Journal of Chromatography, 297 (1984) 351–358
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 345

HIGH-PRESSURE SIZE-EXCLUSION CHROMATOGRAPHY OF ANTICOAGULANT MATERIALS

D. MULLER*, M. NDOUME-NZE and J. JOZEFONVICZ
Laboratoire de Recherches sur les Macromolécules, Département de Chimie, Université Paris-Nord, Avenue J. B. Clément, 93430 Villetaneuse (France)

SUMMARY

A comparative determination of molecular weights of anticoagulant materials is made on different types of high-performance liquid chromatographic stationary phases in aqueous media. The influence of different elution parameters is reported. Narrow-molecular-weight-distribution polystyrenesulphonates are used as standards for the calibration curves. Slight retentions are observed on unmodified silica supports. The commercially available grafted silica columns give similar results for the molecular weight values of heparin. A retention of pentosan polysulphate is observed on a TSK column. However, the size of this anticoagulant polymer is determined on the other grafted silica columns.

INTRODUCTION

The observed correlations between anticoagulant activity and molecular weight of heparin fractions[1–7] has increased the importance of molecular weight determination of anticoagulant polymers by chromatographic methods. It was demonstrated that heparin can be separated by low-pressure gel-permeation chromatography, for example on Sephadex resins, into fractions of different molecular weight and anticoagulant activity[8].

High-performance liquid chromatography (HPLC) was also used to accomplish a fractionation of heparin on unmodified porous silica[9]. Using dextrans as molecular weight standards, Sugisaka and Pètracek[10] have determined the molecular weight of heparin samples on an HPLC silica column. A glycophase column was used to compare the molecular sizes of different fractions of heparin[11,12]. Recently, high-pressure size-exclusion chromatography (HPSEC) was performed with different heparin samples on a grafted TSK silica column using oligosaccharides as calibration standards[13]. These different studies show the importance of the chemical nature of both standards and stationary phases for the determination of molecular weight.

Pentosan polysulphate (PPS) is also an anticoagulant material[14]. The molecular weight of this medicinal product is not well defined.

These different results concerning both heparin and PPS led us to determine the conditions for HPSEC molecular weight calculation of anticoagulant polymers

0021-9673/84/$03.00

TABLE I

CHARACTERISTICS OF HPSEC COLUMNS

V_0 = Void volume; V_t = total exclusion volume; V_p = internal volume.

Column	*Dimensions (mm)*	V_0 *(ml)*	V_t *(ml)*	V_p *(ml)*	*Exclusion limits of dextrans (daltons)*	*Column efficiency (plates/m)*	*Supplied*
LiChrospher Si 100	300 × 4.8	2.20	4.10	1.90	$10^3/7 \cdot 10^4$	8000	Merck
LiChrospher Si 100	150 × 4.8	1.26	2.25	0.99	$2 \cdot 10^3/7 \cdot 10^4$	8000	Merck
LiChrosorb Si 100	300 × 4.8	2.25	4.12	1.87	$10^3/7 \cdot 10^4$	6000	Merck
Protein I 60	300 × 7.8	6.25	10.0	3.75	$6 \cdot 10^2/10^4$	15,000	Waters
Protein I 60 + I 125	2 (300 × 7.8)	12.0	20.0	8.0	$6 \cdot 10^2/3 \cdot 10^4$	15,000	Waters
LiChrospher Si 100 diol	250 × 4.0	1.42	2.83	1.41	$10^3/7 \cdot 10^4$	17,000	Merck
TSK-G SW 2000	600 × 7.5	11.03	21.73	10.70	$10^3/4 \cdot 10^4$	11,000	LKB

on different HPLC stationary phases. On several unmodified and grafted silica columns we studied successively the influence of different factors on the elution properties: the ionic strength of the eluent solution, the amount of injected polymer and the nature of the stationary phase. The selected molecular weight standards are generally sodium polystyrenesulphonates (PSSs), because they have a small polydispersity and a polyanionic structure due to the sulphonate substituents, similar to those of the anticoagulant products.

EXPERIMENTAL

The Waters chromatographic system was an M6000 pump and a U6K injector with a R401 differential refractometer connected to a pen recorder and to a Tectronix tape recorder. The chromatographic data were treated by a GPC program on a Tectronix 4051 calculator to obtain the weight-average molecular weight, M_w, the number-average molecular weight, M_n, the polydispersity, I, and finally the distribution curves of the weight fractions. The samples were also characterized by the molecular weight, M_p, determined at the maximum of each peak.

