Elimination of High Affinity Heparin Fractions and Their Anticoagulant and Lipase Activity

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High and low affinity heparin (HA and LA heparin) were prepared from commercial heparin by affinity chromatography to insolubilized antithrombin III. HA heparin was radiolabeled with $^{35}$S and subdivided by gel chromatography into high molecular weight (HMW, average 17,000–26,000 daltons), intermediate molecular weight (MMW, average 12,000–13,000 daltons), low molecular weight (LMW, average 5,000–7,000 daltons), and very low molecular weight (VLMW, average 4,600 daltons) fractions. The kinetics of lipolytic and anticoagulant activity and protein-bound radioactivity were studied after intravenous injection of these fractions. LA heparin failed to induce anticoagulant activity but released the hepatic triglyceride lipase (H-TGL) and lipoprotein lipase (LPL) activities normally. VLMW and LMW heparin failed to release both lipolytic enzymes and did not induce anticoagulant activity measurable by the activated partial thromboplastin time (APTT). A powerful anticoagulant effect was found in the anti-Xa assay, which disappeared according to a continuously concave curve in semilogarithmic plots, with elimination rates similar to those of the protein-bound radiolabel. The other heparin preparations induced all activities measured. Heparin anticoagulant activity estimated by the two assays disappeared following a convex curve, preceded by a rapid initial elimination phase in semilogarithmic plots. The disappearance rates of plasma protein-bound heparin radioactivity and heparin anticoagulant activity estimated by factor Xa inactivation were similar. Peak values of the two lipolytic activities were attained rapidly. H-TGL activity, as well as LPL activity, disappeared following convex curves in semilogarithmic plots, with elimination rates similar to those of plasma protein-bound heparin radioactivity. On the basis of these kinetics, we suggest that, after intravenous administration of heparin, the two lipolytic enzymes present in plasma are complexed with heparin, analogous to the heparin–antithrombin III complex. Finally, the kinetic data indicate that elimination of these activities is determined by the hepatic part of the complexes, probably by removal of free heparin.

After intravenous administration of commercial heparin, an anticoagulant activity appears in the blood, and lipolytic enzymes are released into the circulation, probably by removal from the endothelial surface. These enzymes comprise hepatic triglyceride lipase (H-TGL) and lipoprotein lipase (LPL). The various activities induced by heparin may be caused by different heparin molecules, as commercial heparin preparations are heterogeneous.

Recently, better characterized heparin fractions have been prepared by using insolubilized antithrombin III and by subsequent subdivision according to molecular size, employing gel chromatography. Antithrombin III inactivation of Xa is preferentially catalyzed by small heparin molecules that have little effect on thrombin neutralization. Larger heparin molecules, on the other hand, catalyze the inactivation of thrombin more readily than Xa.

In a previous article we have described the kinetics of commercial heparin preparations based on their anticoagulant activity. In the present study, we report on the kinetics of heparin anticoagulant activity and the two lipolytic activities after injection of low affinity (LA) heparin, high affinity (HA) heparin, and $^{35}$S-radiolabeled fractions of different molecular weight of HA heparin. The disappearance of the biologic activities of these fractions was compared with the disappearance of the radiolabel.

MATERIALS AND METHODS

Subjects

Fifteen males and two females volunteered in the experiments. They were biochemists, physicians, students, or technicians of the Department of Internal Medicine or the Department of Haematology. The ages of the volunteers ranged from 23 to 43 yr. All had given their consent after careful explanation of the nature, purpose, and possible risks of the experiments. The investigation was approved by the Committee on Medical Ethics of the University Hospital of Utrecht.

