Journal of Chromatography A, 657 (1993) 33–41
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 539

Determination of the molecular mass of heparin samples by size-exclusion chromatography applying non-identical standards

M. Bergman*, R. Dohmen, H.A. Claessens and C.A. Cramers

Laboratory of Instrumental Analysis, Eindhoven University of Technology, P.O. Box 513, 5600 MB Eindhoven (Netherlands)

(First received March 19th, 1993; revised manuscript received July 5th, 1993)

ABSTRACT

The influence of three experimental parameters, temperature, pH and ionic strength of the eluent, on the retention of specific heparin samples and polysaccharides as calibration standards in size-exclusion chromatography was investigated applying chemometric techniques. The results allowed the adjustment of the retention volumes of the studied heparin samples in such a way that the average molecular masses of these compounds could be calculated using polysaccharides as the standards. This approach shows the possibilities of determining average molecular masses of biopolymers using non-identical commercially available standards.

INTRODUCTION

The anticoagulant heparin is a polydisperse polysaccharide that is widely used therapeutically for the prevention of thrombo-embolic disorders. Heparin is a copolymer of uronic acid (glucuronic acid or iduronic acid) and glucosamine, either N-acetylated or N-sulphated. It is highly substituted with O-sulfate residues at the 6-positions of the glucosamine residues and at the 2-positions of the iduronic acid residues [1–3].

It has been shown that a relationship between the molecular mass and the biological activity of heparin [4] exists. Therefore, the characterization of heparin samples by the determination of the average molecular masses and molecular mass distributions of heparin samples is very important. One of the techniques suitable to provide this information is size-exclusion chromatography (SEC) [5–10]. However, a disadvantage of this technique is the lack of well defined heparin standards with narrow molecular mass distributions. In some reports the use of these standards was mentioned to determine the average molecular mass [number-average (M_n) or mass average (M_w)] and the molecular mass distribution of heparin samples [5,7–10]. However, these heparin standards are not yet commercially available.

A possible solution to overcome this general problem is the use of standards not similar to heparin, *e.g.*, polystyrene sulphonates [6]. These standards can be used to obtain an estimate of the average molecular mass (M_n or M_w) and the molecular mass distribution of biopolymer samples such as heparins. A problem may occur when an accurate determination of the average molecular mass or the molecular mass distribution of the biopolymer sample must be made. SEC is based on differences in molecular sizes [10], and not on differences in molecular masses. It is well known that different types of polymers

* Corresponding author.

0021-9673/93/$06.00

of the same molecular mass, may have different conformational structures and consequentially show different molecular sizes. Also, these conformational structures may depend on a number of experimental conditions. Therefore, the calibration of the SEC analysis of biopolymers such as heparins with non-identical standards may lead to erroneous results.

An improvement can be achieved by the use of calibration standards belonging to the same chemical group as the sample substances. For example, from a physico-chemical point of view, commercially available polysaccharides are relative similar to heparin molecules. However, the charged groups on the heparin molecules may influence considerably the hydrodynamic volume of these molecules. This is mainly caused by the mutual electrostatic repulsion of the negatively charged groups on the heparin molecules. Therefore, the hydrodynamic volume of polyelectrolytes can be influenced by the experimental conditions. The effect of ionic strength and pH on the conformation of polyelectrolytes in SEC has already been studied [11–14]. However, polystyrene sulphonates [12–14] and proteins [11] were used.

In this study, an attempt was made to reduce the difference in hydrodynamic volume between a number of standard polysaccharide molecules and the studied heparin molecules by manipulating a number of experimental conditions. Three experimental parameters, ionic strength, pH and temperature of the eluent, were investigated. The influence of the investigated parameters on the retention volume was studied by applying chemometric techniques. Finally, with these data the molecular mass distribution of the studied heparin samples was calculated.

