Automated determination of heparin with chromogenic substrates

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Automated Determination of Heparin with Chromogenic Substrates

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Abstract. Spectrophotometric heparin assays which are based on the catalytic effect of heparin on either the inactivation of thrombin or that of factor Xa by antithrombin III, were adapted for use in a laboratory batch analyzer. Optimal conditions were determined for assays using the chromogenic substrates Chromozym-Th and S-2238 with thrombin, and S-2222 with factor Xa. Inactivation of the clotting enzyme by antithrombin III was stopped by addition of chromogenic substrate. Assays thus obtained appeared to be applicable in a wider range of heparin concentrations and were less dependent on plasma antithrombin III concentration that known manual spectrophotometric methods. The best results were obtained with the methods based on thrombin inactivation and applying a logarithmic reference curve.

Introduction

Several spectrophotometric heparin assays have been described [1–4]; all are manual methods, some of which are claimed to be adaptable to automated analyzers. The aim of this study was to find an automated heparin assay suited for routine laboratory use that assesses the heparin level in the therapeutic range and above, and that is independent of the antithrombin III (AtIII) level in the sample.

Since clinicians frequently aim at plasma heparin levels between about 200 and 800 U/l, it was necessary that the reference curves used would cover that range. The methods should, however, also give acceptable estimates of the heparin level in case of overdosage, and thus the method should render reliable values above the higher limit (up to 1,500 U/l).

For ease of automatic data handling it was important that reference curves would meet a simple mathematical formula. Most authors [1–3] reported their reference curves to be linear from 0 to 600 U of heparin/l plasma, and suggested dilution of samples at higher levels in order to obtain reliable estimates. As dilution is time consuming, however, it is impossible to differentiate immediately be-

tween a small or a large overdose. Moreover, dilution changes reaction conditions since the AtIII in the sample is diluted as well. To find a calibration curve that covers a wider range of heparin concentrations, we included in our study the use of a parabolic and a logarithmic fit through reference measurements.

Most amidolytic heparin assays published negate the influence of the plasma AtIII levels, and the influence of AtIII in the plasma on the outcome of the heparin determination has been reported to be moderate [1]. The linearity of the dose-response curves in the assay with Xa and S-2222 was reported to be dependent on the use of AtIII in the assay [1, 2].

Heparin is preferably measured under conditions of a molar excess of other reactants, i.e. AtIII and either thrombin or factor Xa. An excess of 1,000 times has been suggested as satisfactory [6] to obtain reactions dependent on heparin concentration only. Because heparin increases neutralization of factor Xa by AtIII about 1,300-fold, and that of thrombin about 2,000-fold [5], a 1,000-fold excess of free AtIII, however, will bring the rates of enzyme inactivation by free AtIII and by the AtIII-heparin complex in the same order of magnitude.

Therefore, the contribution of free AtIII to the inactivation of enzyme may not be neglected a priori. Whether the concentration of free AtIII in the assay does influence the outcome of the test depends on the ratio of the concentration of the heparin-AtIII complex to that of free AtIII, hence on the amount of AtIII added.

Therefore, we studied the influence of various AtIII levels in heparinized plasmas on the outcome of the assays and determined analytical conditions to minimize this influence.

Materials

Citrated Plasma. By means of a vacuum system (Venoject) with a multi-sample needle, 1.8 ml blood was drawn from the antecubital vein and used for other purposes. Next, three tubes (Venoject, black stopper, 4.5 ml, 3.8% tri-Na-citrate) were drawn and centrifuged (10 min, 3,500 *g*, 15 °C) within 15 min. The plasma thus obtained contained less than 3×10^9 platelets/l.

Pooled Plasma. Equal volumes of citrated plasma from 30 healthy donors were mixed and stored at −70 °C in aliquots of 1 ml.

Heparin was obtained from Leo, Emmen, Holland. According to the manufacturer, the solution contained 5,000 USP U/ml of the sodium salt. Dilutions were made with buffered saline (147 m*M* NaCl, 6.52 m*M* Na_2HPO_4, 1.62 m*M* KH_2PO_4, pH 7.35).

