Validation protocol of analytical hemostasis systems: measurement of anti-Xa activity of low-molecular-weight heparins

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A standard validation protocol adapted to the chromogenic assay of anti-Xa activity of low-molecular-weight heparins was used in a multicenter study to assess its suitability for comparing and evaluating analytical hemostasis systems. The protocol included: familiarization with the system (repeatability); assessment of limits of linearity, detection limits, and cross-contamination; and validation (reproducibility and accuracy of measurements of treated patients' plasmas). We calibrated the systems with the same range of lyophilized plasmas daily and evaluated repeatability and reproducibility by using a single batch of lyophilized plasmas at three anti-Xa activities. The two automated systems tested [SB 300 (Gilford) and ACL (IL)] and the two semi-automated systems [ST 888 (D. Stago) and Chromotimer (Behring)] gave similar mean values. Dispersion of results was lower with the automated systems than with the semi-automated ones, especially at low anti-Xa activities, a tendency that also was observed for reproducibility. Because each analytical system gave linear results for activities as great as 1000 IU/L, suitable sample dilution is advisable for higher anti-Xa activities. Accuracy was greater in the automated systems. We conclude that this protocol is feasible and is applicable to validation of other analytical hemostasis instruments, in particular the latest generation of fully automated instruments.

INDEXING TERMS: anti-factor Xa • hemostasis • standardization

The Société Française de Biologie Clinique (SFBC) has published several protocols for validation of biochemical analytical systems and methods [1].10 An American conference "on a national understanding for the development of reference materials and methods for clinical chemistry" [2] also yielded recommendations. However, these protocols deal mainly with evaluation of biochemical instruments [1]. The few protocols described for coagulation testing have dealt only with clotting assays [3, 4], prompting us to adapt the general SFBC protocol [1] to specific needs in hemostasis. The increasing availability of automated coagulation instruments means that validation of such protocols is now a real possibility. The enzyme kinetics of coagulation is similar to that observed in biochemical assays. However, in coagulation testing, preanalytical variables, such as sample preparation (i.e., avoidance of platelet protein release), may affect protocol instructions.

Low-molecular-weight heparins (LMWH) are increasingly common in clinical practice. They barely affect the activated partial thromboplastin time (APTT) because their anti-IIa activity is much less than their anti-Xa activity. Unlike unfractionated heparin (UFH), which can be monitored by APTT and (or) heparin activity [anti-IIa and (or) anti-Xa], LMWH therapy can be monitored only by measuring anti-Xa activity [5]. Laboratory automation has been applied to this measurement to

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10 Nonstandard abbreviations: SFBC, Société Française de Biologie Clinique; LMWH, low-molecular-weight heparins; APTT, activated partial thromboplastin time; UFH, unfractionated heparin; Xa, factor Xa; AT III, antithrombin III, and IS, International Standard
We therefore thought it of interest to adapt the SFBC protocol to evaluation of analytical systems that measure anti-Xa activity by chromogenic assays with synthetic substrates or by clotting assays, which are less specific [7–10]. The SFBC recommended a method for measuring the anti-Xa activity of UFH [11]. This method was validated with LMWH [12]. The chromogenic method is based on inhibition of factor Xa by the LMWH-antithrombin III (AT III) complex, with subsequent colorimetric measurement of residual factor Xa [13, 14].

In this study, our aim was to define a simple protocol suitable for systems analyzing hemostasis and to validate it in a multicenter study, using anti-Xa activity of LMWH measurement as an example.

**Materials and Methods**

**Protocol steps.** As recommended in the SFBC protocol [1], the evaluation consisted of three steps. First, a period of familiarization over 3 consecutive days allowed technologists to become acquainted with instrument handling and setup (e.g., room temperature, power supply, humidity) and to determine within-run precision. Second, in a preliminary 2-day period, the analytical range (linearity and detection limits) and the effect or carryover between samples or reagents [15, 16] were studied. During these first two periods, day-to-day precision (reproducibility and accuracy) was assessed in controls to detect any anomaly before more comprehensive studies. Step 3 was the validation period (at least 3 weeks), in which we assessed the accuracy of the assays on patients' plasmas, comparing the results obtained with those of a validated method [12–14]. Precision (reproducibility of the calibration curves, day-to-day precision) was also evaluated during this period. Two expert laboratories performed the validated (“reference”) method to determine that the required criteria had been met.

