

COMPLEMENT INHIBITORY AND ANTICOAGULANT ACTIVITIES OF FRACTIONATED HEPARINS

W.E. Hennink, J.P.A.M. Klerx\*, H. van Dijk\* and J. Feijen  
Department of Chemical Technology, Twente University of Technology,  
P.O. Box 217, 7500 AE Enschede, The Netherlands  
\*Immunology Section, Laboratory of Microbiology, State University of  
Utrecht catharijnesingel 95, 3511 GG Utrecht, The Netherlands

(Received 4.5.1984; Accepted in original form 9.7.1984  
by Editor L. Roka)

ABSTRACT

Almost monodisperse heparin fractions ( $\bar{M}_w/\bar{M}_n < 1.1$ ) were obtained by gel filtration of a commercial heparin. These fractions were assayed for anticoagulant activity (thrombin times and APTT), chromogenic anti-factor Xa activity, inhibitory activity for the human classical complement pathway, carboxyl group content and total sulfate content. Linear relationships were observed between the molecular weight of the heparin fractions and the anti-coagulant activities as determined by thrombin time- and APTT-assay and the classical complement pathway inhibitory activity. On the other hand a hyperbolic-like relationship was observed between the molecular weight of the heparin fractions and the chromogenic anti-factor Xa activity. The heparin fractions did not show significant differences with respect to the carboxyl group and total sulfate content. Low- and high affinity heparin fractions were obtained by affinity chromatography using immobilized AT III. High- and low-affinity fractions greatly differed not only with respect to their APTT activity, but also where their complement-inhibitory activities were concerned. The latter in contrast to literature data available. These differences could not be explained by the observed differences in molecular weight of high and low affinity heparin respectively.

INTRODUCTION

Heparin, a physiological mixture of polysulfated glycosaminoglycans, has a number of biological effects (1). Most pronounced, so far, are the influence on different parts of the coagulation system and the inhibition

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Keywords: heparin, heparin fractions, anticoagulant activity, complement-inhibition, antithrombin III-Sepharose.

of the classical and alternative pathways of the complement cascade (2,3, 4). With respect to the anticoagulant activity, different structure-function relationship studies have appeared using heparin fractions of different molecular weight (e.g. 5) and affinity for antithrombin III (2). As far as complement-inhibition is concerned two comparative studies have been published dealing with heparins of different molecular weight and chemical composition, respectively (6,7). In the latter paper no correlation between anticomplementary activities on the human alternative pathway and anticoagulant activity was observed for heparins with high- and low affinity for antithrombin III. The high- and low affinity fractions were prepared by gel filtration of a heparin-antithrombin III mixture. Cofrancesco et al (8) showed that with respect to the anticomplementary activity of heparin and related polysaccharides the activity depends upon the sulfate content whereas the number of carboxyl groups does not effect this activity. No studies have been published with respect to heparin with high and low affinity for antithrombin III prepared by affinity-chromatography in relation to inhibition of the classical complement pathway.

In this paper inhibition of human complement activity by heparin fractions obtained by gel filtration and affinity chromatography on antithrombin III-linked Sepharose was investigated. The complement inhibition by the heparin fractions, tested in hemolytic assays, was compared to anticoagulant activities as determined by the thrombin time-, the chromogenic anti-factor Xa-, and the activated partial thromboplastin time (APTT) assay.

#### MATERIALS AND METHODS

##### Reagents

Heparin (170 IU/mg) from porcine mucosa was obtained from Diosynth, Oss The Netherlands. Biogel P 200 was a product of Biorad, Richmond, USA. CNBr-activated Sepharose 4B was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Purified human antithrombin III was a generous gift of the American Red Cross. Human serum, used in the complement assays, was of a male blood-group A, rhesus (D) positive volunteer and stored in lyophilized form at 4 °C. Male F1 (BALB/c x Swiss inbred) mice bred and maintained in our laboratory, were donors of mouse erythrocytes (MoE). The mice were bled by orbital puncture under ether narcosis and the blood was diluted 1 : 1 in Alsever's (isotonic glucose-citrate) solution. Sheep blood in Alsever's solution was obtained from the Rijksinstituut voor de Volksgezondheid (RIV, Bilthoven, The Netherlands) and used as source of sheep erythrocytes (ShE). Rabbit anti-ShE serum (hemolytic amboceptor) was also from the RIV. PTT-reagent was obtained from Boehringer, Mannheim, FRG. Coatest Heparin, used for the chromogenic anti-Factor Xa assay, was obtained from Kabi Vitrum, Stockholm, Sweden.