Antithrombin III from KabiVitrum, Amsterdam was used. The contents of each vial (10 U) were solved in 10 ml distilled water. One unit is defined as the activity found in 1 ml of pooled plasma.

Thrombin used was bovine thrombin from Roche, Basel. The contents of each vial (120 USP units) were dissolved in 2.40 ml distilled water. Before use 1 vol thrombin was diluted with 5 vol buffer.

Factor Xa used was bovine factor Xa from Kabi Vitrum, Amsterdam. The contents of each vial (71 nkat) were dissolved in 10 ml distilled water.

Heparin Kits. Coatest/Heparin was obtained from KabiVitrum, Amsterdam. TC-Heparin was obtained from Boehringer-Mannheim, Amsterdam. These assays were performed according to the instructions supplied for kinetic measurement (table I).

Chromogenic substrates used were Tos-Gly-Pro-Arg-pNA (Chromozym-Th, Boehringer-Mannheim, Amsterdam), Benz-Ile-Glu-Gly-Arg-pNA · HCl and H-*D*-Phe-Pip-Arg-pNA · 2HCl (S-2222 and S-2238 both from KabiVitrum, Amsterdam). Each of the substrates was dissolved in distilled water.

Buffer for automated assays (pH 8.4 and I 0.2) contained di-Na-EDTA (7.5 m*M*), Tris · HCl (50 m*M*) and NaCl (175 m*M*).

Methods

Assays were performed on a 'Corona' batch analyzer (Clinicon, Amsterdam). This analyzer has a built-in microprocessor that checks on the linearity of

change in optical density during the measuring period. Conditions were set so as to generate an error message on deviations of more than 2 milliextinctions from linear, i.e. at deviations larger than 2% of the slowest rate observed in practice. Prediluted plasma samples were fed into the instrument and there diluted in duplicate and pipetted into polystyrene cuvettes. The cuvette was transported into a tunnel of constant temperature and (after the preincubation time) Xa or IIa solution was added. After a programmed incubation time a chromogenic substrate solution was added and with a small delay the remaining Xa or IIa activity was measured. The change in absorbance was calculated, stored in memory and printed.

We varied the heparin assay conditions to suit the following demands: (1) Standard deviation of the reference measurements about the calculated reference line has to be less than 5% of the range covered. (2) At the highest heparin level measured, at least half of the original enzyme should be left at the end of the incubation time so as to ensure excess of this reactant during the reaction. (3) The same rationale goes for the antithrombin-III concentration. (4) At the end of the measuring time the reaction rate should be within 2% of the starting rate.

The final procedures of the heparin assays are given in table I. Reference samples were made of pooled normal plasma spiked with known amounts of the heparin of the same batch as we desired to determine. Artificial variations in the AtIII level were simulated by variations in the amount added in the predilution stage, as indicated in table II. The reference curve was obtained by a logarithmic fit through reference measurements. Alternatively, a linear and a parabolic fit were studied.

Results and Discussion

The spectrophotometric determination of heparin can most simply be explained as a reverse titration of clotting enzyme. A fixed

Table I. Analytical conditions of the heparin assays

	Assay				
	Chromozym-Th manual[1]	S-2222 manual[2]	Chromozym-Th automated	S-2222 automated	S-2238 automated
Predilution, µl					
Buffer I	–	800	550	800	550
Plasma	–	100	50	100	50
AtIII (1.0 U/ml), µl	–	100	400	100	400
Diluted plasma, µl	10	200	60	100	75
Buffer I, µl	–	–	140	100	125
Preincubation time, s	–	180	456	456	456
Temperature, °C	25	37	25	25	25
Enzyme amount, µl	1,000	100	100	200	100
Xa, nkat/ml	–	7.1	–	7.1	–
IIa, USP units/ml	0.03	–	8.33	–	8.33
Incubation time, s	180	45	33	33	33
Substrate, µl	100	200	100	100	100
Concentration, mmol/l	1.5	2.0	0.95	1.0	0.75
Measuring time, s	30	30	22	22	22
Wavelength, nm	405	405	405	405	405