EXPERIMENTAL

Equipment

The chromatographic system consisted of a Model 100A HPLC pump (Beckman, Palo Alto, CA, USA) with a Model R-401 differential refractometer detector (Waters Millipore, Milford, MA, USA). All experiments were performed on a Zorbax GF-250 column (200 × 9.4 mm I.D.) (Rockland Technologies, Newport, DE, USA). The column was packed with 5-μm silica-based particles with a 150 Å pore size (pH range 3–8.5). The column was thermostated with a laboratory-constructed water-jacket, connected with an ultra-thermostat (Colora, Germany). Injections were made with a Model 7125 injector (Rheodyne, Cotati, CA, USA) equipped with a 20-μl injection loop. A guard column (PL Aquagel, 75 × 7.5 mm I.D.) (Polymer Laboratories, Church Stretton, Shropshire, UK) was placed between the injector and the Zorbax column.

Chemicals

The heparin samples and heparin standards used were a kind gift from Professor Dr. H.C. Hemker of the Department of Biochemistry, University of Limburg, (Maastricht, Netherlands). Two different heparin samples were used, Fraxiparine (Sanofi, Maassluis, Netherlands) with an average molecular mass of 5090 g/mol [15], and Calparine (Sanofi) with an unknown average molecular mass. The heparin standards, with mass-average molecular masses of 3000, 4200, 6000, 9200 and 11200 g/mol, were prepared by fractionation of a low-molecular-mass heparin (enoxaparin) by gel permeation chromatography [16]. The polysaccharide standards of M_w 5800, 12 200, 23 700, 48 000 and 100 000 g/mol were obtained from Polymer Labs. Sodium dihydrogenphosphate and sodium chloride were purchased from Merck (Darmstadt, Germany). Water was demineralized with a Milli-Q water-purification system (Waters Millipore). The eluents were filtrated over a 0.45-μm membrane filter and degassed with helium prior to use.

In all analyses, the concentration of the samples and the injection volume were kept constant.

RESULTS AND DISCUSSION

In the linear range of a specific size-exclusion column, the retention volume V_r is a parameter for the molecular size of a compound. Therefore, in this work the influence of the ionic strength, pH and temperature of the eluent on

TABLE I

EXPERIMENTAL SETTINGS USED IN THE CENTRAL COMPOSITE DESIGN FOR THE DETERMINATION OF THE INFLUENCE OF THE IONIC STRENGTH, TEMPERATURE AND pH OF THE ELUENT ON THE RETENTION VOLUME OF HEPARIN

Setting No.	Temperature (°C)	pH	Ionic strength
1	25	4	0.01
2	25	4	1
3	25	7	1
4	45	7	1
5	45	4	1
6	45	7	0.01
7	25	7	0.01
8	45	4	0.01
9	35	5.5	0.1
10	35	5.5	2
11	35	5.5	0.005
12	35	7.45	0.1
13	35	3.55	0.1
14	48	5.5	0.1
15	22	5.5	0.1

TABLE II

RESULTS OF THE ANALYSIS OF VARIANCE (ANOVA) OF THE RETENTION MODEL FOR HEPARIN

The total sum of squares [SS(Total)] is calculated by comparing the experimental and calculated data. The total sum of squares can be divided into a contribution of the mean [SS(Mean)] and a contribution of the correction [SS(Corrected)]. The latter can be considered as the sum of the contribution of the factors [SS(Factors)] and a residual sum of squares [SS(Residue)]. The latter value can be separated into a sum of squares caused by pure experimental error [SS(Pure Error)] and by lack of fit [SS(Lack of Fit)]. DF = degrees of freedom.

Sums of squares (SS)	Fraxiparine (M_w = 5090 g/mol)	Heparin standard (M_w = 9200 g/mol)
SS(Total)	4742.7 (41 DF)	2274.1 (22 DF)
SS(Mean)	4658.85 (1 DF)	2250.69 (1 DF)
SS(Corrected)	83.85 (40 DF)	23.45 (21 DF)
SS(Factors)	83.7861 (9 DF)	23.41 (4 DF)
SS(Residue)	0.05868 (31 DF)	0.03605 (17 DF)
SS(Pure Error)	0.04048 (15 DF)	0.01280 (9 DF)
SS(Lack of Fit)	0.01820 (16 DF)	0.02325 (8 (DF)

the retention volume of heparins and polysaccharides was investigated.