##### Fractionation of Heparin

Heparin fractions of different molecular weight and simultaneously with narrow molecular weight distributions were prepared by gel filtration using Biogel P 200. Heparin (2.5 g) was dissolved in 50 ml of an isotonic Tris buffer pH=7.5 (0.17 M NaCl-0.03 % NaN<sub>3</sub>-0.025 M Tris/HCl). One fourth

of this solution was applied to a pre-equilibrated column of Biogel P 200 (95 x 2.6 cm). Elution was performed with the same buffer at a flow rate of about 15 ml/hr at room temperature and fractions of 3.5 ml were collected. The remaining 37.5 ml heparin solution was fractionated in three different runs in the same way as described above. Corresponding fractions were combined. The heparin concentration of the different fractions was determined with the metachromic assay as described by Jaques et al (9), modified as described previously (10). Subsequent fractions were pooled till a dry weight of 0.5 g was expected. Consequently five different fractions were obtained. The pooled fractions were dialyzed against water and lyophilized. Antithrombin III was immobilized onto CNBr-activated Sepharose as described previously (10). A sample of heparin (4 ml containing 1 mg heparin per ml Tris-buffer) was passed over the AT III-Sepharose column and eluted with 2 column volumes of buffer yielding low affinity heparin fractions. High affinity heparin fractions were obtained by eluting the column with Tris-buffer containing 2.0 M NaCl. The fractions were dialyzed against water and lyophilized.

#### Determination of the Molecular Weights ( $\bar{M}_n$ and $\bar{M}_w$ ) of Heparin Fractions

Heparin molecular weights were determined by high performance liquid chromatography using a TSK SW 3000 column (600 x 7.5 mm, Toyosoda, Japan). Elution was performed at room temperature at a flow rate of 1.0 ml/min using 0.1 M NaAC buffer, adjusted with HAC to pH 6.0, as mobile phase. The SW 3000 column was calibrated with heparin fractions of known  $\bar{M}_n$ ,  $\bar{M}_w$  and molecular weight distribution. These values were obtained by a combination of GPC (gel permeation chromatography) and LALLS (low angle laser light scattering) measurements (11). Average molecular weights (weight average ( $\bar{M}_w$ ) and number average ( $\bar{M}_n$ )) of the heparin fractions used in this study were calculated from the chromatographic data according to the procedure described by Yau et al (12).

#### Determination of the Carboxyl Group- and Total Sulfate Content of Heparin Fractions

The carboxyl group- and sulfate contents of the heparin fractions were determined by conductimetric titration as described by Ebert et al (13).

#### Complement Inhibition

For testing of the classical pathway activity in human serum ShE were used. They were washed 3 times with isotonic sodium iodide (14) to elute adsorbed serum proteins (15,16). The cells were resuspended in veronal-buffered (5 mM) saline, pH=7.4, containing 0.15 mM  $\text{Ca}^{2+}$  and 0.5 mM  $\text{Mg}^{2+}$  (VSB $^{2+}$ ; 17,18) to a final number of  $3 \times 10^8$ /ml. The suspension was mixed with an equal volume of 1:2,000 diluted, heat inactivated (56 °C, 30 min) hemolytic amboceptor and incubated for 10 min at room temperature (19). After washing, the sensitized ShE (ShEA) were resuspended to a number of  $1.5 \times 10^8$  per ml in VSB $^{2+}$ .

For determination of human alternative pathway activity unsensitized

MoE were used. After washing for three times in isotonic sodium iodide, they were suspended to a concentration of 2% (v/v) in veronal buffered (5 mM) saline containing 8 mM ethyleneglycol-bis(aminoethyl)tetracetic acid (EGTA) and 2.5 mM Mg<sup>2+</sup>, pH=7.4 (EGTA-VB;20,21).