[1] Boehringer Diagnostica: Produkt informatie TC Heparine, 1980.
[2] Kabi Diagnostica: Determination of heparin in plasma with S-2222, laboratory instruction, April 1977.

amount of clotting enzyme (E) is added in excess. The complex (HA) formed (eq. 1) by heparin (H) and AtIII (A), binds and inactivates this enzyme (eq. 2), and the remaining enzyme activity is measured. Because enzyme inactivation by AtIII without heparin (eq. 3) is much slower than by the AtIII-heparin complex, the influence of unbound AtIII seems neglectable. Available spectrophotometric heparin kits are based on this model [1, 4]:

$$H + A \xrightarrow{K_1} HA, \quad (1)$$

$$HA + E \xrightarrow{K_2} HAE, \quad (2)$$

$$A + E \xrightarrow[K_2 \gg K_3]{K_3} AE, \quad (3)$$

where H = heparin, A = antithrombin III, and E = enzyme (thrombin or Xa).

Although AtIII is available in molecular excess (> 20x) over heparin in the Kabi heparin kit, we found enzyme inactivation to be strongly influenced by both the amount of AtIII and the amount of heparin at low heparin concentrations, whereas at high heparin concentrations AtIII appeared to be the main determinant of the test result (fig. 1). As in the Boehringer kit the plasmas are tested without predilution, different AtIII levels are not easily simulated, but for this assay an even larger influence of AtIII may be expected because no extra AtIII is added.

The influence of AtIII is not explained by the titration model above, but it can be explained by a more complex model of the action of heparin as has been found by *Markwardt and Walsmann* [7], and confirmed by others [8–10]. In this complex model heparin

Table II. Dilution procedure for reference samples

U/l	Plasma, µl	AtIII, µl	HEP 200 U/l, µl	Buffer, µl
For automated heparin assays with S-2222				
0	100	100	0	800
200	100	100	100	700
400	100	100	200	600
600	100	100	300	500
800	100	100	400	400
To simulate plasma AtIII concentrations of				
50%: AtIII was 50 µl and buffer was 50 µl more				
150%: AtIII was 150 µl and buffer was 50 µl less				
200%: AtIII was 200 µl and buffer was 100 µl less				
For automated heparin assays with S-2238 or Chromozym-Th				
0	50	400	0	550
200	50	400	50	500
400	50	400	100	450
600	50	400	150	400
800	50	400	200	350
To simulate plasma AtIII concentrations of				
50%: AtIII was 375 µl and buffer was 25 µl more				
150%: AtIII was 425 µl and buffer was 25 µl less				
200%: AtIII was 450 µl and buffer was 50 µl less				

enhances the formation of a complex between AtIII and a clotting enzyme, but leaves this complex after it has been formed:

$$HAE \xrightarrow[K_2 > K_4]{K_4} H + AE. \quad (4)$$

This extra reaction predicts a multiphase behavior of enzyme inactivation. In a first transient phase, concentrations of HA and HAE are built up. In a second steady phase, there is constant production of free heparin (H) and hence constant input in reaction 1, and thus constant inactivation of enzyme as well. Next, reactions slow down by the increasing influence of decreasing AtIII and enzyme levels. In the transient and steady phase, enzyme inactivation is mainly dependent on the heparin concentration and little on the concentrations of AtIII or enzyme, whereas in the next phase the reactant present in the lowest concentration is rate limiting. Therefore, heparin determinations should be carried out in the transient and steady phase, as much as practically possible.

The transient steady phase model predicts that the AtIII present in the assay as well as the incubation time remain important. Any heparin determination should therefore be tested on its dependency on AtIII concentration and on incubation time. So we studied the influence of various incubation periods and of extra AtIII added.