Heparin model

First the influence of the experimental parameters on the retention volume of both Fraxiparine and the heparin standard with an average molecular mass of 9200 g/mol was investigated. Then the remaining heparin standards were used to validate the method. A central composite design was used to determine these influences more rapidly. The design is shown in Table I. All levels were measured twice, except for the centre point, which was measured ten times. The pure experimental error could be calculated from the measurements in the centre point. The influences of the investigated parameters were calculated from the results of the experiments under different conditions. For Fraxiparine (M_w = 5090, with a peak maximum at molecular mass 4500 g/mol [15]), the equation describing the dependence of the retention volume of this heparin on these parameters is

$$V_R = 25.845 - 0.422T - 5.131 \cdot \mathrm{pH} + 0.889 \log I - 0.0517 \cdot \mathrm{pH} \cdot \log I + 0.0126T^2 + 0.971(\mathrm{pH})^2 - 0.424(\log I)^2 - 0.120 \cdot 10^{-3}T^3 - 0.060(\mathrm{pH})^3 \quad (1)$$

where V_R is the retention volume (ml) of Fraxiparine under the experimental conditions, T the temperature (°C) and I the ionic strength of the eluent. With the assumption of homoscedasticity, the model can be checked with a goodness-of-fit (GOF) and a lack-of-fit (LOF) test. The results of these tests for the calculated model of Fraxiparine are given in Tables II and III.

The experiments with the heparin standard (M_w = 9200 g/mol) resulted in the following equation for the retention volume as a function of the experimental parameters:

$$V_R = 11.410 - 0.0208 \cdot \mathrm{pH} + 1.162 \log I - 0.0458 \cdot \mathrm{pH} \cdot \log I - 0.148(\log I)^2 \quad (2)$$

The results of the GOF and LOF tests are given in Tables II and III. To obtain a more detailed insight into the calculated models, the influence

TABLE III

RESULTS OF THE VALIDATION OF THE MODEL FOR HEPARIN WITH A GOODNESS OF FIT AND A LACK OF FIT TEST

The calculated F-values are compared with F-values in ref. 17 at the 95% confidence level (F^*). The degrees of freedom (DF) are calculated with the number of independent measurements (f), the number of model parameters (p) and the number of experiments (n).

Parameter	Fraxiparine (M_w = 5090 g/mol)	Heparin standard (M_w = 9200 g/mol)
F_{GOF}	4918.43	2760.33
F^*	2.56	3.68
DF ($p-1, n-p$)	9, 31	4, 17
F_{LOF}	0.42	2.04
F^*	2.85	4.10
DF ($f-p, n-f$)	16, 15	8, 9

of the ionic strength, pH and temperature on the retention volume of heparin are plotted separately in the Figs. 1, 2 and 3, respectively. The results show that the ionic strength of the eluent has a relatively large influence on the retention volume of heparin, while the influence of the temperature and pH of the eluent on the retention volume of heparin is of minor importance in the investigated pH and temperature ranges.

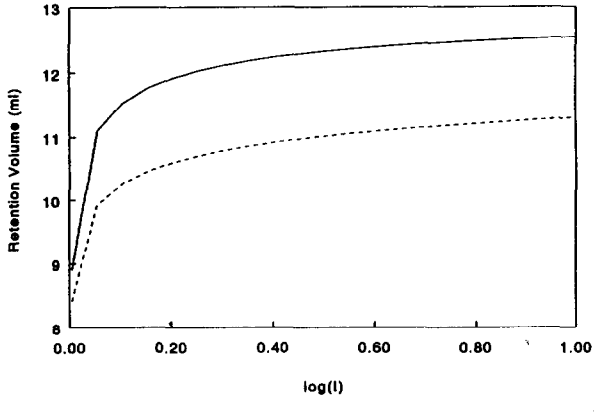

Fig. 1. Calculated influence of the ionic strength of the eluent on the retention volume (ml) of two specific heparin samples, M_w = 5090 (solid line) and 9200 (dotted line). The temperature and pH of the eluent were 35°C and 5.5, respectively.