The characteristics of the different columns are listed in Table I. The laboratory-prepared unmodified silica columns were packed by the slurry method[15]. The grafted silica columns used were the Merck LiChrospher Si 100 diol, the Waters Protein I 60 and I 125 and the TSK SW 2000 provided by LKB.

The eluents, 0.2 M $NaNO_3$ or 0.2 M NaCl, were used at flow-rate 1 ml/min and 25°C.

Chemicals

Distilled water was passed through a mixed-bed ion exchanger. The solutions were degassed and filtered through a 0.45-μm Millipore membrane. Sodium chloride and sodium nitrate were analytical grade. The molecular weight standard polystyrenesulphonates (PSSs) were provided by Pressure Chemical Co. (Pittsburg, PA, U.S.A.).

The heparin (HEP) samples were kindly provided by D. Labarre or by R. D. Rosenberg or by Choay S.A. (Paris, France).

Pentosan polysulphonate (PPS) lot Y 10 is a medicinal-grade anticoagulant, kindly provided by Sanofi (Toulouse, France).

RESULTS AND DISCUSSION

Typical chromatograms of anticoagulant materials HEP and PPS are shown in Fig. 1.

Influence of ionic strength

The influence of ionic strength on elution is shown in Fig. 2. As previously noted[10], this effect reflects the expansion of the anionic macromolecule in water due to intramolecular electrostatic repulsions[9,17]. This is also reflected by the variation in viscosity of such polymers (Fig. 3). The nature of the sodium salt (NaCl, $NaNO_3$ or Na_2SO_4), at the same ionic strength, does not seem to affect the elution volume.

The polyanionic character of the anticoagulant materials led us to work with controlled ionic strength solutions (0.2 M NaCl or 0.2 M $NaNO_3$).

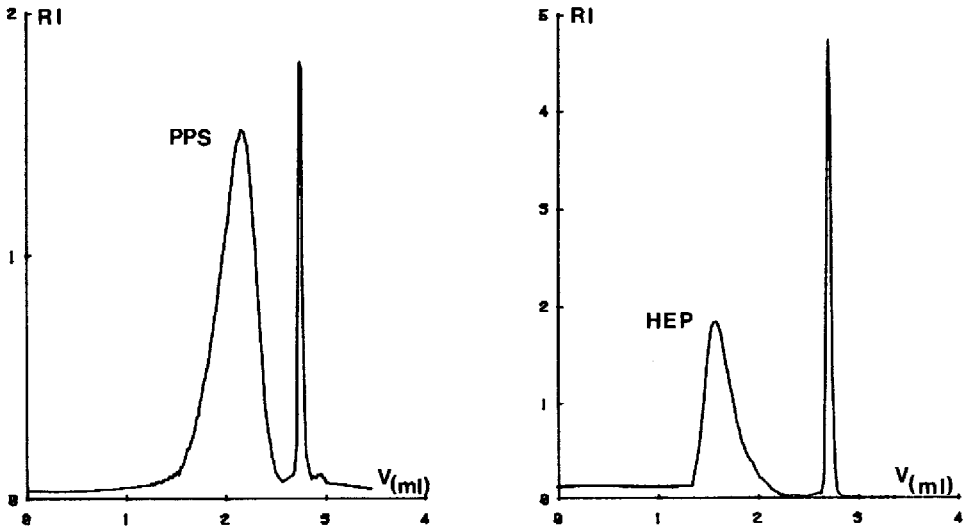

Fig. 1. Elution curves of anticoagulant materials (20 μg of each). Column: LiChrospher Si 100 diol. Eluent: 0.2 *M* NaCl, 1 ml/min. Temperature: 25°C. RI = Refractive index; *V* = elution volume.

Influence of the polymer load

The elution volume depends also on the amount of polymer injected into the chromatographic system (Fig. 4). Similar behaviour was previously observed[18,19], even with low concentrations of polymer. This effect could be explained by a longitudinal stretching of the macromolecule with the solvent flow in the pores of the stationary phase[20]. Consequently, the elution volumes for the molecular weight de-

