>
</tr>
</tbody>
</table>

CI, confidence interval.

* Passing-Bablok method comparison was used to test for agreement between both methods of sampling; x = tube 2 (“gold standard” method), and y = tube 1 (method under investigation). Bland-Altman bias plots were generated to investigate bias for individual samples. Statistical differences in values between sampling methods (tube 2 vs tube 1) were investigated using the Wilcoxon signed rank test for paired samples.
coagulation assays tested, only the APTT showed a significant
difference in clotting time between sampling methods (tube
2 vs tube 1) with a mean prolongation of the APTT of 0.5
seconds ($P = .001$) in tube 1. However, the individual bias of
paired samples (range, –6.5% to 8.8%) remained lower than
the critical difference. For the PT, no statistically significant
difference was found between the samples, and individual
bias (range, –3.7% to 4.7%) was lower than the calculated
critical difference.

For the specialized coagulation assays, differences
between the sampling methods were found for antithrombin
and protein C. For antithrombin, the mean concentration in
tube 1 was 0.7 U/dL lower than in tube 2 ($P = .002$). Passing-
Bablok analyses showed good agreement between sampling
methods with a slope of the regression equation of 1. The
individual bias of the paired samples (range, –6.2% to 6.3%)
remained within the critical difference. For protein C, the
mean concentration in tube 1 was 0.8 U/dL lower than in
tube 2 ($P < .0001$), and the Passing-Bablok regression
equation showed a proportional bias in the slope. However, the
individual bias of the paired samples (range, –8.2% to 3.3%)
remained within the critical difference.

All other specialized coagulation assays tested showed
no statistically significant differences in measured values
between sampling methods, and bias of the paired samples
was lower than the critical difference. Ranges for the relative
differences between tubes 1 and 2 were as follows: factor
II, –12.1% to 8%; factor V, –10.0% to 11.1%; factor VIII,
–15.4% to 9.0%; factor IX, –9.8% to 10.7%; and factor X,
–11.0% to 11.8%.

**Discussion**

In this study, we have shown small, statistically signifi-
cant but not clinically relevant differences between the discard
tube and the second tube for 3 assays. These data imply that
drawing a discard tube is not necessary for routine and certain
specialized coagulation assays. Although we have not tested
this for all coagulation factors and inhibitors, we have no rea-
tion to expect that this conclusion does not apply to coagula-
tion factors VII, XI, and XII and protein S, especially because
there is no proof that says otherwise.

The findings for the routine coagulation parameters (PT
and APTT) are in line with those previously reported,\textsuperscript{1,4-10}
with a mean and maximum difference for the PT measure-
ment between tubes that are comparable to those found by
Yawn et al\textsuperscript{1} (mean, 0.05 second; maximum, 3.8 seconds) and
Gottfried and Adachi\textsuperscript{5} (mean, 0.1 second; maximum, 3.8 sec-
onds). In the latter study, the APTT also was measured, and
regression analysis was performed. For the APTT, the mean
difference (0.48 second) was equal to that found in our study.

The maximal difference (32.2 seconds), however, was worse
compared with that in our study. The equations of the regres-
sion lines were also comparable.\textsuperscript{3}

A limitation of the study is that critical differences were
calculated using intra-assay CVs determined by our labora-
tory and intersubject CVs derived from the literature. This
may have resulted in critical differences that are not com-
pletely valid for our population. Most important, nearly all
individual relative differences remained below the maximum
of 10% as recommended by the CLSI. For all samples with
results above this maximal difference, the clinical relevance
was investigated, and it was found that none were clinically
relevant.\textsuperscript{[Table 3]} For the determination of the range of rela-
tive differences, 1 sample for factor II and 1 for factor VIII
were excluded (43.5% and 19.0% differences, respectively).
In all other statistical tests, however, these samples (sample
IDs 59 and 82) have been included. In box-whisker plots
(data not shown), these samples are identified as far outli-
ers, but the absolute differences are not clinically relevant.
Inclusion of these samples would have led to a range of
relative differences that, in our opinion, does not give a good
representation of reality.

The previous CLSI guideline advocating a discard tube
was based on the hypothesis and circumstantial proof at
best that the tissue thromboplastin released at venipuncture
will contaminate the first tube drawn, thereby activating
the clotting cascade.\textsuperscript{1} Indeed, we found a small decrease
of antithrombin and protein C concentrations and a small

**Table 3**

<table>
<thead>
<tr>
<th>Parameter/ Sample ID</th>
<th>Difference (%)</th>
<th>Value (U/dL)</th>
<th>Clinically Relevant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tube 1</td>
<td>Tube 2</td>
</tr>
<tr>
<td>Factor II 59</td>
<td>43.5\textsuperscript{1}</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>1</td>
<td>–12.1</td>
<td>101</td>
<td>114</td>
</tr>
<tr>
<td>Factor V 80</td>
<td>–11.1</td>
<td>94</td>
<td>105</td>
</tr>
<tr>
<td>Factor VIII 82</td>
<td>19.0\textsuperscript{1}</td>
<td>103</td>
<td>85</td>
</tr>
<tr>
<td>89</td>
<td>–15.4</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>41</td>
<td>–11.8</td>
<td>40</td>
<td>45</td>
</tr>
<tr>
<td>Factor IX 34</td>
<td>10.7</td>
<td>79</td>
<td>71</td>
</tr>
<tr>
<td>Factor X 5</td>
<td>–11.0</td>
<td>86</td>
<td>96</td>
</tr>
<tr>
<td>78</td>
<td>–10.9</td>
<td>104</td>
<td>116</td>
</tr>
<tr>
<td>2</td>
<td>–10.7</td>
<td>97</td>
<td>108</td>
</tr>
<tr>
<td>25</td>
<td>–10.1</td>
<td>122</td>
<td>135</td>
</tr>
<tr>
<td>52</td>
<td>11.8</td>
<td>9</td>
<td>8</td>
</tr>
</tbody>
</table>

\textsuperscript{1} The difference is calculated as percentage to the mean of the duplicate.
\textsuperscript{1} These samples were identified as far outliers and all other samples as near outliers in box-whisker plots (not shown).
prolongation of the APTT in tube 1 as compared with tube 2. Although statistically significant, this difference was not clinically relevant. The decreases that were found for the individual clotting factors, with the largest decrease found for factor VIII, were too small to be statistically or clinically relevant. Moreover, the individual bias between samples was generally smaller than the critical difference for each assay calculated using the in-house laboratory variance (Table 1). However, according to CLSI guidelines, a 10% difference between samples is acceptable and differences of more than 10% are said to be of clinical relevance. Most samples fulfilled these criteria, but a few individual samples did not, and these samples were evaluated in more detail (Table 3). It was noticed that based on the differences between reported values for tubes 1 and 2, clinical diagnosis would not have been affected. Especially at low levels, small absolute differences may result in a large bias when this is expressed as a percentage to the mean of both values. For example, the difference of 43.5% between one sample of factor II results from an absolute bias of 5 U/dL at a level of around 10 U/dL (14 vs 9 U/dL). Values of either tube 1 or tube 2 would have led to the same conclusion: the patient has factor II deficiency.

The findings of our study support the new CLSI guideline that a discard tube is unnecessary for routine coagulation assays and provide evidence that drawing a discard tube can also be abandoned for certain plasma-based specialized coagulation tests.

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References