Automated Amidolytic Method for Determining Heparin, a Heparinoid, and a Low-M₄ Heparin Fragment, Based on Their Anti-Xa Activity

Hugo ten Cate, Roy J. Lamping, Ch. Pieter Henky, Arle Prins, and Jan W. ten Cate

Using the chromogenic substrate S-2222, we have optimized and automated an amidolytic assay for heparin. The assay is based on the detection of anti-Xa activity generated by heparin in plasma. The method is reproducible (intra- and interassay CVs of 2.4 and 3.3%, respectively) and reliable in antithrombin III-deficient plasma. Results of this assay, obtained for plasma samples from patients and volunteers treated with heparin, correlate well (r = 0.899) with those of the test for activated partial thromboplastin time. Upon administration of a low-M₄ heparinoid (Org 10172) and heparin fragment (Kabi 2165), however, the activated partial thromboplastin time failed to detect anticoagulant activity, whereas the chromogenic heparin assay revealed anti-Xa activity. This automated amidolytic assay for heparin is therefore suitable not only for monitoring standard therapy with heparin but also for measuring the activity of recently developed heparin fractions.

Additional Keyphrases: synthetic chromogenic substrates • antithrombin III • clotting assays • coagulation factors • activated partial thromboplastin time compared

The main action of heparin on the coagulation system is to enhance the biological activity of the main coagulation inhibitor, antithrombin III (AT-III). This rapidly inactivates activated clotting factors, particularly coagulation factors Xa (EC 3.4.21.6) and IIa (EC 3.4.21.5).

Heparin therapy is routinely monitored with a clotting assay, such as the activated partial thromboplastin time (APTT), on the assumption that the anticoagulant response is predictive of the antithrombotic effect. The recent clinical application of heparin of low relative molecular mass (low-M₄ heparin) (7) and of a heparinoid (2, 3) requires another kind of therapy monitoring, because these substances exert specific anti-Xa activity without appreciable effect on, and hence detection by, cloting tests (3, 4). The development of chromogenic substrates has made feasible the selective measurement of heparin- or heparinoid-antithrombin anti-Xa activity of AT-III in plasma (5-7). For dose-adjustment studies with these new heparins, therefore, automated methods that are convenient and reliable and have a large throughput are desirable.

Moreover, an automated assay for anti-Xa would reduce costs, allow large-scale monitoring of patients (e.g., those undergoing hemodialysis, cardiac surgery with extracorporeal circulation, or thrombosis treatment), and improve accuracy.

Here we report the optimization of a chromogenic assay for anti-Xa activity and discuss its application in patients and in volunteers receiving heparin, the heparinoid Org 10172, and the heparin fragment Kabi 2165.

Materials and Methods

Materials

Venous blood samples were collected in polystyrene tubes containing as anticoagulant either 1 mL of trisodium citrate dihydrate (0.12 mmol/L) or 15 mg of solid K₂EDTA per 10 mL of blood. Citrated blood was centrifuged at 2400 × g for 10 min at room temperature; to obtain platelet-poor plasma, we recentrifuged these samples at 13 000 × g for 4 min at room temperature. Blood containing EDTA was directly rendered platelet-poor by centrifugation at 13 000 × g.

Blood was also collected in tubes containing 0.30 mL of "Edinburgh cocktail," prepared from equal volumes of Na₂EDTA (100 g/L) prostaglandin E₁, (30 μmol/L), and theophylline (30.0 mmol/L). Platelet-rich plasma was obtained by centrifuging citrated blood at 340 × g for 10 min at room temperature.

The pooled plasma used for comparisons was prepared by pooling platelet-poor citrated-plasma samples from 40 healthy subjects (equal numbers of both sexes), then freezing small aliquots at -70 °C.