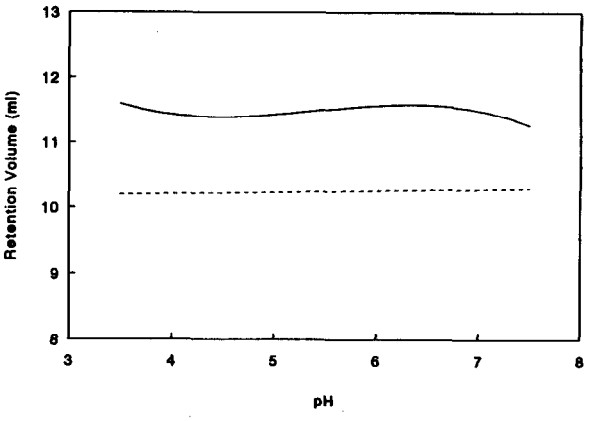

Fig. 2. Calculated influence of the pH of the eluent on the retention volume (ml) of two specific heparin samples, M_w = 5090 (solid line) and 9200 (dotted line). The temperature and ionic strength of the eluent were 35°C and 0.1, respectively.

Polysaccharide model

The influence of the ionic strength, pH and temperature of the eluent on the retention volume of five polysaccharide standards of M_w = 5800, 12 200, 23 700, 48 000 and 100 000 g/mol was also investigated. A 3^3 factorial design was used, which indicates that three factors were measured at three different levels. The factorial

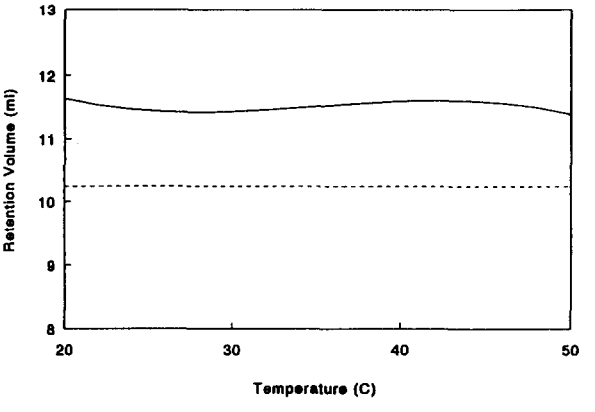

Fig. 3. Calculated influence of the temperature of the eluent on the retention volume (ml) of two specific heparin samples, M_w = 5090 (solid line) and 9200 (dotted line). The pH and ionic strength of the eluent were 5.5 and 0.1, respectively.

TABLE IV

EXPERIMENTAL SETTINGS USED IN THE FACTORIAL DESIGN FOR THE DETERMINATION OF THE INFLUENCE OF THE IONIC STRENGTH, TEMPERATURE AND pH OF THE ELUENT ON THE RETENTION VOLUME OF POLYSACCHARIDES

Setting No.	Temperature (°C)	pH	Ionic strength
1	25	4	0.01
2	25	4	1
3	25	7	1
4	45	7	1
5	45	4	1
6	45	7	0.01
7	25	7	0.01
8	45	4	0.01
9	35	5.5	0.1

design is given in Table IV. All levels were measured twice, except the centre point (35°C, pH 5.5, ionic strength = 0.1), which was measured ten times. The pure experimental error could be calculated from the measurements in the centre point.

From the experimental results, the influences of the three parameters on the retention volume of the polysaccharide standards could be calculated. In the investigated experimental framework, no significant influence of the ionic strength, pH and temperature of the eluent on the retention volume of the polysaccharide standards with average molecular masses of 12 200, 23 700 and 100 000 g/mol was observed.

The results from the calculations of the influence of the three parameters under study on the retention volume of the polysaccharide standard with an average molecular mass of 5800 g/mol can be expressed as

$$V_R = 12.192 + 0.00154 \log I \quad (3)$$

From the results for the polysaccharide standard with an average molecular mass of 48 000 (g/mol), a slightly different equation was derived:

$$V_R = 9.929 + 0.04875 \log I \quad (4)$$

These two expressions were validated with a GOF and an LOF test. The results of these tests are given in Table V. From eqns. 3 and 4 it can be concluded that the temperature, pH and ionic strength of the eluent have almost no influence on the retention volume of the polysaccharide standards.