Complement activities were determined as described (18,22). In brief: 200 µl of human serum, serially diluted in VSB<sup>2+</sup> (classical pathway) or EGTA-VB (alternative pathway) were mixed with 100 µl of ShEA in VSB<sup>2+</sup> (classical pathway) or MoE in EGTA-VB (alternative pathway) and incubated in a waterbath for 60 min (classical pathway) or 30 min (alternative pathway) at 37 °C. To stop the lytic reaction 2.2 ml of ice-cold VSB<sup>2+</sup> (classical pathway) or EDTA-GVB (alternative pathway) were added, the intact cells and ghosts were spun down and the extinction at 412 nm (E412) of the supernatant (I) was measured. The percentage of lysis was calculated by the formula:

$$Y=(I-II)/(III-II)\times 100 \%$$

in which II further refers to the E412 of a buffer control (0 % lysis) and III to the E412 of a water lysed control for 100 % hemolysis. To determine the amount of a serum giving rise to 50 % hemolysis (one unit), microliters of serum were logarithmically plotted against log Y/(100-Y) according to van Krogh (17). The activities of the classical and alternative complement pathway were expressed in CH50 and AP50 units, respectively, per ml serum. All tests were performed in duplicate. To quantify complement inhibition, the assay was performed in the absence or presence of logarithmically increasing amounts of unfractionated heparin or heparin fractions. Complement inhibitory activity of (the) heparin (fractions) was expressed in equivalents causing 50 % decrease of classical complement pathway activity (IC50 units) or alternative complement pathway activity (IA50 units) per mg lyophilized material (23).

#### Anticoagulant Activity of Heparin Fractions

The anticoagulant activity of the heparin fractions obtained with Biogel P 200 was determined using the thrombin time assay (10) and the activated partial thromboplastin time (APTT) assay (10). The chromogenic anti-factor Xa assay was performed as described by Teien et al (24). Anticoagulant activity of low- and high affinity heparin was determined with the APTT assay only. Unfractionated heparin (170 IU/mg) was used as reference.

### RESULTS

Heparin was fractionated by gel filtration over a Biogel P 200 column. The elution pattern and the areas corresponding to the five final fractions are shown in figure 1.

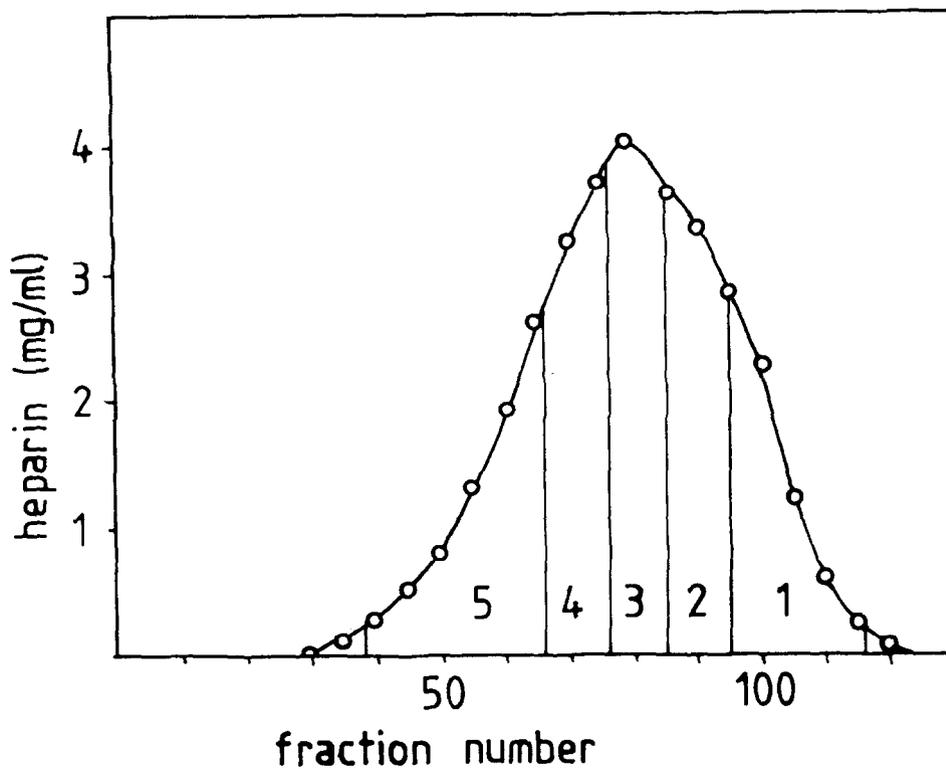


FIG. 1.

Elution profile of heparin on Biogel P 200. 1 to 5 correspond to the final pooled fractions.

The molecular weights ( $\bar{M}_n$  and  $\bar{M}_w$ ) of unfractionated heparin and the five fractions as well as the biological activities and the carboxyl group- and sulfate content are given in table 1.