Blood Collection, Storage, and Transport

Blood was collected from an indwelling catheter (Abbocath-T, 16, from Abbott Ireland Ltd., Sligo, Ireland) positioned in a forearm vein at 2, 5, 10, 15, 30, 45, and 60 min, and subsequently, at 30-min intervals, until 4 hr after injection (or 9 hr after administration of high anticoagulant heparin). After discarding the first milliliter, 9 parts of blood were drawn into 1 part of 0.129 M trisodium citrate. Platelet-poor plasma was obtained by centrifuga-
tion (3,000 g, 15 min at 4°C) and stored at −70°C. Frozen plasma samples for the lipase activity assays were transported in dry ice.

**Heparin**

Commercial heparin was obtained from Organon (Oss, The Netherlands). Heparin with high and low anticoagulant potency was prepared by affinity chromatography using insolubilized antithrombin III, as has been described previously. HA 35S-heparin fractions of different molecular weight were prepared separately from two batches of HA heparin. During this study, two batches containing, respectively, 900 and 700 mg of HA heparin were radiolabeled with 35S by the Radioc hemical Centre, Amersham, England. After the radiolabeling procedure, 140 and 300 mg, respectively, of HA 35S-heparin was recovered, both of which retained more than 90% affinity for antithrombin III. The labeled heparin fractions were further subdivided by gel chromatography on AcA 44 Ultrogel (as previously described) into 3 and 4 fractions, respectively, with different molecular weight. Details of the heparin preparations used are presented in Table 1. In order to obtain measurable levels of heparin anticoagulant activity after intravenous administration of radiolabeled HA heparin, unlabeled HA heparin of corresponding molecular weight and specific anticoagulant activity was added to the labeled heparin fractions of the first batch (781025). All labeled heparin fractions prepared from the second batch of high anticoagulant heparin were incubated with 125I-labeled antithrombin III before injection. The second batch had lower specific anticoagulant activity, but this did not affect the binding to antithrombin III or the kinetic behavior. The kinetics of 125I-radioactivity will be described in a separate article. All preparations were sterilized before injection by passing them through a 0.22-μ or a 0.45-μ Millipore filter in the presence of a human albumin solution (GPO plasma substitute, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam).

**Heparin Anticoagulant Activity Assays**

Heparin anticoagulant activity in plasma was measured by a diluted activated partial thromboplastin time according to Marder7 with slight modifications. The heparin-containing test plasma was diluted (1:4, 1:10, 1:20, or 1:40) with normal plasma, depending on the expected heparin anticoagulant activity. A quantity of 0.1 ml of diluted test plasma and 0.1 ml of a mixture of equal parts of cephalin and anticoagulant-free bovine plasma was added. The clotting time was recorded. The test was performed in duplicate. Clotting times were converted into heparin anticoagulant activities by means of a calibration curve, constructed as described above. The variation coefficient of the assay was 10% over the whole range of 0.01–5.0 U/ml.

**Radioactivity Counting**

Radioactivity counting was measured separately as hepatic triglyceride lipase activity and lipoprotein lipase activity, as described before.11 Ten microliters of plasma was incubated for 2 hr at 4°C with 10 μl of hepatic lipase antiserum. Five hundred microliters of the assay mixture, containing 3.2 mM acyl-1-14C triolein emulsion, 40 mM Tris-HCl buffer, pH 8.4, 0.1 M NaCl, 2.5% bovine serum albumin, and 50 μl of normal human serum, was added and the tubes were incubated at 37°C for 60 min. The released free fatty acid radioactivity was determined with the liquid–liquid partitioning system described by Belfrage and Vaughan.13 In the assay of hepatic lipase, the lipoprotein lipase was inhibited by raising the NaCl concentration to 1.0 M and omitting the human serum. The preincubation with antiserum was also omitted. The variation coefficient for the lipoprotein lipase and hepatic lipase determinations were 5.0% and 4.0%, respectively.