We then analyzed data as recommended in the general SFBC protocol. The limits of acceptability of statistical criteria had already been set according to our experience of routine LMWH monitoring.

**Analytical systems and reagents.** An analytical system comprises the instrument, the appropriate reagents, and the method. The kinetic analyzers evaluated in the described protocol were those routinely used in the participating laboratories (Table 1): two automated instruments, SB 300 (Gilford Instruments, Oberlin, OH) and ACL 300 (Instrumentation Laboratory, Milan, Italy) [17]; and two semiautomated instruments, ST 888 (D. Stago, Gennevilliers, France) [18] and Chromotimer (Behring Institute, Rueil-Malmaison, France) [19]. The “reference” method was performed with the SB 300, which had been used in a previous French collaborative study [12], and with the ST 888. Each manufacturer was informed of the study and ensured that the instrument was operating well.

Behring Institute (Behringwerke) provided Berichrom® heparin, and D. Stago gave Stachrom® heparin to two centers (Table 1). These reagents were those routinely used in each center.

**Calibrations.** The first International Standard (IS) for LMWH (National Bureau of Standards and Controls, NIBSC 85/602 [20], 1680 IU of anti-Xa per milligram, was a generous gift from T.W. Barrowcliffe (NIBSC, Potters Bar, UK). Aliquots of the IS were dispatched to all four centers.

**Plasma samples.** We used two types of plasma samples, lyophilized (same batch used throughout the study) and frozen. For precision studies, the lyophilized samples were Hepanorm LMWH (batch 921 031; D. Stago) at two anti-Xa activity concentrations, medium (0.38 kIU/L) and high (0.78 kIU/L); and a low anti-Xa activity plasma (0.23 kIU/L) especially processed by Institut Serbio (Gennevilliers, France).

Caliplasma LMWH (batch 606 302 A; bioMérieux, Marcy L'Etoile, France) was used to construct the calibration curves, containing anti-Xa at 0.05, 0.30, 0.90, and 1.30 kIU/L. An intermediate 0.60 kIU/L concentration of anti-Xa was prepared on-site by mixing the 0.30 and the 0.90 kIU/L plasmas in equal volumes. To obtain two additional samples, at 0.20 and 3.00 kIU/L, we added appropriate amounts of the first IS of LMWH to a “zero” plasma [a normal plasma without heparin (Heparin calibrator, batch IB 205; D. Stago)].

To validate the analytical systems, we used them to assay frozen plasma samples from blood collected from 100 patients receiving LMWH in Fragmin® (AB Kabi, Sweden), Fraxiparin® (Sanofi, France), or Lovenox® (Rhone-Poulenc, France). Blood was collected in CATD solution [21]—citric acid, theophylline, adenosine, and dipryridamole (109 mmol/L citrate)—1 volume of anticoagulant per 9 volumes of blood. After two centrifugations (5000g, 4 °C), the collecting laboratory essayed each platelet-free plasma sample to define the reference values and stored the remainder in 500-µL aliquots, frozen at −80 °C. The samples were grouped according to anti-Xa activity: (a) 0.20–0.40 kIU/L, (b) 0.41–0.60 kIU/L, (c) 0.61–0.80 kIU/L, and (d) 0.8–1.0 kIU/L. Each center received two aliquots of each sample for the accuracy study.
Table 2. Within-assay precision: anti-Xa activity measurement (kIU/L) of LMWH on lyophilized samples.