Fig. 4 shows the retention volume of the polysaccharide standards as a function of the molecular mass of the polysaccharide standards. Under the assumption that the polysaccharide standard with an average molecular mass of 100 000 g/mol is totally excluded from the pores of the stationary phase of the size-exclusion column, the measured values can be fitted with the following logarithmic function:

$$\log M_w = -0.282 V_R + 7.209 \quad (5)$$

TABLE V

RESULTS OF THE VALIDATION OF THE RETENTION VOLUME EXPRESSION OF THE POLYSACCHARIDE STANDARDS WITH A GOODNESS OF FIT AND A LACK OF FIT TEST

Parameters defined as in Table III.

Parameter	Polysaccharide standard (M_w = 5800 g/mol)	Polysaccharide standard (M_w = 48 000 g/mol)
F_{GOF}	24.24	10.83
F^*	5.76	5.72
DF ($p-1, n-p$)	1, 24	1, 24
F_{LOF}	0.44	0.87
F^*	3.77	3.77
DF ($f-p, n-f$)	15, 9	15, 9

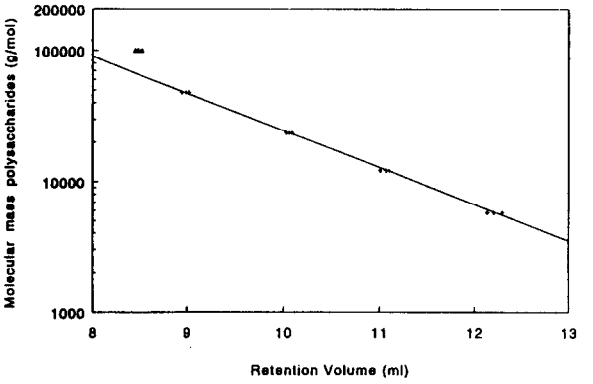

Fig. 4. Calibration graph for the polysaccharide standards. The experiments were performed with water as the eluent at 35°C. The polysaccharide standard with an average M_w = 100 000 is excluded from the pores in the column.

So far the results showed that the molecular size of heparins can be controlled by the composition of the mobile phase, while the retention volumes of polysaccharide standards are hardly influenced by the eluent composition. In principle, this observation offers the possibility of calculating the optimum composition of the eluent at which the retention volume of heparin samples approaches that of a polysaccharide standard with the same molecular mass.

Calculation of optimum ionic strength

Eqn. 5 expresses the relationship between the molecular mass and the retention volume of polysaccharide standards. In order to be able to calibrate the average molecular mass of heparin samples, the retention volume of a heparin standard of a certain average molecular mass should be the same as that of a polysaccharide with the same molecular mass under specific conditions. To determinate the molecular mass of heparin samples from eqn. 5, the optimum eluent composition must be calculated from eqns. 1 and 2, in order to manipulate the molecular sizes of the heparin compounds to values comparable to those of the polysaccharide standards. However, the error made by the interpolation should also be taken into account [18]. The results from these calculations are given in Table VI, where it can be seen that two different optimum values for the ionic strength of the eluent were obtained. We assume that this may be due to the different nature of the heparins. However, because the difference between the two optimum ionic strengths is small, the mildest separation condition ($I = 1.42$) was selected for the calibration of the heparin samples.

At this calculated optimum ionic strength, the retention volume of the heparin standards can be measured, and the molecular masses of the heparin standards can be calculated from eqn. 5. The results are summarized in Table VII. As can be seen, this method provides a fairly good estimate of the molecular masses of the heparin standards. All the calculated molecular masses are slightly higher than the true values but especially for the higher molecular masses the difference between the true and calculated values is small.

With the help of the presented model, the molecular mass distribution of a commercially

TABLE VI

RESULTS OF THE CALCULATION OF THE OPTIMUM IONIC STRENGTH OF THE ELUENT FOR THE STUDIED HEPARIN SAMPLES

Temperature = 35°C; pH of the eluent = 5.5.

Sample	V_R (calculated) (ml)	Standard deviation ($n = 36$) of calculated V_R (ml)	Ionic strength (calculated)
Fraxiparine (M_w = 5090 g/mol)	12.62	0.0872	1.42
Heparin standard (M_w = 9200 g/mol)	11.52	0.0847	1.77

TABLE VII

RESULTS FROM THE VALIDATION OF THE CALIBRATION MODEL FOR HEPARIN

Retention volumes measured at 35°C and an eluent pH of 5.5.