Bovine factor Xa, chromogenic substrate S-2222, and human AT-III concentrate (Coastest®/Heparin kit) were obtained from KabiVitrum B.V. Diagnostica, Amsterdam, The Netherlands. We dissolved one vial of factor Xa in 10 mL of distilled water, obtaining an activity of 7.1 μkat/L (1 μkat = 60 U). One vial of substrate S-2222 [benzoyl-Ille-Glu-(γ-OR)-Gly-Arg-p-nitroanilino • HCl, where R consists of equal proportions of H and CH₃] was reconstituted with 10 mL of distilled water, to a concentration of 1.5 g/L. To one vial of AT-III concentrate we added 10 mL of distilled water to provide an activity of 1 plasma equivalent unit (PEU) per milliliter.

Heparin ("Thromboliquine," batch no. RVG 00341, in 5-mL ampoules containing 5 × 10⁶ int. units/L) and the heparinoid Org 10172 (8) (batch no. CP 081083, in 2-mL ampoules containing 800 kilo-int. units of anti-Xa activity per liter) were provided by Organon, Oss, The Netherlands. The heparin fragment Kabi 2165 (batch no. DzN17, in 5-mL ampoules containing 5 × 10⁶ int. units of anti-Xa activity per liter) was a gift from KabiVitrum.

Anti-Xa activity is expressed in international units, defined in terms of the 3rd International Reference Heparin Preparation (9). Buffer (pH 8.4) contained 50 mmol of Tris, 7.5 mmol of EDTA, and 200 mmol of NaCl per liter. To prepare the reaction buffer, we added 10 mL of factor Xa (71 nkat) to 25 mL of buffer.

Lechitin was obtained from Central Soya, Chicago, Ill.
Washed kaolin was from Fisher Scientific Co., Fair Lawn, NJ.
Barbital buffer (pH 7.35) contained 125.5 mmol of NaCl, 39.2 mmol of sodium barbital, and 0.1 mmol of HCl per liter.
All other chemicals were of analytical grade, from E. Merck, Darmstadt, F.R.G.
Subjects

Six apparently healthy male volunteers (ages 19–45, mean 26 years) participated in the study. Their body weights varied from 65.6 to 92.0 kg (mean 78.2). For 10 days before and during the study, they refrained from smoking and from taking drugs known to interfere with hemostasis.

We also obtained blood from six patients (three men and three women) on chronic hemodialysis. Their ages were 47–72 years (mean 57), and their weights ranged from 42.8 to 72.4 kg (mean 55.1). They received no medication interfering with hemostasis. All participants were informed of the experiment according to the Convention of Helsinki–Tokyo before the study, and the study was approved by the Scientific and Medical-Ethical Committees of the University Hospital.

Procedures

Preparation of standards. Mix 30 µL of the pooled plasma with 0.10 mL of AT-III solution and increasing quantities of heparin, Org 10172, or Kabi 2165 to final concentrations ranging from 0 to 100 units/L. Adjust the final volume of each standard to 0.50 mL with buffer. Figure 1 illustrates a representative standard curve. We estimated the potency of each heparin or related compound by comparison with a standard curve prepared from the specific product given to a patient.

Assay. Table 1 summarizes the steps of both the manual and automated assay procedures. For the latter we used an Automated Kinetic Enzyme and Substrate Analyzer (Vitatron, Dieren, The Netherlands). For the manual determination of anti-Xa activity, we used a Model 550 spectrophotometer with a thermostated cuvet holder and connected to a Coleman 5-120 enzyme calculator, which was started with

the addition of the substrate; results were printed by a Coleman 5-050 printer (all equipment from Perkin-Elmer, Beun-de Ronde BV, Amsterdam, The Netherlands).

If the anti-Xa activity recorded exceeds 800 int. units/L, we dilute the plasma with the pooled plasma and re-test.

Determination of activated partial thromboplastin time (APTT). For comparison, we determined APTT with use of kaolin (10, 11). We dissolved 0.10 g of lecithin in 100 mL of barbital buffer and stored 1-mL aliquots at −20 °C. Washed kaolin, 4 g, was suspended in 100 mL of NaCl, 150 mmol/L, and stored at 4 °C. Equal volumes of lecithin and kaolin preparations were mixed just before starting the assay. After diluting 0.10 mL of citrated plasma with 0.10 mL of barbital buffer, we added 0.10 mL of the lecithin/kaolin suspension and incubated for 5 min at 37 °C. We then added 0.1 mL of CaCl2 (33 mmol/L) and determined the clotting endpoint visually. Standards consisted of various amounts of heparin, heparinoid, or heparin fragment added to the pooled plasma, to give a final activity concentration of 0, 200, 400, 600, and 800 int. units/L. By comparison with results for these standards we could express the clotting time of a given sample in terms of international units of heparin activity per liter.