<table>
<thead>
<tr>
<th>Target activity of</th>
<th>Automated instruments</th>
<th></th>
<th>Semi-automated instruments</th>
<th></th>
<th></th>
<th>Upper limit of SD,* kIU/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin HBPM, kIU/L</td>
<td>No. of assays</td>
<td>n</td>
<td>Mean</td>
<td>SD</td>
<td>CV,%</td>
<td>Mean</td>
</tr>
<tr>
<td>Low (0.23)</td>
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<td>4.8</td>
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<tr>
<td></td>
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<td>0.009</td>
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</tr>
<tr>
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<td>3</td>
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<td>7.5</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.22</td>
<td>0.014</td>
<td>6.4</td>
<td>0.21</td>
<td>0.020</td>
</tr>
<tr>
<td>Medium (0.38)</td>
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<td>20</td>
<td>0.35</td>
<td>0.015</td>
<td>4.4</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>2</td>
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<td>0.006</td>
<td>1.8</td>
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<tr>
<td></td>
<td>3</td>
<td>0.35</td>
<td>0.020</td>
<td>5.9</td>
<td>0.38</td>
<td>0.015</td>
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<tr>
<td></td>
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<td>0.35</td>
<td>0.015</td>
<td>4.5</td>
<td>0.37</td>
<td>0.022</td>
</tr>
<tr>
<td>High (0.78)</td>
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<td>0.016</td>
<td>2.1</td>
<td>0.78</td>
<td>0.017</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.73</td>
<td>0.033</td>
<td>4.6</td>
<td>0.79</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.76</td>
<td>0.014</td>
<td>1.9</td>
<td>0.82</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.75</td>
<td>0.028</td>
<td>3.8</td>
<td>0.79</td>
<td>0.024</td>
</tr>
</tbody>
</table>

*Upper limit of SD fixed in the protocol.

**Assay procedure.** Following the recommended method for UFH [11] and LMWH [12, 14], we incubated the test sample (25 μL) with factor Xa (250 μL) for 30 s at 37 °C. To assess residual factor Xa activity, we added its chromogenic substrate, CBS 3139 (50 μL), and measured the absorbance at 405 nm every 10 s for 30 s at 37 °C. No exogenous AT III was added. We adapted the method for use with the SB 300 and the semi-automated ST 888 instrument.

**Evaluation methods.** Within-run precision was determined over 3 consecutive days by replicating (n = 20) the anti-Xa assay of the three lyophilized controls. We assessed the reproducibility of the calibration curves at least 18 times with the lyophilized Caliplasma HBPM plasmas for each series of anti-Xa determination. We assayed the control samples at the beginning and end of each series (18 days) to assess day-to-day precision of the systems. We subjected each of the three lyophilized within-run precision control plasmas to 36 anti-Xa assays. The upper limits of the SD had already been fixed at 0.02, 0.04, and 0.06 kIU/L for the three activity concentrations of 0.2, 0.4, and 0.8 kIU/L, respectively (corresponding to CVs of 10%, 10%, and 7.5%), according to clinical interpretation.

We assessed linearity by adding the IS for LMWH to the "zero" plasma to yield anti-Xa activity of 1.30 kIU/L. This was further diluted in the zero plasma to obtain additional activity concentrations, according to the protocol instructions. We assayed each of the 14 diluted samples in triplicate during the 2 days of the preliminary period.

We determined the limit of detection (represented by 3 SD from the mean absorbance) with 20 replications of the anti-Xa assays of the zero plasma.

To assess carryover, we analyzed on 2 consecutive days the samples prepared at low (L) and high (H) concentrations, according to the sequence H, H, L, L, H, H, L, L, H, H, L, L, H, H, L, L, H, H, L, L, H, H, L, L, H, H, L, L, H, H, L, H, H, L, L. We then calculated any differences between the absorbances of samples L (possibly contaminated) and L,

We tested analytical accuracy by comparing the systems’ results over 4 days for treated patients’ samples. Each participating laboratory assayed each plasma twice; in case of discrepancy between duplicate data or with reference data, the laboratory concerned received another aliquot. According to the criteria for reproducibility, a discrepancy was defined as a difference >20% of the lower duplicate value or a difference >20% of the reference value.