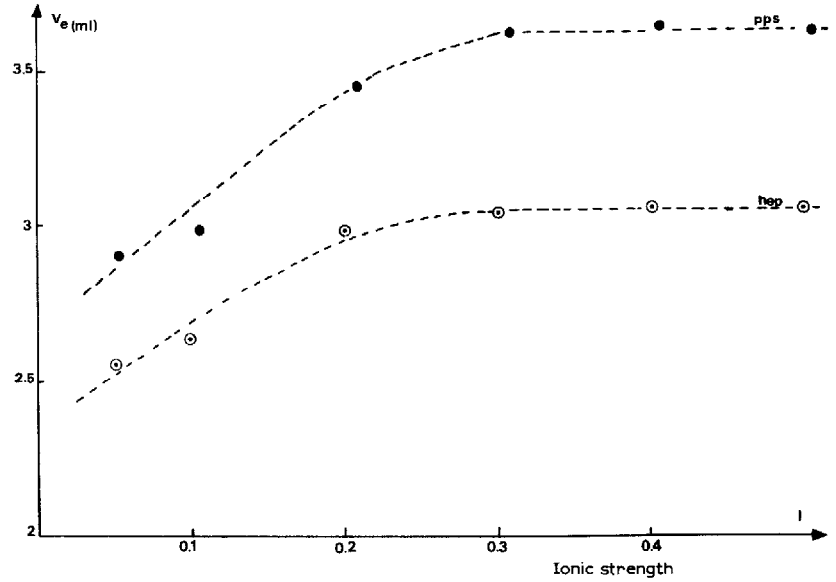

Fig. 2. Influence of ionic strength on elution volume (V_e) of pentosan polysulphate (●) and heparin (⊙). Column: LiChrospher Si 100. Eluent: NaCl. Temperature: 25°C.

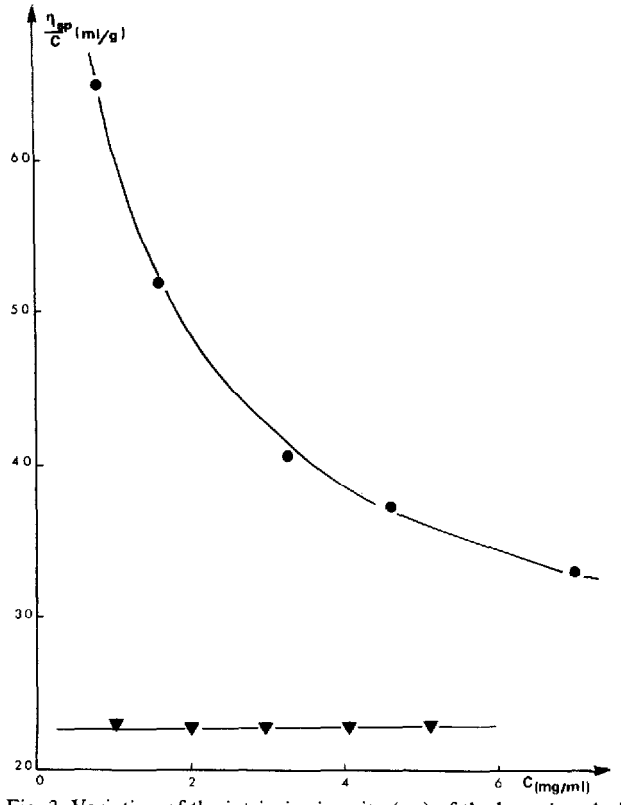

Fig. 3. Variation of the intrinsic viscosity (η_{sp}) of the heparin solution with the concentration (C) at 25°C. Flow-rate, 1 ml/min. ●, Water; ▼, 0.2 M NaCl.

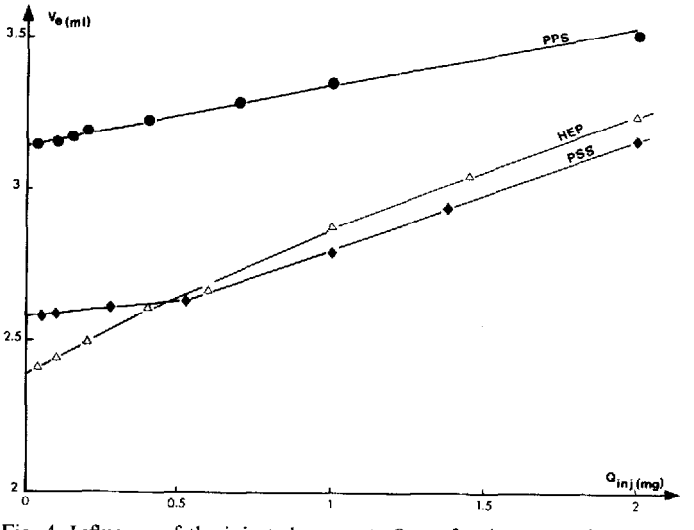

Fig. 4. Influence of the injected amount, Q_{inj}, of polymer on the elution volume (V_e) of pentosan polysulphate (●), heparin (△) and polystyrenesulphonates (◆). Conditions as in Fig. 1.