Results

Assay Optimization and Characteristics

Influence of anticoagulant. We collected 37 venous blood samples from four heparin-treated patients, using collection tubes containing either citrate or EDTA. After correction of the measured anti-Xa activities for differences in hematocrit as previously described (12), the correlation between the activities determined in EDTA-treated and citrated plasma was quite good (r = 0.965).

Influence of different concentrations of supplemental AT-III and Xa. We used different concentrations (100–300 PEU/L) of AT-III to prepare standard curves. When AT-III exceeded 200 PEU/L, the slopes of the curves decreased (Figure 2), confirming the results of Teien and Lie (6). For the assay we used an AT-III concentration of 200 PEU/L. To investigate the effect of different concentrations of Xa, we prepared standard curves with Xa concentrations (in the Xa-buffer dilution) of 0.2–3.0 µkat/L (Figure 3). Standard curves for Xa concentrations <2 µkat/L had low sensitivity, but increasing the Xa concentration did not substantially

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[Table and figure images are included here but not transcribed due to the limitations of the text-only format.]
improve the slope of the curve; therefore, for economy, we chose an initial Xa-concentration of 2 μkat/L. Figure 4 illustrates our calculations of the \(K_m\) value (13) for the chromogenic substrate S-2222. Our result of 0.23 mmol/L does not essentially differ from the \(K_m\) listed for the Coatest kit, 0.30 mmol/L. We used a final substrate concentration of 0.36 mmol/L.

**Accuracy of the automated method.** The intra- (n = 10) and interassay (n = 10, over six days) CVs for determinations with plasma samples containing 600 int. units of heparin per liter were 2.4 and 3.3%, respectively.

**Influence of the concentration of AT-III in the test sample.** To investigate the effect of variations in the plasma AT-III content on the assay results, we compared standard curves prepared with either the pooled normal plasma, containing about 1000 PEU of AT-III per liter, or plasma from a patient with a severe AT-III deficiency (110 PEU/L). No significant differences were observed within a heparin concentration range of 300–800 int. units/L. At lower heparin concentrations, however, there were some differences (Figure 5).

Increasing the initial AT-III concentration to 300 PEU/L corrected the discrepancy between the normal standard curve and the AT-III-deficient standard curve, confirming that this discrepancy was caused by the different concentrations of AT-III in plasma.

**Influence of platelets.** To investigate the effects of disruption of platelets, either in the course of blood sampling or during further handling of the samples, we performed the following experiments:

a) Four persons were injected with 2500 int. units of heparin ("Thromboliquine"). After 10 and 60 min we collected blood at exactly the same time from left and right forearm veins, with either evacuated collection tubes ("Venject"; Terumo Europe, Leuven, Belgium) or a "Butterfly-19" infusion set (Abbott Ireland Ltd., Sligo, Republic of Ireland). With either method the collection tubes contained 1.5 mg of EDTA per milliliter of blood. From the mean anti-Xa activities and ranges obtained (Table 2), we conclude that the use of a reduced pressure blood-collection system does not significantly (p > 0.05) affect anti-Xa activities.

b) Under similar conditions as in (a), we also collected blood into tubes containing either EDTA or "Edinburgh cocktail" to study whether addition of platelet-release inhibitors in excess would influence anti-Xa results. The latter set of samples was put without delay on melting ice and centrifuged (0 °C, 30 min, 3400 × g). EDTA-treated blood was handled as described in Materials and Methods. The two sets of samples showed no differences in anti-Xa activities (data not shown), thus excluding the significance of the

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**Table 2. Effect of Blood-Sampling Technique on Anti-Xa Activity in Four Volunteers Receiving 2500 Int. Units of Heparin**