We found the inactivation of enzyme to be in first approximation linearly related to the incubation time, both with and without heparin (fig. 2a–c). In the absence of heparin, only reaction 3 is responsible for enzyme inactivation. In the presence of heparin, enzyme inactivation is dependent on heparin concentration. Extrapolation to zero time (fig. 2) indicates the occurrence of a transient phase of rapid, heparin-dependent enzyme

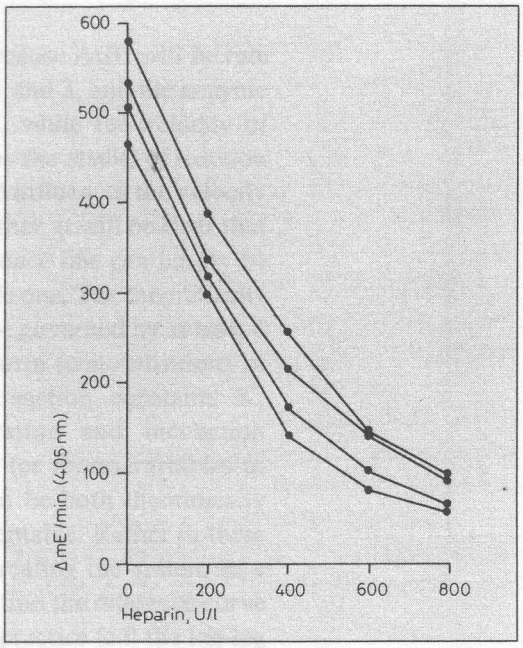


Fig. 1. Reference curves of the manually performed heparin kit: Coatest/Heparin (S-2222). From top to bottom 4 curves with different AtIII concentrations: 50%, 100%, 150% and 200% of normal plasma.

inactivation. The system therefore behaves as predicted by the second model.

During the second phase the rate of enzyme inactivation is governed by the rate of heparin release from the AtIII-enzyme-heparin complex. The enzyme kinetics of this mechanism resemble those of active site titration of hydrolytic enzymes [11]. As a practical consequence, heparin will continue to bind AtIII to the clotting enzyme during the incubation time. To ensure that the reaction rate is sufficiently independent of the AtIII concentration, the latter reactant will have to be present in excess (80 times the concentration of the other reactant).

Still at higher heparin concentrations (above 600 U/l plasma in fig. 4), a slight deviation of the linear shape of the reference