**Table 1. Characteristics of Heparin and Heparin Fractions**

<table>
<thead>
<tr>
<th>Heparin</th>
<th>Batch</th>
<th>Mean Molecular Weight</th>
<th>Number of Experiments</th>
<th>Weight (mg) of Administered Frac.</th>
<th>Specific Radioactivity (μCi/mg)</th>
<th>Anticoagulant Potency (U/mg)</th>
<th>APTT Anti-Xa</th>
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<tbody>
<tr>
<td>High affinity heparin</td>
<td>NaE13A</td>
<td>1</td>
<td>40</td>
<td>—</td>
<td>370</td>
<td>370*</td>
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<tr>
<td></td>
<td>NaE13B</td>
<td>1</td>
<td>40</td>
<td>—</td>
<td>370</td>
<td>370*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>781027</td>
<td>1</td>
<td>32</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Low affinity heparin</td>
<td>NaE13A</td>
<td>1</td>
<td>40</td>
<td>—</td>
<td>370</td>
<td>370*</td>
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<tr>
<td></td>
<td>NaE13B</td>
<td>1</td>
<td>40</td>
<td>—</td>
<td>370</td>
<td>370*</td>
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<tr>
<td></td>
<td>781027</td>
<td>1</td>
<td>32</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
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<tr>
<td>HA HMW 35S</td>
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<td>280</td>
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<td>430</td>
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<tr>
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<td>9.1</td>
<td>4.8</td>
<td>10</td>
<td>239</td>
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*The three preparations were prepared separately from the same original batch. The values for the low affinity preparations are approximate.
used after correction had been made for $^{35}$S radioactivity not bound to protein. This was achieved by passing the plasma samples through a filter (Diaflo Ultrafilters, type PM10, Amicon, Lexington, MA). The counts passing through the filter were subtracted. Plasma $^{35}$S radioactivity that passed the filter varied from 0.7% to 2.7%, 3.3% to 30.4%, 15.2% to 62.3%, 34.1% to 88.3%, and 38.7% to 89% at 2, 60, 120, 180, and 240 min, respectively. In case of simultaneous injection of $^{125}$I-antithrombin III and $^{35}$S-heparin, the liquid scintillation data for $^{35}$S were corrected for the contribution of $^{125}$I. No cross-talk existed for $^{35}$S in the $^{125}$I channel. The cross-talk of $^{125}$I in the $^{35}$S was 11.3%. The $^{35}$S data were corrected for this cross-talk. These radioactivity estimations were performed with 2.5 ml of plasma and counted in duplicate. The counting time was 10 min. When $^{35}$S was present alone 0.5-ml samples were used.

**RESULTS**

**Disappearance of Heparin Anticoagulant Activity**

Representative elimination curves of heparin anticoagulant activity based on factor Xa inactivation are shown for HA heparin and LA heparin in Fig. 1, and HA MMW $^{35}$S-heparin supplemented with cold HA MMW heparin and HA VLMW $^{35}$S-heparin in Fig. 2.

Injection of LA heparin induced poor anticoagulant activity both when measured in the APTT and in the anti-Xa assay. The APTT and anti-Xa elimination curve of HA heparin conformed to what we have previously observed with crude commercial heparins. In semilogarithmic plots, an initial fast phase preceded a convex curve, which was described by postulating the combination of a saturable elimination mechanism with an exponentially removing elimination mechanism. In addition, differences between disappearance rates of APTT and anti-Xa activity for HA heparin were similar to what was previously noted for crude heparin⁹ (Fig. 3A). APTT activity was eliminated faster than anti-Xa activity in the initial phase of rapid clearance. Below a heparin concentration of 0.8 U, the ratio between the two activities remained constant (ratio 1.49).