Ionic strength	Real average molecular mass (g/mol)	Calculated average molecular mass (g/mol) from eqn. 5
1.42	3000	$4.5 \cdot 10^3$
	4200	$5.4 \cdot 10^3$
	6000	$7.0 \cdot 10^3$
	9200	$10.7 \cdot 10^3$
	11 200	$12.7 \cdot 10^3$
1.77	6000	$7.1 \cdot 10^3$
	9200	$10.2 \cdot 10^3$
	11 200	$13.1 \cdot 10^3$

available heparin, Calparine, was calculated from a chromatogram of a Calparine sample (Fig. 5). The chromatogram was divided into slices, and for each slice the corresponding molecular mass was calculated using both the polysaccharide and the heparin calibration graph. Fig. 6 shows the results of both calibration methods for the calibration of Calparine. The data from these experiments are in good agreement with the expected results. The molecular mass of the different oligosaccharides of commercially available heparin, such as Calparine, normally ranges from 2000 to 40 000 g/mol [2].

CONCLUSIONS

It has been shown that by adjusting experimental parameters such as the pH, ionic strength and temperature of the eluent, the molecular masses of specific heparin samples can be calculated by applying other biopolymers, *e.g.*, polysaccharides, as standards. An advantage of this approach is that these calibrations

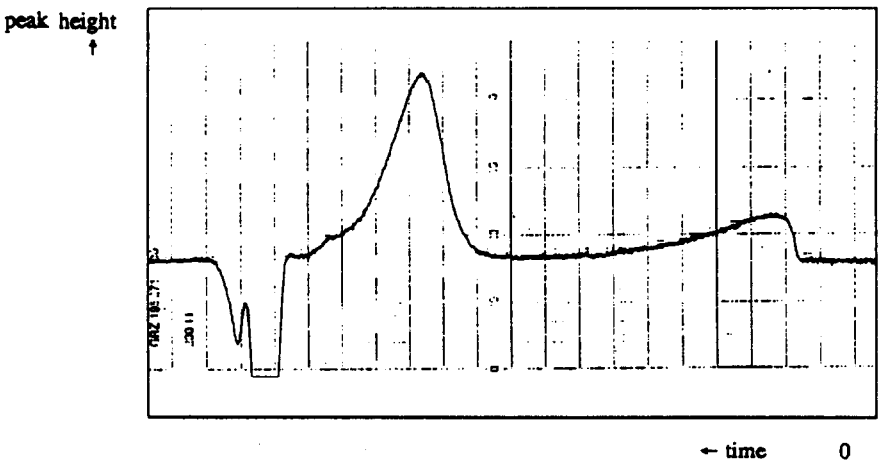

Fig. 5. Size-exclusion chromatogram of a specific heparin sample (Calparine) on a Zorbax GF-250 column (200 mm × 9.4 mm I.D.) with RI detection. Flow-rate, 1.0 ml/min; temperature, 35°C; buffer composition, Na_2HPO_4–NaCl; pH of buffer, 5.5; ionic strength of buffer, 1.42.

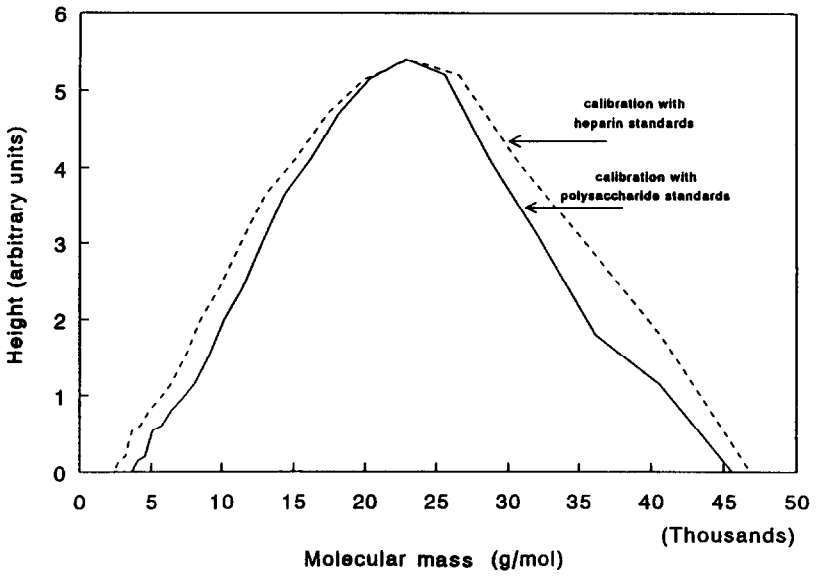

Fig. 6. Molecular mass distribution of a specific heparin sample (Calparine), calculated from a polysaccharide and a heparin calibration graph.