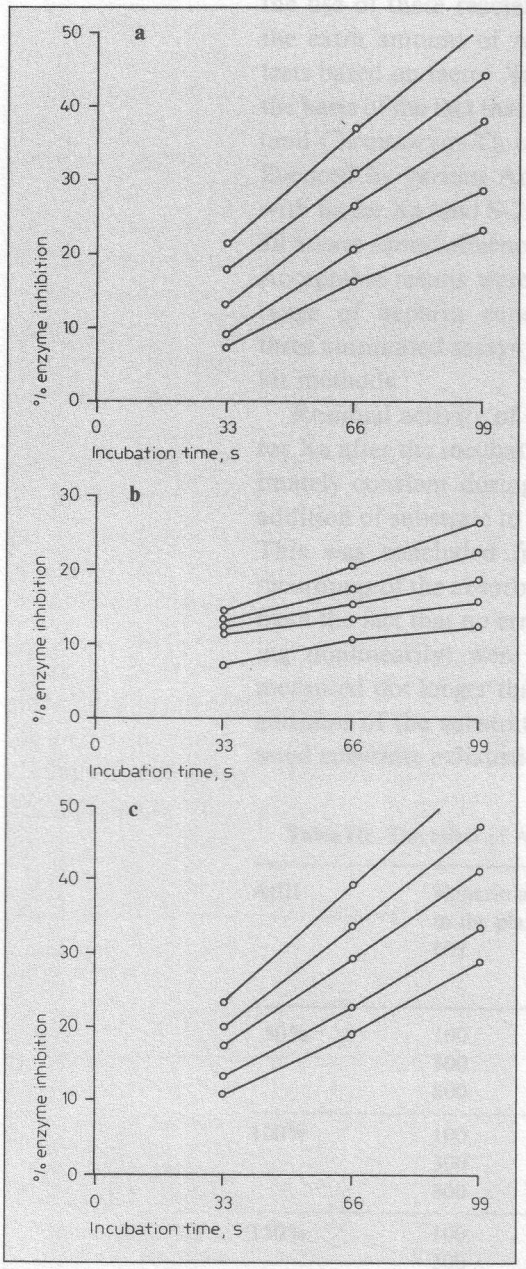


Fig. 2. Relative enzyme inhibition at 3 incubation times for 5 heparin concentrations and 3 chromogenic substrates. Curves from top to bottom: 200 U/l, 150 U/l, 100 U/l, 50 U/l, 0 U/l heparin. **a** Chromozym-Th. **b** S-2222. **c** S-2238.

line was observed. Because AtIII will be rate limiting in reactions 1 and 3, and the enzyme in reactions 2 and 3, while the velocity of reaction 4 is limited by the results of reaction 2 on the one hand and influences the velocity of reaction 1 on the other, it will be clear that the shape of the reference line can hardly be expected to be a simple one. The theoretically correct shape would be governed by at least 6 variables besides heparin (concentrations of enzyme and AtIII, reaction constants K_1 through K_6, temperature and incubation time). Trying to fit 6 (or more) variables to our data points would be both theoretically and practically unacceptable. Rather in these cases one resorts to treating the system as a black box and tries to find the reference curve that is most useful in practice (c.f. the log-log reference curve of the traditional bioassay). As most desk calculators and even many laboratory analyzers can handle formulas as a logarithmic and a parabolic fit through the reference points, we studied whether the latter two could improve accuracy of readings from the reference curve at higher heparin concentrations.

Enzyme inactivation by AtIII without heparin at the used concentration was about the same as the inactivation caused by increasing the concentration of heparin by 150 U/l (fig. 2). So, variations in AtIII in the assay (A in eq. 1–4) may have considerable influence on the result. Therefore, conditions in our assays were chosen so that the AtIII in the sample hardly contributed to the AtIII in the assay. In the assays based on thrombin inactivation, only a maximum of 1/8 of the total AtIII present at the reaction originates from the sample. Increasing AtIII in the assay based on factor Xa inactivation, however, requires a comparably high concentration of factor Xa and thus large amounts of

S-2222 as well. Economical constraints on the use of these reactants forced us to limit the extra amount of AtIII to a factor 1, in tests based on factor Xa inactivation. This is the basis of the fact that assays with thrombin (and Chromozym-Th or S-2238) are less influenced by various AtIII levels than assays with factor Xa (and S-2222) are (table III). In all assays some influence was found however. Acceptable results were obtained in a wider range of heparin concentrations with the three automated assays than with the manual kit methods.

Residual activity of thrombin and of factor Xa after the incubation time was approximately constant during at least 1 min after addition of substrate to the reaction mixture. This was concluded from the linearity of recordings of the absorbance (not shown) and from the fact that no error messages (indicating nonlinearity) were obtained. Since we measured not longer than until 28 s after the addition of the substrate, we never encountered substrate exhaustion in the assays.

From the results shown in figure 2, we conclude that there is a linear, time-related inactivation of enzyme during at least thrice the incubation time. Since the enzyme activity continuously decreases during incubation before adding substrate, but remains constant after this addition, we conclude that inactivation of enzyme ceased after addition of substrate solution. The inhibitory effect of synthetic substrates has been reported before [3, 8]. It could be the consequence of greater affinity of the enzyme for the substrate than for AtIII. The Lineweaver-Burk plots shown in figure 3 suggest that V_{max} rather than K_m remains constant at various levels of inhibitor (chromogenic substrate) present at the inactivation of thrombin by AtIII. This favors the opinion of competitive inhibition of AtIII by the chromogenic substrate.