**Statistical analysis.** We assessed the precision of the mean, SD, and CV with statistical Statview software and compared the mean absorbances of L and L samples from the carryover study by Student’s t-test. If the differences were significant, we calculated the contaminant effect ic with the formula: ic % = (L1 - L2)/(H1 - H2) × 100. We determined linearity by plotting the measured activities against the theoretical activities. For each analytical system, we also used another representation, expressing the ratio (measured value/theoretical value) × 100 as a function of the theoretical activities for each heparin concentration. Relationships between data sets were evaluated by linear regression analysis (Deming). The differences between the tested (y) and comparison (x) methods were also plotted against the comparison methods’ values [22]. Limits of acceptance were ±15% of the comparison method value, according to clinical interpretation. Correlation between systems was also assessed by one-way ANOVA with repeated measures.

**Results**

**Precision.** For within-run precision, anti-Xa assays for the three heparin concentrations (Table 2) showed dispersions of 2–10% for the automated instruments A and B, 4–15% for system C, and 12–27% for semi-automated system D. Dispersion was greater at low concentrations. Mean activity was similar in three systems and slightly higher in system D. Day-to-day imprecision (Table 3) was generally greater with semi-automated systems (CVs 5–23% vs 3–17% for the automated systems), although similar results were obtained with automated system A and
semiautomated system C. Means were similar to those obtained in the precision study.

The reproducibility study with calibration curves (Table 4) revealed similar dispersions in absorbances for systems A, B, and C.

**Analytical range.** As Fig. 1 shows, for all four systems, the regression line deviates slightly from the line of identity for anti-Xa values exceeding \( \sim 1.00 \text{ kIU/L} \). This is more obvious when the percentage deviation from theoretical values is represented for each heparin concentration. Greater dispersion was noted with the semiautomated instruments, regardless of the amount of anti-Xa activity. In contrast, automated instruments showed only variations at low and high concentrations of heparin. The detection threshold for anti-Xa was \(<0.05 \text{ kIU/L} \) for all four systems.

**Carryover.** We found no contaminant carryover effect, as shown by the lack of significant difference by Student's \( t \)-test between the absorbances of the potentially contaminated and the non-contaminated samples (see Materials and Methods).

**Table 3. Between-assay precision: anti-Xa activity (kIU/L) measurement of LMWH on lyophilized samples.**

<table>
<thead>
<tr>
<th>Target activity of Hepanorm HBPM, kIU/L</th>
<th>Automated Instruments</th>
<th>Semiautomated Instruments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gifford (A)</td>
<td>ACL (B)</td>
</tr>
<tr>
<td></td>
<td>Mean SD CV, %</td>
<td>Mean SD CV, %</td>
</tr>
<tr>
<td>Low (0.23)</td>
<td>B 18 0.25 0.042 16.7</td>
<td>0.22 0.023 10.5</td>
</tr>
<tr>
<td></td>
<td>E 18 0.21 0.021 10</td>
<td>0.20 0.026 13.0</td>
</tr>
<tr>
<td></td>
<td>36 0.23 0.036 15.8</td>
<td>0.21 0.026 12.6</td>
</tr>
<tr>
<td>Medium (0.38)</td>
<td>B 18 0.35 0.027 7.3</td>
<td>0.36 0.023 6.5</td>
</tr>
<tr>
<td></td>
<td>E 18 0.36 0.034 9.5</td>
<td>0.36 0.024 6.7</td>
</tr>
<tr>
<td></td>
<td>36 0.36 0.031 8.7</td>
<td>0.36 0.024 6.6</td>
</tr>
<tr>
<td>High (0.78)</td>
<td>B 18 0.79 0.051 6.5</td>
<td>0.78 0.024 3.1</td>
</tr>
<tr>
<td></td>
<td>E 18 0.78 0.047 6.1</td>
<td>0.78 0.024 3.1</td>
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<td></td>
<td>36 0.78 0.047 6.1</td>
<td>0.78 0.024 3.1</td>
</tr>
</tbody>
</table>

* Upper limit of SD fixed in the protocol.

Measurements were carried out at the beginning (B) and at the end (E) of each series of assays.

**Accuracy.** We chose system A as the reference for the three other analytical systems (Fig. 2a, b, c) and system C as the reference for the semiautomated instruments only (Fig. 2d). The range of acceptable measurement was set as the interval between the limit of linearity and the limit of detection (1.00 and 0.05 kIU/L, respectively) and was used to evaluate statistically the results for 85 treated patients' plasmas.