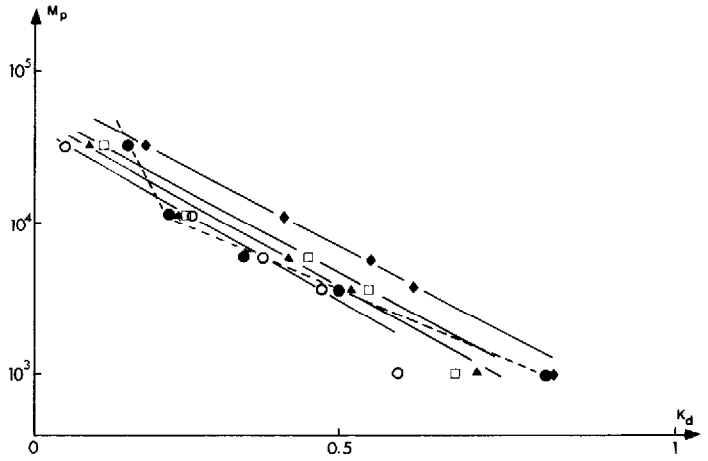

Fig. 5. Calibration curves with sodium polystyrenesulphonate standards on LiChrospher Si 100 (○), LiChrosorb Si 100 (●), protein I 60 + I 125 (▲), TSK SW 2000 (□) and LiChrospher Si 100 diol (◆) columns. Eluent: 0.2 *M* NaCl, 1 ml/min. Temperature: 25°C. $K_d = (V - V_0)/V_p$.

termination were extrapolated at infinite dilution and the elution curves were obtained with small loads of polymer.

Influence of the nature of the stationary phase

The effects of ionic strength and load are similar for the different HPLC columns. Consequently, we chose standard elution conditions (0.2 *M* NaCl, 25°C, flow-rate 1 ml/min, load 20 μg) to study the influence of the nature of the stationary phase on elution of anticoagulant samples.

It is relatively difficult to compare the chromatographic performances of the different columns (Table I) because of their different geometries and the unknown nature of the grafted molecule for some commercial grafted silica columns. The calibration curves for PSS standards on the different phases are shown in Fig. 5. It should be noted that retention is observed for PSSs, on the TSK support, in contrast to dextran standards[21].

The molecular weights of anticoagulant samples were determined with the different columns (Table II). The smaller molecular weight values of HEP and PPS on the pure silica columns, LiChrospher and LiChrosorb Si 100, indicate abnormal retentions, probably due to hydrogen interactions between silanol groups of the silica beads and the macromolecule. The regular geometry of LiChrospher silica does not

TABLE II

MOLECULAR WEIGHTS, M_p AT THE MAXIMUM OF EACH PEAK, ON DIFFERENT HPLC COLUMNS

Elution conditions: 0.2 *M* NaCl; flow-rate 1 ml/min; load 20 μg.

Sample	*LiChrospher Si 100*	*LiChrosorb Si 100*	*Protein I 60 + I 125*	*LiChrospher Si 100 diol*	*TSK SW 2000*
HEP RB 19207	15,000	15,500	22,000	21,000	20,000
PSP 10	3000	2700	4100	4000	2500

improve elution. The different grafted columns give similar molecular weight values for the same heparin sample.

The molecular weight of PPS, determined on the LiChrospher Si 100 diol and on Protein I 60 + I 125 columns, are similarly around 4000 daltons. Retention is observed on TSK resin, and the apparent molecular weight is abnormally small. This could be explained by hydrophobic interactions taking place between the grafted silica column and the PPS polyanion[22]. However, the molecular weight for PPS is near the exclusion limit of the different columns (see Table I). Thus, the determined values of M_p are not very accurate.

The average molecular weights[23]; polydispersity and anticoagulant activity of different heparin samples, determined on a LiChrospher Si 100 diol column, are presented in Table III. Although comparison of the properties of the different heparin samples does not lead to any definitive conclusion, it is nevertheless possible to observe that the most active samples are in the range of 20,000 daltons. Moreover, the influence of isomolecularity observed, for example for heparin R2, probably reflects the importance of purification for the anticoagulant activity of heparin.

CONCLUSIONS

Comparison of the molecular weight of anticoagulant materials (HEP and PPS) demonstrates clearly the existence of interactions between the polyanionic macromolecule and the unmodified silica support. These interactions are probably due to silanol groups on the surface of the silica.

The different commercial grafted silica columns, under the selected experimental conditions (ionic strength, concentration), give similar molecular weight values for heparin. The anticoagulant activity of the different heparin samples seems to be related to both the molecular weight and the purification procedure of the anticoagulant polymer. Pentosan polysulphate appears to be a smaller-molecular-weight anticoagulant material, around 4000 daltons.