The deviation of the reference measurements about the calculated linear calibration line was smaller for the automated than for the manual methods (fig. 4). When a parabola is fitted through the reference points this

Table III. The effect of AtIII levels on heparin assays

AtIII	Heparin added to the plasma U/l	Heparin automatic assay using			Heparin manual assay using S-2222 U/l
		S-2238 U/l	Chromozym-Th U/l	S-2222 U/l	
50%	100	116	112	57	50
	300	286	286	206	214
	800	769	776	598	831
100%	100	151	120	144	160
	300	298	285	355	353
	800	723	790	856	780
150%	100	131	140	193	202
	300	321	311	358	325
	800	809	850	> 800 off curve	≫ 800 off curve

The results of the 9 measurements by each assay were read from the same reference line.

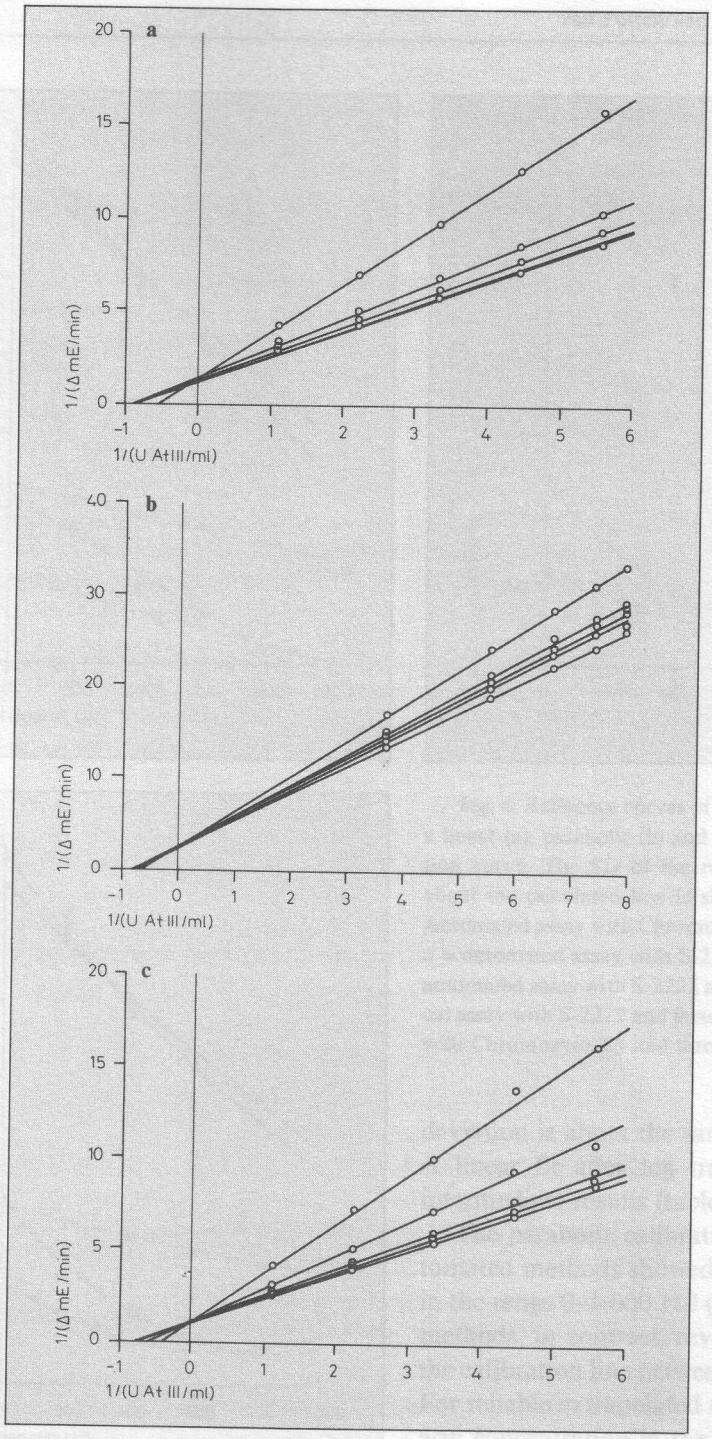
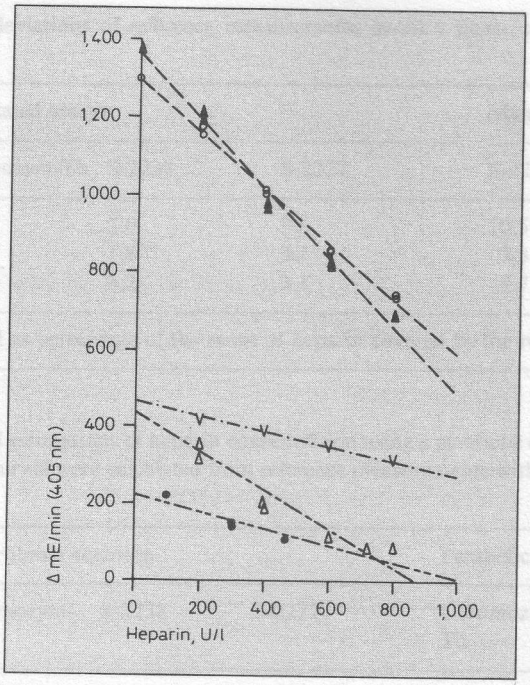