<table>
<thead>
<tr>
<th>Anti-Xa Acty, Int. units/L</th>
<th>After 10 min</th>
<th>After 60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venject</td>
<td>360</td>
<td>100</td>
</tr>
<tr>
<td>Range</td>
<td>150–520</td>
<td>20–200</td>
</tr>
<tr>
<td>Butterfly</td>
<td>340</td>
<td>80</td>
</tr>
<tr>
<td>Mean</td>
<td>150–540</td>
<td>30–110</td>
</tr>
</tbody>
</table>

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**Fig. 3.** Effect of Xa concentrations on the standard curve for heparin Xa concentrations in the reaction buffer: \(\bullet\), 3000; \(\square\), 2000; \(\Delta\), 1000; \(\bigcirc\), 500; and \(\triangle\), 200 μkat/L.

**Fig. 4.** Determination of the \(K_m\)-value (0.23 mmol/L) for the chromogenic substrate, S-2222 (S).

**Fig. 5.** Standard curves for heparin prepared from either pooled normal plasma (\(\bigcirc\)) or AT-III-deficient plasma (\(\square\)). The lower curve (\(\triangle\)) represents values for AT-III-deficient plasma supplemented to normal values by addition of AT-III concentrate.
release of heparin-neutralizing Platelet Factor 4 after blood sampling.

(c) To eliminate disruption of platelets (accidently present in the test plasma) during freezing and thawing of plasma as a source of artificial reduction of anti-Xa activity, we performed the following experiment. To part of a preparation of platelet-rich plasma (385 × 10^6 platelets per liter) we added two amounts of heparin (final concentrations 200 and 600 int. units/L). The other part of the platelet-rich plasma was centrifuged at 13 000 × g for 4 min, after which the supernatant was similarly mixed with heparin and used as a control. Both platelet-rich and control plasma were freezethawed twice and subsequently assayed in duplicate for anti-Xa activity.

We prepared the standard curve for this comparison from citrated plasma from the same blood donor. Table 3 summarizes the results for anti-Xa activity.

**Influence of temperature.** Anti-Xa activities in heparinized (200 or 600 int. units/L) plasma after centrifugation at room temperature or at 4 °C did not differ (data not shown). Moreover, anti-Xa activity in heparinized platelet-poor plasma (same heparin concentrations) incubated at room temperature or on melting ice for 2 h was no less than that of fresh platelet-poor plasma (data not shown).

**Influence of bilirubin.** Addition of as much as 500 mg per liter of bilirubin to pooled plasma samples containing 600 int. units of heparin per liter did not alter the anti-Xa values (data not shown).

**Clinical Studies**

Six male volunteers were injected with a single intravenous dose of 5000 int. units of heparin, 3500 anti-Xa units of Org 10172, or 5000 anti-Xa units of Kabi 2165. As Figure 6 shows, the anti-Xa activities and the APTT values determined at intervals after the administration of heparin were highly correlated (r = 0.899).

In volunteers receiving Org 10172 the APTT changed little, whereas the chromogenic assay revealed a distinct increase in anti-Xa activity. Thus the correlation between the anti-Xa assay and the APTT was poor (r = 0.105). Similar results were obtained for the heparin fragment Kabi 2165, the correlation between the two methods being r = 0.372.

For six patients subjected to hemodialysis with either Org 10172 or heparin, we monitored the anti-Xa activity with the chromogenic assay. A representative experiment (Figure 7) showed almost no effect of Org 10172 on the APTT, in contrast to the effect of heparin. Nevertheless, at these anti-Xa activities, there was no clotting in the extracorporeal circuit.

**Discussion**

Although the clinical benefit of monitoring heparin therapy is not entirely established, especially its potential for avoiding bleeding complications (14), various authors do stress its importance (15–17). Currently, general clotting tests such as the APTT are used. The increasing development and clinical application of low-M₄ heparin fractions (1) and heparinoids (2, 3), however, demand a different kind of laboratory control, because these preparations commonly fail to prolong general clotting tests sufficiently (2, 4). The increased ratio for anti-Xa/anti-IIa activity in these new preparations, as compared with crude heparin, can be applied in specific anti-Xa assays, such as the chromogenic method originally described by Teien et al. (5, 6).

