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Determination of Low Molecular Weight Heparin in Clinical Laboratory

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Key Words. Heparin · Low molecular weight heparin · Heparin standardization · Reference curves · Monitoring heparin therapy

Abstract. The applicability was investigated of automated spectrophotometric heparin assays and three clotting assays for determination of two low molecular weight (LMW) heparin fractions: Org 10172 and DxN10 and two infrafractionated commercially available heparins. The relative activity of the two commercially available heparins was similar in the anti-Xa assay, in the anti-IIa assay and in 3 clotting assays. The LMW heparins showed markedly different relative activity in all 5 assays. The activities of those heparin preparations relative to the standard heparin were compared in the 5 assays, but standardization against a standard heparin preparation appeared impossible. Methods of heparin determination can be used to monitor treatment with a heparin preparation only if the same preparation is used as a reference substance.

Introduction

Heparin fractions separated on basis of molecular weight or affinity to antithrombin III (AtIII) show variations in their catalytic activity on the inactivation of factor Xa and of thrombin [1-3]. Much research work is being done on this subject. Crude heparin is suspected to contain many inactive or even harmful components and it is generally expected that heparin fragments can be isolated that have a specific antithrombotic action

more pronounced than that of crude heparin. Selected fractions of heparin of low molecular weight (LMW) are expected to be available for clinical use in the near future. Clinicians will then ask for monitoring tests or for quantification of these heparins in patient plasmas.

We therefore investigated the applicability of automated spectrophotometric heparin assays and three clotting assays for determination of several commercial heparins and of Org F10172 and DxN10 in plasma. For each

heparin preparation, we compared the relative activity in normal human pooled plasma in 3 clotting assays and in 2 spectrophotometric heparin assays, based on factor Xa and on factor IIa inactivation.

Materials and Methods

Citrated Plasma. By means of a vacuum system with a multisample needle, blood was drawn from the antecubital vein. The first tube was used for other purposes. A second tube (Venoject, black stopper, 4.5 ml, 3.8% tri-Na-citrate) was drawn for routine clotting determinations. Within 15 min, the latter sample was centrifuged (10 min, 3,500 g, 15 °C). The plasma thus obtained contained less than 3×10^9 platelets/l.

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

Antithrombin III from KabiVitrum, Amsterdam contained 10 U per vial. One unit is defined as the activity found in 1 ml normal plasma. The contents of each vial were dissolved in 10 ml distilled water. A small amount of standard heparin was added to titrate heparin neutralisation [5].

Thrombin (Roche, Basel). The contents of each vial (120 USP Units of Bovine Thrombin) were dissolved in 2.4 ml distilled water. Before use 1 vol thrombin was diluted with 5 vol buffer I.

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

Heparins were obtained from Leo, Emmen, Holland (heparin Leo batch No. 81L1414 and heparin standard 181021) and from Organon, Oss, Holland (Thromboliquine batch No. 128386). According to the manufacturers, the solutions contained 5,000 USP U/ml of the sodium salt, dilutions were made with buffered saline (147 mM NaCl, 6.52 mM Na_2HPO_4 , 1.62 mM KH_2PO_4 , pH 7.35).

Heparin fractions of low molecular weight (MW 5,000) were donated by KabiVitrum, Stockholm (DxN10, 170 anti-Xa Units/mg) and by Organon, Oss (Org 10172 high affinity heparinoid, 100 mg/ml). Dilutions were made with buffered saline (147 mM NaCl, 6.52 mM Na_2HPO_4 , 1.62 mM KH_2PO_4 , pH 7.35).

APTT Reagents. (1) 'Kaolin/platelet substitute' from Diagnostic Reagents Ltd., Thame, Oxon, England. (2) 'Actin-activated cephaloplastin reagent' from Dade, Salm en Kipp, Breukelen, Holland. (3) 'APTT Automated' from General Diagnostics, Amsterdam, Holland.

Chromogenic substrates used were: Benz-Ile-Glu-Gly-Arg-pNA-HCl and H-D-Phe-Pip-Arg-pNA-2HCl (S-2222 and S-2238 both from KabiVitrum, Amsterdam). The substrates were dissolved in distilled water.

Buffer I (pH 8.4 and I 0.2) contained di-Na-EDTA (7.5 mM), Tris-HCl (50 mM) and NaCl (175 mM).

Clotting assays were performed as indicated by the manufacturer of the reagent, on a 'KC10' coagulometer (Salm en Kipp, Breukelen, The Netherlands). Plasma was incubated with the APTT-reagent in plastic tube. After recalcification, the clotting time was measured.

Automated chromogenic heparin assays were performed on a 'Corona batch analyser' (Clinicon Amsterdam) as reported before [4].

Results

A reference curve of pooled plasma spiked with known amounts of a standard heparin (in USP units/l) was made for each of the 2 spectrophotometric heparin assays and the 3 APTT assays. Response curves based on units or milligrams (as indicated by the manufacturers) were constructed with the other preparations (fig. 1-5).

The anti-Xa and anti-IIa activities of the different heparin dilutions were read from the standard reference curve and divided by the heparin concentration (U or mg) indicated by the manufacturer and corrected for the dilution. The obtained ratios (table I) express the activities of the different heparins relative to the standard heparin in the assay concerned.