Fig. 3. Lineweaver-Burk plots revealing the inhibitory effect of chromogenic substrates on the inactivation of thrombin by AtIII. Curves from top to bottom: 30%, 10%, 3%, 1% and 0% of 'inhibitor'. The substrate concentration at the measuring stage (table I) is considered 100% inhibitor. **a** Chromozym-Th. **b** S-2222. **c** S-2238.



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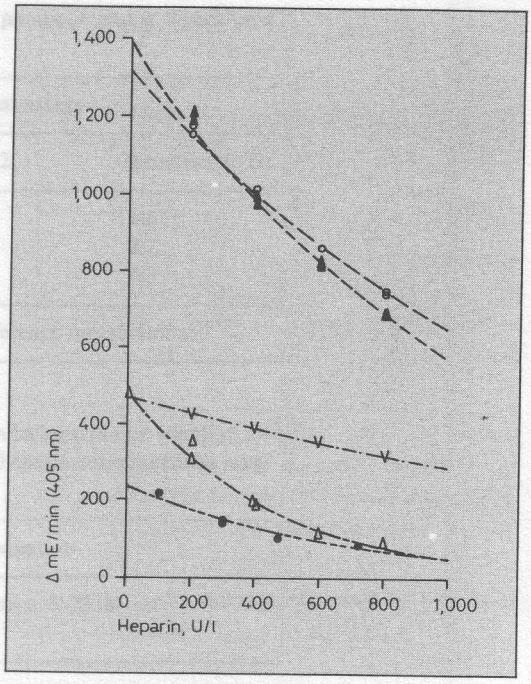

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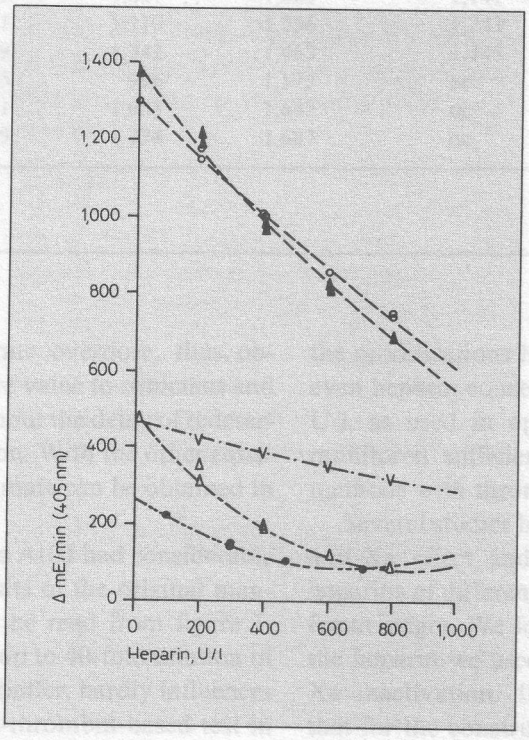

b

Fig. 4. Reference curves of the heparin assays with a linear (**a**), parabolic (**b**) and logarithmic (**c**) calibration curve. The SD of the reference measurements about the calculated line is shown in table IV. ▲ = Automated assay with Chromozym-Th and thrombin; o = automated assay with S-2238 and thrombin; v = automated assay with S-2222 and factor Xa; △ = manual assay with S-2222 and factor Xa; ● = manual assay with Chromozym-Th and thrombin.