Automation of the chromogenic anti-Xa assay, as described here, improves the value of this method in several ways:

- The amount of reagents used is about half that for the manual assay, thereby reducing costs (mainly of the substrate).

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**Table 3. Effect of Freezing–Thawing on Recovery of Anti-Xa Activity after Addition of Heparin to Platelet-Rich or Control Plasma**

<table>
<thead>
<tr>
<th>Heparin added (final concen), int. units/L</th>
<th>Range of anti-Xa act recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>int. units/L</td>
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<tr>
<td>Platelet-rich plasma</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>600</td>
<td>150–160</td>
</tr>
<tr>
<td>Control plasma</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>160–200</td>
</tr>
<tr>
<td>600</td>
<td>590–620</td>
</tr>
</tbody>
</table>

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**Fig. 6. Correlations between APTT and chromogenic assay values for anti-Xa, determined for three different heparin preparations**

- Upper curve (I), heparin (n = 40, r = 0.899); middle curve (II), Kabi 2165 (n = 29, r = 0.372); lower curve (III), Org 10172 (n = 27, r = 0.105). APTT is expressed in int. units/L. Instead of seconds by comparison with a standard curve (see text)

**Fig. 7. Anti-Xa activities (solid lines) and APTT values (broken lines)**

before and after administration of heparin (□) or heparinoid (○, Org 10172) to six patients undergoing hemodialysis.
• Accuracy is good, as illustrated by the small coefficients of variation.
• Routine monitoring of many patients in a brief time is possible.

This study further confirms the observation of Teien and Lie (6) that the concentration of AT-III in plasma influences anti-Xa activity in the low heparin concentration range (200 int. units/L). If using the anti-Xa method to determine the actual heparin concentration in plasma, one must compensate for all possible variations in the plasma content of AT-III by adding excess inhibitor in the test system. In the present method, this would require AT-III addition of at least 300 PEU/L. Increasing the supplemental inhibitor concentration from 200 to 300 PEU/L, however, markedly decreased the sensitivity of the anti-Xa method (Figure 2).

There being no general agreement on this particular problem (18), we prefer adding 200 PEU of inhibitor per liter in the automated method, which suffices to compensate for various concentrations of AT-III in plasma within the therapeutic range for heparin (300–800 int. units/L). Monitoring heparin values at lower values than that has no clinical relevance.

Although the presence of platelets as such does not induce loss of recovery of heparin (anti-Xa) activity, destruction of platelets by freezing–thawing produced a considerable loss of anti-Xa activity after addition of heparin (200 or 600 int. units/L) to a freeze–thawed platelet-rich plasma originally containing $3.85 \times 10^8$ platelets per liter. It therefore seems advisable to prevent activation of platelets in the first place and, secondly, to reduce the number of platelets present as much as possible. Using “Butterfly-19” needles for blood sampling and centrifuging the blood at $13,000 \times g$ for 4 min (preparing platelet-poor plasma) appears to help.

Preparation of standards for heparin in fresh pooled plasma for a standard curve showed that (a) addition of platelet-release inhibitors (as in “Edinburgh-cocktail”) did not change the anti-Xa activities from those measured in plasma containing only EDTA as anticoagulant, (b) different blood-sampling methods did not influence anti-Xa results, and (c), in contrast to other reports (7), there was no effect of temperature on heparin recovery. Therefore we do not recommend the routine use of additional platelet-release inhibitors to reduce heparin-neutralizing activity, as suggested by others (7).

The use of a “Venoven” blood-sampling system apparently does not cause measurable loss of anti-Xa activity, which improves the value of this assay as a practical clinical test.

In conclusion, the main advantages of this automated assay for anti-Xa activity are its practical application in routine determination of heparin activity and its potential for determining anti-Xa activity of new heparin derivatives, which will be increasingly used in clinical practice.

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References