The HA LMW and HA VLMW heparin induced no activity accurately measurable with the APTT, but induced a considerable activity when measured in the anti-Xa assay. The elimination of this activity followed a continuously concave curve in a semilogarithmic plot (Fig. 2). The HA MMW and HA HMW heparin induced similar activities measured with the APTT as...
Fig. 3. (A) Relationship between the anticoagulant activity estimated with the APTT and anti-Xa assay in vivo after injection of 40 mg of HA heparin. (B) Relationship between the anticoagulant activity estimated with the APTT and anti-Xa assay in vivo after injection of 15.6 mg of HA MMW ³⁵S-heparin (O-O) and after 19.6 mg of HA HMW ³⁵S-heparin (O-O).

with the anti-Xa assay (Fig. 2). The APTT and anti-Xa elimination profile for both heparin fractions had the same shape as for total HA heparin or crude heparin. The ratio between APTT and anti-Xa activity remained constant along the entire curve for both heparin fractions. The ratios were 0.99 for HA MMW heparin and 0.60 for the HA HMW heparin preparation of molecular weight >26,000 (Fig. 3B).

Disappearance of Heparin Radioactivity Bound to Plasma Protein

Elimination curves of plasma protein-bound radioactivity after administration of HA MMW ³⁵S-heparin and HA VLMW ³⁵S-heparin are shown in Fig. 4. The shape of HA HMW ³⁵S-heparin and HA LMW ³⁵S-heparin curves conformed to those of HA MMW ³⁵S-heparin and HA VLMW ³⁵S-heparin, respectively. All curves are similar to the elimination curves of heparin anticoagulant activity estimated by factor Xa inactivation, as is demonstrated in Fig. 5, in which anti-Xa values after injection of HA MMW ³⁵S-heparin are plotted against the plasma protein-bound radioactivity data.

Response and Disappearance of Lipolytic Activity

Representative curves of H-TGL activity and LPL activity after injection of HA heparin and LA heparin are shown in Fig. 6 and of HA MMW ³⁵S-heparin and HA VLMW ³⁵S-heparin in Fig. 7. HA LMW ³⁵S-heparin and HA VLMW ³⁵S-heparin failed to release cpm ³⁵S radioactivity/ml.

Fig. 4. Elimination curve of protein-bound ³⁵S-heparin radioactivity after injection of 16 mg of HA MMW ³⁵S-heparin (O-O) or of 9.1 mg of HA VLMW ³⁵S-heparin (I-I). Similar curves as for HA MMW ³⁵S-heparin were obtained for HA HMW ³⁵S-heparin. Similar curves as for HA VLMW ³⁵S-heparin were obtained for HA LMW ³⁵S-heparin.

Fig. 5. Relationship between the protein-bound ³⁵S-heparin radioactivity and anticoagulant activity estimated by the anti-Xa assay after injection of 16 mg of HA MMW ³⁵S-heparin. Such relationship lines were observed after administration of all radiolabeled fractions.
both lipolytic enzymes. LA heparin has no anticoagulant activity, but released H-TGL and LPL activity to levels comparable to those induced by the other HA heparin preparations.

After a sharp initial rise, maximal H-TGL activity and LPL activity were attained at 2–10 min and 10–30 min, respectively, after injection. Maximal LPL activities were 10%–50% of the maximal H-TGL activities. H-TGL activity and LPL activity disappeared in parallel, according to a convex curve, similar to the convex part of the elimination curves of HA heparin radioactivity bound to plasma proteins and heparin anticoagulant activity. The elimination data of H-TGL activity and plasma protein-bound heparin radioactivity are plotted against each other in Fig. 8, indicating similar elimination rates. Similar curves can be made for LPL versus radioactivity and for H-TGL and LPL versus anticoagulant activities.

**DISCUSSION**

The anticoagulant activity of crude heparin estimated in vitro with the APTT and the anti-Xa is the same. This was also true for HA heparin, but heparin fractions of different molecular weight differed in both assays (Table 1). Particularly, low molecular weight heparin was exceptional because it had a high anticoagulant potential in the anti-Xa assay but no activity estimated by the APTT or thrombin time. Heparin anticoagulant activity after intravenous administration of several commercial heparin preparations disappears according to nonexponential kinetics. In semilogarithmic plots, heparin anticoagulant activity (as measured in the APTT or in the anti-Xa assay) disappeared according to a convex curve, mostly preceded by a rapid elimination phase. The convex
part of the curve could be explained by assuming a model based on the combination of a saturable and an exponentially removing elimination mechanism.