The activity of Heparin Leo is in all assays comparable to the activity of the standard,

Fig. 1. Reference curves of the spectrophotometric heparin assay with S-2238 and thrombin with 5 heparins. For curve $\square$, 100 U correspond to 500 mg of the heparinoid. $\circ$ = Heparin Leo; $\triangle$ = thromboliquine; $\bullet$ = heparin standard; $\blacktriangle$ = heparin DxN10; $\square$ = heparinoid, Org. 10172.

Fig. 2. Reference curves of the spectrophotometric heparin assay with S-2222 and factor Xa with 5 heparins. For curve $\square$, 100 U correspond to 50 mg of the heparinoid. See legend to figure 1 for symbol explanations.

Fig. 3. Reference curves of the activated partial thromboplastin time with 'Actin' with 5 heparins. For curve $\square$, 100 U correspond to 62.5 mg of the heparinoid. All preparations clotted at concentrations up to 800 U/l. See legend to figure 1 for symbol explanations.

Fig. 4. Reference curves of the activated partial thromboplastin time with 'kaolin-cephalin' with 5 heparins. For curve □, 100 U correspond to 62.5 mg of the heparinoid. Heparin Leo, thromboliquine and heparin standard did not form a clot at concentrations above 600 U/l. See legend to figure 1 for explanations of symbols.

Fig. 5. Reference curves of the activated partial thromboplastin time with 'APTT automated' with 5 heparins. For curve □, 100 U correspond to 62.5 mg of the heparinoid. Only heparin standard and heparin fragment DxN10 would clot at concentrations of 600 U/l. None would clot at higher concentrations. See legend to figure 1 for symbol explanations.

also from Leo. The same holds for the heparin activity of thromboliquine, although the relative activity appears higher than the expected 1.0 in the spectrophotometric assays.

The relative activities of the LMW heparins depend strongly on the assay and reagents used. The relative anti-Xa activity of heparin fraction DxN10 varies from 1.12 to 1.42 and the relative anti-thrombin activity varies from 2.69 to 3.07. The least difference in clotting activity is observed in the APTT with actin, the relative activity in the kaolin-cephalin APTT is smaller than 1.0.

The activity of the heparinoid Org 10172 is not given by the manufacturer. In our experiments dilution was chosen to obtain comparable response curves. Consequently, the relative activities are expressed in U/mg. The relative anti-thrombin activity of heparinoid Org. 10172 is dependent on the dilution. The relative anti-Xa activity ranges from 2.08 to 2.86. The relative activities in the clotting assays are strongly dependent on

Table I. Relative heparin activities: heparin activities determined with the anti-IIa assay and with the anti-Xa assay expressed as ratio to the activity of heparin standard

Dilution	Heparin Leo	Thromboliquine	KabiVitrum DxN10	Org. 10172, U/mg
<i>Anti-IIa assay</i>				
1:20	1.24	1.30	1.42	0.81
2:20	2.04	1.10	1.17	0.52
3:20	1.04	1.16	1.14	0.40
4:20	0.94	1.17	1.12	0.33
<i>Anti-Xa assay</i>				
1:10	0.85	0.85	2.75	1.16
2:10	1.18	1.28	6.07	1.04
3:10	1.28	1.35	3.01	1.01
4:10	1.26	1.20	2.69	1.43

the reagents used. As with heparin DxN10 the relative activity is quite similar in the APTT with actin (fig. 4) and smaller than 1.00 in the APTT with kaolin cephalin.

Discussion

For the measurement of heparin concentrations, it is a condition sine qua non that the ratio of the activities of the heparin preparations being compared is independent of the dilution used; condition of similarity [6]. For the standardisation of heparin preparations, the relative activities should also be independent of the assay used [6]. As both requirements seemed to be met by the commercial nonfragmented heparin, the concentrations of these preparations can be determined and expressed in U/l.

The LMW heparins reveal contradictory properties in the 5 tested methods. Therefore, the concentrations of these preparations cannot unambiguously be expressed in U/l. Moreover, the relative anti-thrombin activity of Org. 10172 is also dependent on the

dilution used and cannot even be determined. In the five assays the response curves of the APTT with actin (fig. 3) are most similar and one can hypothesize that units of the heparin preparations as indicated by the manufacturer had been determined in an assay similar to the APTT with actin. It is, however, highly questionable if this assay or any other will reflect the antithrombotic effect in vivo. It would be in no way justified to conclude that two different heparin-like substances with the same activity in some test system, will have a similar clinical effect. Therefore, the therapeutic range will have to be determined for each preparation.

To determine the therapeutic range one should be able to monitor the therapy. It appears quite feasible to construct for each heparin preparation a curve steep enough to give reliable readings in a desired range. It is obvious that clinicians can be provided with data on the amount of heparin in the patients' plasma only relative to the activity of the heparin preparation used for the reference curve. This makes it possible for clinicians to study the relation between a given

dose and the achieved plasma concentration as well as the relation between the plasma concentration and clinical effect.

A related pharmacokinetic problem is still whether each of the components of a heparin preparation will behave similarly after injection. If there is a difference in clearance between heparin fraction of various molecular weights, the heparin preparation used for establishing the reference curves might well differ from the heparin encountered in the plasma samples drawn.

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Introduction

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