The slopes of the regression lines were close to unity for the four systems \( (r = 0.936–0.967) \) (Fig. 2). Despite these acceptable data, we observed negative intercepts, mainly with the semiautomated instruments. Worthy of note were the lower and more scattered results given by the semiautomated systems, especially system D (Fig. 2). Means of the anti-Xa activities of the 85 frozen plasmas analyzed by the automated systems were similar \((0.552 \text{ and } 0.547 \text{ kIU/L, SD = 0.19 for both instruments})\), whereas those determined by the two semiautomated systems, mainly system D, were lower \((mean 0.511 \text{ and } 0.490 \text{ kIU/L, SD = 0.19 and 0.20, respectively})\). No statistical difference (by ANOVA) was observed in the results obtained with the two automated systems, whereas each of the semiautomated systems was significantly different \((P < 0.05)\) from the reference system A and from each other.

Similar statistical conclusions could be drawn for the lowest-activity samples \((<0.40 \text{ kIU/L})\). However, for medium activities \((0.41–0.80 \text{ kIU/L})\), results were statistically comparable between the two automated instruments and between the two semiautomated instruments. At high anti-Xa activity \((0.80–1.0 \text{ kIU/L})\), there was no significant difference in results among the four systems.

**Discussion.**

A simplified protocol, derived from the general SFBC protocol and specially defined to validate analytical systems of hemostasis, was validated by a multicenter study of chromogenic measurements of anti-Xa activity in LMWH, the variable chosen as an example. Although clinical trials have noted that LMWH monitoring is unnecessary \([23, 24]\), it still may be clinically useful in some clinical cases, i.e., renal insufficiency, obesity,
Fig. 1. Linearity study: Each panel corresponds to an analytical system being evaluated. Upper graph in each panel: regression line (dashed line = identity line). Lower graph in each panel: results expressed as percentage of theoretical values.
Fig. 2. Accuracy of analytical systems: Panels a, b, c, and d correspond to evaluating centers and analytical systems A, B, C, and D in Table 1. Upper graph in each panel: correlation $y = f(x)$ between two analytical systems, where $x$ is the reference system (dashed line = line of identity). Lower graph of each panel: plot of the difference ($y - x$) between the two instruments vs the result by the comparison analytical system. Outer lines represent the protocol-set limits for accuracy ($\pm 15\%$ of $x$).
bleeding diathesis, or risk of over- or underdosing [25, 26]. The Reference Method for this variable, as proposed by the French Hemostasis Committee [11], has been adapted to automated and semiautomated systems. One would expect the same reagents to be used with the four instruments evaluated, but the best performances should be obtained with the use of appropriate reagents for each instrument. This led us to compare the various part of the systems, including the instrument, the reagent, and the adaptation.

In this preliminary study we dealt with very few systems and centers, so that the feasibility, practicability, and simplicity of the proposed protocol could be evaluated. As stipulated in this protocol, the limits of acceptability of different statistical criteria must be clearly fixed beforehand. Interpretation of results depends on whether LMWH is used prophylactically (range of LMWH activities: 0.2–0.4 kIU/L) or therapeutically (range: 0.5–1.0 kIU/L). We therefore chose the limits of acceptability in light of the clinical context, rather than the laboratory criteria for spectrophotometric methods.

We used lyophilized plasmas at three anti-Xa activities to determine within- and between-assay precision. Regardless of the amount of heparin activity, the mean values recorded over the two first periods with each system were similar to the target values for each control sample.

Data dispersion with repeated measurements on the automated instruments were within the limits of acceptability of the protocol, which had been assigned according to the clinical interpretation [5, 27]. Greatest dispersions were noted at low anti-Xa activity, especially for semiautomated instruments. Similarly, the semiautomated instruments gave slightly higher mean values than did the automated systems, especially for samples with low anti-Xa activities. This observation is especially relevant for system D at any anti-Xa concentration and must be considered when using semiautomated instruments to monitor prophylactic treatment with LMWH [28].