TABLE 1

Molecular Weights, Anticoagulant Activities, Chromogenic Anti-Factor Xa Activity, Inhibitory Activity for the Human Classical Complement Pathway And Carboxyl Group- and Total Sulfate Content of Unfractionated Heparin (Diosynth) and Heparin Fractions.

fraction number	$\bar{M}_n$	$\bar{M}_w$	$\bar{M}_w/\bar{M}_n$	APTT (IU/mg)	thrombin time (IU/mg)	anti-Xa (IU/mg)	classical pathway inhibition (IC50 units/mg)
1	6.100	6.900	1.13	27	17	88	50
2	10.000	10.600	1.06	117	52	157	69
3	13.400	13.900	1.04	177	201	182	84
4	16.600	17.100	1.03	227	268	206	138
5	20.800	21.500	1.03	261	352	201	133
Diosynth	11.100	13.800	1.24	170	170	170	100

fraction number	carboxyl group content ( $\mu\text{mol/mg}$ )	total sulfate content ( $\mu\text{mol/mg}$ )
1	1.57 $\pm$ 0.17	2.91 $\pm$ 0.11
2	1.42 $\pm$ 0.10	2.96 $\pm$ 0.10
3	1.42 $\pm$ 0.13	3.05 $\pm$ 0.12
4	1.43 $\pm$ 0.13	3.06 $\pm$ 0.10
5	1.42 $\pm$ 0.08	3.10 $\pm$ 0.05
Diosynth	1.50 $\pm$ 0.10	3.13 $\pm$ 0.07

Between the number average molecular weight ( $\bar{M}_n$ ) of the heparin fractions and the APTT-, thrombin time- and complement-inhibitory (classical pathway) activities, linear relationship exists. The correlation coefficients are 0.982, 0.982 and 0.938 respectively. A nonlinear more hyperbolic type relationship is observed for the anti-factor Xa activity of the heparin fractions as function of the molecular weight (both  $\bar{M}_n$  and  $\bar{M}_w$ ). With respect to the correlation coefficient between either the APTT-, the thrombin time- or the anti-factor Xa activities and the classical complement pathway inhibition of the heparin fractions, the APTT test gave the highest value ( $r=0.934$ ). Figure 2 shows the APTT activity and classical complement inhibitory activity as a function of the number average molecular weight of the heparin fractions.

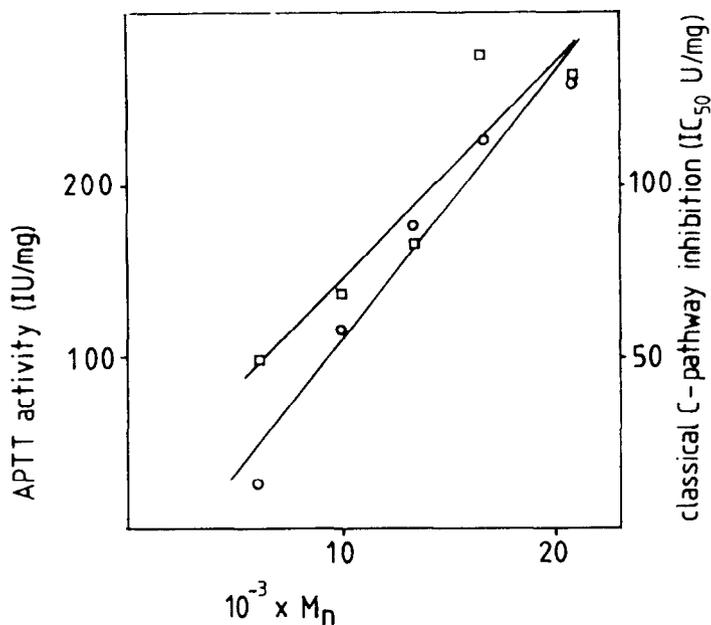


FIG. 2

Relation between the number average molecular weight ( $\bar{M}_n$ ) of heparin fractions and the classical complement inhibitory activity ( $\square$ ) and the APTT activity ( $\circ$ ).

In further experiments heparin was separated over AT III-Sepharose in a high- and low affinity fraction. The yields of these fractions were about 40 and 60 percent (w/w) of unfractionated heparin respectively. Low- and high affinity heparin were assayed for APTT activity, classical and alternative complement inhibitory activity, carboxyl group- and sulfate content. In addition the molecular weight of these fractions were determined. The results are given in table 2.

TABLE 2.