Similar elimination patterns of heparin anticoagulant activity were seen after injection of all but the smallest molecular weight fractions (HA VLMW and HA LMW heparin). The complex shape of the anticoagulant disappearance curve of crude heparin is thus not the result of a summation of different anticoagulant disappearance curves of the various heparin fractions. The anticoagulant activity of the lowest molecular weight fractions, possessing only anti-Xa activity, disappeared according to a continuously concave curve in a semilogarithmic plot. The proportion of VLMW heparin present in HA heparin was approximately 10%, and this is so low that the aberrant elimination kinetics have no influence on the overall shape of the elimination curve of crude or HA heparin.

After injection of HA heparin, the anticoagulant activity estimated by the APTT disappeared faster in the initial clearance phase than the anticoagulant activity estimated by the anti-Xa assay, whereas the ratio between APTT and anti-Xa activity remained constant in the later phase of the elimination. These differences were similar to those previously found for crude heparin (ratio anti-Xa/APTT of 1.49 for HA heparin and 1.45 for crude heparin). Possibly, heparin elimination mechanisms have a preference for large heparin molecules, carrying more APTT than anti-Xa activity, and causes these to be removed earlier than small molecules with a higher anti-Xa/APTT activity ratio. The relatively low anti-Xa/APTT ratios calculated for HA MMW heparin and the >26,000 molecular weight HA HMW heparin preparation (0.99 and 0.60 respectively) are consistent with this idea. The constant ratios found for these two heparin fractions are probably due to the fact that these fractions are quite homogeneous.

Because heparin is almost completely bound to plasma proteins and becomes progressively desulfated in vivo, we have based our kinetic data on radioactivity bound to plasma proteins. This was achieved by subtracting the amount of radioactivity passing through a filter with a pore size of 10,000 daltons from the raw radioactivity data. Intense protein binding of heparin was indicated by the fact that only 2% of the radioactivity present in plasma shortly after injection of the heparin fractions with molecular weight below 10,000 daltons was demonstrated in the filtrate. During elimination, filtrable radioactivity increased to levels that were comparable to desulfation data of others. Our present results show that heparins with and without affinity for antithrombin III were equally effective in releasing both lipases, which is in agreement with in vitro data of Bengtsson on binding of heparin to insolubilized lipoprotein lipase. Our data further show that release of lipolytic enzymes requires a heparin chain of sufficient length.

Elimination of lipase activities following convex curves in semilogarithmic plots dictates dose-dependent kinetics with increasing half-lives after administration of increasing submaximal doses of heparin. Such dose-dependent half-lives of H-TGL and LPL activity have been reported. Their kinetics might therefore be described by elimination mechanisms similar to what we postulated for heparin anticoagulant activity.

Although it is generally accepted that binding of heparin to lipase precedes the release of lipase, it is not precisely known whether heparin–lipase complexes circulate or whether heparin leaves the complex as soon as lipase has been released. The disappearance of lipase activity in parallel with the disappearance of protein-bound 35S-heparin radioactivity favors the concept of circulating heparin–lipase complexes. This concept is supported by data of Wallinder, who showed that partially purified rat hepatic lipase, and also a lipoprotein lipase derived from rat adipose tissue, binds avidly to isolated nonparenchymal liver cells, probably endothelial cells, that had lost their lipase content during the isolation procedure.

The observation that the lipase activity disappeared faster after administration of HA heparin than after LA heparin, even though similar amounts were released, was interesting. This observation is unexplained. A possible reason might be that LA heparin is removed faster, but this has not been studied directly.

REFERENCES

4. La Rosa JS, Levy RI, Windmueller HG, Frederickson DS:
Elimination of high affinity heparin fractions and their anticoagulant and lipase activity

CA de Swart, B Nijmeyer, LO Andersson, E Holmer, L Verschoor, BN Bouma and JJ Sixma