deviation is about the same for all methods. A linear fit after log transformation gives intermediate results (table IV).

The parabolic calibration lines of the automated methods showed no horizontal part in the range 0–1,000 U/l (fig. 4). The manual methods, in contrast, revealed zero slope of the calibration line between 700 and 900 U/l. For reliable extrapolated estimations of heparin concentration in the range 1,000–1,500 U/l, the logarithmic equation may be applied for the automated methods (table V). Infor-

Table IV. Standard deviations of reference measurements about a linear, a parabolic and a logarithmic calibration curve

	Automated assays			Manual assays	
	Chromozym-Th	S-2238	S-2222	S-2222	Chromozym-Th
Linear	3.5	2.0	3.6	10.5	14.6
Parabolic	2.4	1.8	3.1	3.3	2.2
Logarithmic	3.0	3.2	3.1	9.7	4.3

The SD are expressed as percentage of the range of heparin covered by the reference measurements.

Table V. Extrapolated estimations of heparin concentration using a parabolic and a logarithmic equation for the reference curve (the curves were calculated from reference measurements with heparin concentrations ranging 0–800 U/l)

Actual heparin concentration U/l	Logarithmic equation			Parabolic equation		
	Chromozym-Th	S-2238	S-2222	Chromozym-Th	S-2238	S-2222
1,000	1,153	1,027	1,081	1,141	oc	oc
1,200	1,211	1,210	1,256	1,243	oc	oc
1,400	1,419	1,342	1,463	1,445	oc	oc
1,600	1,675	996[1]	1,573	oc	oc	oc
1,800	2,161	1,635	1,642	oc	oc	oc
2,000	2,369	1,774	1,687	oc	oc	oc

oc = Off curve.
[1] Bad duplicates.

mation on a moderate overdose, thus obtained, will be of more value to clinicians and can be presented without the delay of redetermination after dilution. With the other equations, no reliable estimate can be obtained in this range.

Dilution of plasma AtIII had considerable influence on the results of the original manual methods, as can be read from figure 1. Table VI shows that up to 40-fold dilution of samples by saline or buffer, hardly influences the result of the anti-thrombin-based test in the modifications here described. Therefore, even heparin concentrations of up to 10,000 U/l, as used in open-heart surgery can be monitored sufficiently with the automated methods with thrombin.

Several studies have shown a difference in anti-Xa effect and anti-IIa effect between heparins of different molecular weight or different origin. We found a catalytic action of the heparin we used, on both thrombin and Xa inactivation. It should be emphasized that for the construction of reference curves

Table VI. Independency on the plasma AtIII concentration: heparin concentration after dilution with buffer (measured with Chromozym-Th and thrombin)

	Actual heparin concentration					
	10,000 U/l	5,000 U/l	2,000 U/l	1,000 U/l	500 U/l	250 U/l
Dilution	1+39	1+19	1+7	1+3	1+1	1+0
Result, U/l	250	232	245	249	249	259

the same heparin is to be used as that in the unknown samples. Both automated methods are subject to further investigation both for the determination of different heparins, and for the determination of heparin in actual patient plasmas.

In conclusion, it can be said that the automated spectrophotometric methods for heparin determination we present in this article are more reliable than the available manual chromogenic methods and allow heparin to be determined over a wider range. The methods based on thrombin inactivation are to be preferred over those using factor Xa inhibition because the antithrombin methods are hardly influenced by the plasma AtIII concentration.

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