APTT Activities, Anti-Complement Activity,  $\bar{M}_w$ ,  $\bar{M}_n$ , Carboxyl Group and Total Sulfate Content of Heparin Fractionated By Affinity Chromatography on AT III-Sephrose.

heparin fraction	$\bar{M}_w$	$\bar{M}_n$	$\bar{M}_w/\bar{M}_n$	APTT (IU/mg)
low affinity ×	12.600	10.100	1.25	15
high affinity ××	15.000	12.800	1.17	273
unfractionated +	N.D.	N.D.		181
reconstituted mixture ++	N.D.	N.D.		141

heparin fraction	complement classical pathway (IC50 U/mg)	inhibition alternative pathway (IA50 U/mg)	carboxyl group content ( $\mu\text{mol/mg}$ )	sulfate content ( $\mu\text{mol/mg}$ )
low ×	98	15	1.68±0.12	3.26±0.12
high ××	186	91	1.67±0.16	3.28±0.15
unfractionated +	118	28	N.D.	N.D.
reconstituted mixture ++	124	34	N.D.	N.D.

× yield about 60 % of unfractionated material

×× yield about 40 % of unfractionated material

+ Diosynth heparin after dialysis

++ consisting of 60 % (w/w) low- and 40 % (w/w) high affinity material

Compared with the  $\bar{M}_w$  and  $\bar{M}_n$  unfractionated heparin (table 1), high affinity heparin has a slightly higher molecular weight (both  $\bar{M}_w$  and  $\bar{M}_n$ ), whereas these values are somewhat decreased for low affinity heparin. In agreement with literature data, high- and low affinity heparin have a high and low APTT activity respectively. Surprisingly, low- and high-affinity heparin also have different anti-complementary (both classical and alternative) activities.

#### DISCUSSION

This paper describes the comparison of the anticoagulant effect and the inhibition of human complement by different heparin fractions prepared by

gel filtration and affinity chromatography on AT III-Sepharose. Inhibition of the classical complement pathway may be based upon different phenomena, such as calcium chelation (25), binding of C1 (26,27) so that the attachment and activation of C4 and C2 is prohibited and potentiation of C1-inhibitor (28,29). Heparin is also a regulator of the human alternative complement pathway, a phenomenon ascribed to potentiation of the regulatory activity of factor H(4) and to interference with binding of factor B to activated C3 in the formation of the alternative pathway C3-convertase (3). Our results with respect to the anticoagulant activity of heparin fractions are greatly in line with those of others, both when heparin is separated in fractions of different molecular weights obtained by preparative gel filtration (5,6,30,31) and when fractions of different affinity for antithrombin III are concerned (6,32,33).

With respect to complement inhibition by heparin fractions of different molecular weight, Bianchini et al (6) demonstrated that fractions with higher molecular weights have higher anti-complement (classical pathway) activity, which is in agreement with our results. However, since Bianchini et al used another technique for the determination of the molecular weight of heparin fractions, no quantitative comparison with our data can be given. Cofrancesco et al (8) demonstrated that the total sulfate content is an important parameter for the anti-complement activity of heparin and related glycosaminoglycans. The heparin fractions obtained by gel filtration do not differ significantly from each other with respect to total sulfate content, whereas significant differences in anti-complement activity between these fractions are observed (table 1). This indicates that other factors than the total sulfate content seem to govern the anti-complementary activity of heparin.

When heparin is fractionated by affinity chromatography on immobilized AT III, a low- and high affinity heparin fraction is obtained. As described before, these fractions differ substantially from each other with respect to anticoagulant activity (6,32,33). Besides a fractionation in affinity for AT III, also a fractionation in molecular weight is obtained (table 2), using the AT III affinity chromatography technique. This is also observed by Laurent et al (30) and can be explained by the results of Danielsson et al (34) who showed that the ratio high affinity/low affinity of heparin samples is a function of the molecular weight. Our data with respect to the anti-complementary activities of high- and low affinity heparin fractions are at variance with those of Kazatchkine et al (7), who did not find differences in the alternative complement inhibitory activity of high- and low affinity heparin. In addition the differences in anti-complementary activity of high- and low affinity heparin cannot be related to differences in molecular weights or differences in carboxyl group- and/or sulfate content between low- and high affinity heparin (table 2). We find a close correlation between the anticoagulant activity and anti-complement activities of low- and high affinity heparin. This suggests that the structure(s) of heparin involved in binding to AT III may also be responsible for high anticomplementary activity. Another explanation, which is only valid for the classical complement pathway inhibition, might be that complexes of high affinity heparin and AT III function as a C1-esterase inhibitor (35) resulting in a higher specific anti-complement activity for high affinity heparin compared with low affinity heparin.

Our results stress that inhibition of human complement may be an useful additional parameter for heparin.

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