TABLE III

AVERAGE MOLECULAR WEIGHTS, POLYDISPERSITY AND ANTICOAGULANT ACTIVITY OF DIFFERENT ANTICOAGULANT MATERIALS DETERMINED BY HPSEC

Column: LiChrospher Si 100 diol. Eluent: NaCl 0.2 *M*; flow-rate 1 ml/min. Temperature: 25°C.

Sample	$\bar{M}_w$	$\bar{M}_n$	$I = \bar{M}_w/\bar{M}_n$	*Anticoagulant activity*
HEP RB 19207	22,800	17,700	1.29	156*
HEP RB 19227	21,000	12,800	1.64	34*
HEP RB 19229	18,000	12,400	1.45	115*
HEP P 191CH	7500	4700	1.60	70*
HEP R1	13,950	10.950	1.27	187**
HEP R2	17,600	15,300	1.15	264**
HEP DL1	16,700	9100	1.8	
HEP DL2	9400	3300	2.8	
PSP	3700	3900	1.46	

* Anticoagulant activity of 1 mg of sample (U/mg) determined by United States Pharmacopoeia method[25].

** Anticoagulant activity of 1 mg of sample (U/mg) determined with S2160 (Ortho Diagnostics) and pure proteins[6,24].

REFERENCES

1 T. C. Laurent, *Arch. Biochem. Biophys.*, 92 (1961) 224.
2 J. A. Cifonelli, *Carbohyd. Res.*, 37 (1974) 145.
3 L. H. Lam, J. E. Silbert and R. D. Rosenberg, *Biophys. Biochem. Res. Commun.*, 69 (1976) 570.
4 L. O. Andersonn, T. W. Barrowcliffe, E. Holmer, E. A. Johnson and G. E. C. Sims, *Thromb. Res.*, 9 (1976) 575.
5 M. Höök, I. Björk, J. Hopwood and U. Lindahl, *FEBS Lett.*, 66 (1976) 90.
6 L. Thunberg, U. Lindahl A. Tengblad, T. C. Laurent and C. M. Jackson, *Biochem. J.*, 181 (1979) 241.
7 E. Sache, M. Maillard, H. Bertrand, M. Maman, M. Kunz, J. Choay, J. Fareed and H. Messmore, *Thromb. Res.*, 25 (1982) 443.
8 T. W. Barrowcliffe, E. A. Johnson and D. P. Thomas, *Brit. Med. Bull.*, 34 (1978) 143.
9 F. A. Buytenhuys and F. P. B. van der Maeden, *J. Chromatogr.*, 149 (1978) 489.
10 N. Sugisaka and F. J. Petracek, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 36 (1977) 89.
11 H. J. Rodriguez and A. J. Vanderwielen, *J. Pharm. Sci.*, 68 (1979) 588.
12 L. N. L. Teng and D. C. Teller, in R. L. Lundblad, W. V. Brown, K. G. Mann and H. R. Roberts (Editors), *Chemistry and Biology of Heparin*, Elsevier, Amsterdam, 1981, p. 65.
13 J. Harenberg and J. X. de Vries, *J. Chromatogr.*, 261 (1983) 287.
14 A. M. Fischer, R. E. Merton, N. A. Marsh, S. Williams, P. F. Gaffney, T. W. Barrowcliffe and D. P. Thomas, *Thromb. Haemostasis*, 47 (1982) 109.
15 R. E. Majors, *Anal. Chem.*, 44 (1982) 1722.
16 D. Labarre, *Thesis*, Paris, 1977.
17 M. Rinaudo and J. Desbrieres, *Eur. Polym. J.*, 16 (1980) 849.
18 T. Bleha, D. Bakos and D. Derex, *Polymer*, 18 (1977) 897.
19 A. Rudin, *Polym. J.*, 11 (1979) 123.
20 J. P. Cotton, *Thesis*, Paris, 1973.
21 D. Muller, M. Ndoume-Nze and J. Jozefonvicz, in preparation.
22 F. Roumeliotis and K. K. Unger, *J. Chromatogr.*, 218 (1981) 535.
23 P. J. Flory, *The Principles of Polymer Chemistry*, Cornell University Press, Ithaca, NY, 1953.
24 R. E. Jordan, L. V. Favreau, E. N. Braswell and R. D. Rosenberg, *J. Biol. Chem.*, 257 (ø1982) 400.
25 D. R. Bangham and P. M. Woodward, *Bull. W.H.O.*, 42 (1970) 129.