Day-to-day precision was greater than within-run precision, whatever the anti-Xa activities. Only system B was in agreement with the limits assigned by the protocol, despite the predilution of sample required with this system. Even if poor precision is of little consequence for prophylaxis, performances observed with systems A, C, and D at high anti-Xa concentrations may have practical implications in monitoring therapy with LMWH. Furthermore, the large between-assay variations of calibration curves (see Table 4) with semiautomated instruments suggest the necessity of plotting a calibration curve before each series of assays. For the automated instruments, the calibration check is highly dependent on instrument performance.

Absence of carryover from sample to sample and a low detection threshold (<0.05 kIU/L) were appreciable characteristics of all four systems.

The upper limit of the analytical range was ~1.00 kIU/L (maximum). The scatter of values around the identity line was greater for the two semiautomated systems (Fig. 1, C and D) than for the automated ones. This upper limit of linearity is important to consider clinically for monitoring LMWH therapy, given that heparin concentrations ~1.50 kIU/L are not rare [5]. Laboratory technologists should be aware of this problem, because the highly heparinized patients' samples must be diluted in a nonheparinized plasma so that the result will lie in the linear part of the calibration curve and equilibrium of the heparin–AT III complex will be adequate. The current tendency of some manufacturers to provide calibration plasmas titrating to 1.30 kIU/L (e.g., Caliplasma), without a recommendation for sample dilution, is questionable.

Considering only the regression lines between the four systems, we found acceptable accuracy when comparing anti-Xa measurements for patients' frozen plasma samples. Careful analysis of the differential plots and ANOVA confirmed the similarity of data collected with the two automated instruments (Fig. 1A), although 15% of the values were outliers with respect to the protocol-defined limits. Semiautomated instruments, especially system D, gave systematically lower and more scattered results (27–37% were outliers) for all activities <0.60 kIU/L. Thus, the data scatter noted in the study of lyophilized samples was also seen with frozen samples.

With system D, anti-Xa activities measured in high-activity lyophilized samples were overestimated in comparison with the target value, whereas those from frozen samples were lower than with other systems. The fact that the lyophilized plasmas, which had a higher absorbance than the frozen plasmas tested, were used for calibration suggests a problem of interference from plasma turbidity in system D, as reported in spectrophotometric assays [29]. Thus, fresh, or at least deep-frozen, samples should be used whenever possible to evaluate protocols applied to coagulation. Lyophilized plasmas are also more likely to give more broadly dispersed values than non-deep-frozen ones [30]. The origin of the calibrating specimens and (or) the controls is therefore worth considering in any protocol [31].

This study tested analytical systems by assessing the instrument, the reagents, and the method adaptation. Theoretically, the protocol was evaluated under the best conditions (routinely used instrument and reagents, technician familiar with the instrument), which had been checked before the study. Compared with results of previous reports [12, 18, 19], the magnitude of dispersion found with the semiautomated instruments for anti-Xa measurement may be surprising. In fact, semiautomated instruments require the presence of a technician and rigorous respect of the method. The kinetics of enzymatic reactions in coagulation involving chromogenic substrates are sensitive to variations in such assay conditions as temperature (37 °C) and (short) incubation times, which are difficult to ensure manually. The choice of reagents (for a one- or two-stage assay), and then their adaptation to the instrument, could affect the reliability of the results. In conclusion, we adapted a general biochemical protocol to a coagulation variable that is awkward to measure, given that it involves a two-stage assay. The protocol proved feasible, simple, and practicable. Indeed, the instruments tested were already well known and routinely used. Our study confirmed that automated instruments were more accurate than semiautomated instruments, at least for some chromogenic assays. Moreover, application of this protocol revealed that all four of the instruments failed to meet some criteria, in particular criteria of accuracy according to clinical interpretation. Results of the protocol also emphasized the need
for full automation in measurement of anti-Xa activity of heparin. Investigation of carryover between assays involving contaminant reagents (e.g., thrombin), ease of handling of the analytical system, acceptability of different reagents, "open" ("random") access, expression of the results, computerization, and ergonomics is also mandatory in the general protocol. This validation of the study protocol means that it may be applied to chromogenic assays performed with the new generation of fully automated instruments currently being released onto the market. It would be of interest to evaluate the application of this protocol to clot-based assays [32].

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References