can be performed with commercially available standards, consisting of other chemical substances, when standards of the target compounds are not available. A satisfactory agreement in the molecular mass distribution of a Calparine sample was observed for both the calibration with polysaccharide standards and the calibration with heparin standards. However, it should be mentioned that, because of possible secondary separation mechanisms, the results presented in this study may only be valid for this size-exclusion column.

The use of chemometrics was found to be very helpful in setting up the experimental design and the calculation of the data. As a consequence, the effects of the different parameters could be investigated with a relatively small number of experiments. Applying chemometric techniques to the results also allowed the calculation of the influence of the separation variables and additionally the determination of the interaction effects of these variables.

The approach described here for measuring the molecular masses of heparin samples by applying standards of other chemical classes as standards may also be useful for other biopolymers.

ACKNOWLEDGEMENT

The authors gratefully acknowledge Professor Dr. H.C. Hemker of the Department of Biochemistry, University of Limburg (Maastricht, Netherlands), for providing the heparin samples and standards.

REFERENCES

1 B. Casu, *Adv. Carbohydr. Chem. Biochem.*, 43 (1985) 51–134.
2 D.A. Lane, I. Björk and U. Lindahl, *Heparin and Related Polysaccharides (Advances in Experimental Medicine and Biology*, Vol. 313), Plenum Press, New York, London, 1992.
3 L. Oscarsson, G. Pejlel and U. Lindahl, *J. Biol. Chem.*, 264 (1989) 296–304.
4 R. Lasito, H. Gattiker and G. Bilodeau, *J. Chromatogr.*, 226 (1981) 61–67.
5 J. Harenberg and J.X. de Vries, *J. Chromatogr.*, 261 (1983) 287–292.
6 D. Muller, M.N. Doume-Nze and J. Jozefonvicz, *J. Chromatogr.*, 297 (1984) 351–358.
7 J.X. de Vries, *J. Chromatogr.*, 465 (1989) 297–304.
8 H.I. Kirstinsen, E.M. Tomborg, J.R. Nielsen, J.I. Nielsen, K.B. Johansen and P.B. Ostergaard, *Thromb. Res.*, 64 (1991) 131–141.
9 L. Rosenfeld, M.T. Prior and L.M. Girardi, *Thromb. Res.*, 64 (1991) 203–211.

10 W.W. Yau, J.J. Kirkland and D.D. Bly, *Modern Size Exclusion Liquid Chromatography*, Wiley, New York, 1979.
11 M. Potschka, *J. Chromatogr.*, 441 (1988) 239–260.
12 P.K. Dubin and M.N. Tecklenburg, *Anal. Chem.*, 57 (1985) 275–279.
13 P.L. Dubin, C.M. Speck and J.I. Kaplan, *Anal. Chem.*, 60 (1988) 895–900.
14 E. Pérez-Payá, L. Braco, A. Campos, V. Soria and C. Abad, *J. Chromatogr.*, 461 (1989) 229–242.
15 S. Béguin, S. Wielders, J.C. Lormeau and H.C. Hemker, *Thromb. Haemostasis*, 67 (1992) 33–41.
16 A.V. Bendetowicz, E. Pacaud, S. Béguin, A. Uzan and H.C. Hemker, *Thromb. Haemostasis*, 67 (1992) 556–562.
17 D.L. Massart, B.G.M. Vandeginste, S.N. Deming, Y. Michotte and L. Kaufman, *Chemometrics, a Textbook*, Elsevier, Amsterdam, 1988.
18 J.C. Miller and J.N. Miller, *Statistics for Analytical Chemistry*, Ellis Horwood, Chichester, 2nd ed., 1992.