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# CHARACTERIZATION OF HEPARINS BY HIGH-PERFORMANCE SIZE EXCLUSION LIQUID CHROMATOGRAPHY

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## SUMMARY

A method for the determination of the molecular weight distribution of heparins using heparin oligosaccharides as calibration standards and high-performance size exclusion liquid chromatography has been developed. An organic microparticulate stationary phase (TSK 3000), an aqueous mobile phase and UV detection at 206 nm were adopted. Commercial heparins, low-molecular-weight heparins and polysulphated mucopolysaccharides with heparin-like activities were characterized by this procedure. Average molecular weights [weight average ($M_w$), number average ($M_n$), *z* average ($M_z$)] and molecular weight distribution (cumulative weight fraction and differential weight fraction) were calculated from the chromatographic data.

## INTRODUCTION

Heparin, which is widely used as an antithrombotic agent, is a polydispersed sulphated mucopolysaccharide with a wide molecular weight range between 3000 and 40,000 daltons[1]. Heparin functions as an anticoagulant, binding to antithrombin III and accelerating the rate at which this protein complex inactivates the serine proteases of the haemostatic system[2]. The molecular weight distribution varies according to origin, purification and isolation procedures, fractionation and treatment with reagents[3,4]. Commercially available heparin has been separated into fractions of different molecular weights[5,6]. Low-molecular-weight heparins and other polysulphated mucopolysaccharides are characterized by a higher antifactor Xa/aPTT ratio *in vitro* and *ex vivo*[7–12] in comparison with unfractionated heparin. Thus the advantage in the clinical use of these preparations is assumed to be an increase in the antithrombotic/bleeding ratio. Therefore, a knowledge of the molecular weights and the molecular weight distribution is particularly important, especially with regard to the standardization of the new heparin preparations.

For the determination of the molecular weight of heparins, high-performance liquid chromatographic methods have been described[13–15]. However, all techniques used heparins with poorly defined molecular weights or dextrans with wide molecular weight ranges for calibration. We now report the characterization of heparins and

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some other mucopolysaccharides employing high-performance size exclusion chromatography using heparin oligosaccharides with known molecular weights for calibration. The column was packed with a microparticulate phase using 0.1 *M* sodium chloride solution as eluent and the UV absorbance was read at 206 nm. The average molecular weights and the molecular weight distributions were calculated from the chromatographic data[16].

## EXPERIMENTAL

### *Apparatus*

A system consisting of a pump (Model M6000A, Waters, Königstein, G.F.R., or Model 2150-002, LKB Gräfelfing, G.F.R.), an injector (Rheodyne Model 7125; 20 $\mu$l loop, LKB Model 2154-100), a column (Ultrapac TSK G 3000 SW, 600 × 7.5 mm I.D., particle size 10 ± 2 $\mu$m; LKB No. 2135-360), a detector [Uvicord S 2138 B (LKB) at 206 nm and an 8-$\mu$l cuvette] and a two-channel recorder (Model 2210-022, LKB) were used. A pre-column [Ultrapac TSK G SWP, 75 × 7.5 mm I.D., 10 $\mu$m (LKB)] was connected between the injector and the pump. UV spectra were measured with a Model 810 spectrophotometer (Kontron, Eching, G.F.R.). Tritium counting was effected using a TriCarb scintillation counter (Packard, Frankfurt, G.F.R.) with Pico-Fluor TM 15 (Packard) as scintillator. All reagents were of analytical-reagent grade.

### *Samples*

Samples 1–3 and 5–7 were a gift from Dr. F. Fussi (Hepar Industries, Franklin, OH, U.S.A.). Sample 4 was from Medac (Hamburg, G.F.R.), 8 from Luitpold (Munich, G.F.R.), 9 from Organon (Organon, Oss, The Netherlands) and 10 from Kettelhack Riker (Borken, G.F.R.). Heparins 11–13 were kindly supplied by Dr. G. H. Barlow (Reese Research Foundation, Chicago, IL, U.S.A.). A mixture of nitrous acid-treated heparins, reduced with tritiated sodium borohydride, and containing 1, 4, 8, 12 and 16 monosaccharide units, was generously provided by Dr. R. D. Rosenberg (Sidney Farber Cancer Institute, Boston, MA, U.S.A.).

All heparins were of pig mucosa origin, except heparin 3, which was from bovine lung substance. All were sodium salt preparations.

### *Chromatographic conditions*

The eluent used was aqueous 0.1 *M* sodium chloride (filtered and degassed before use) at a rate of 1.0 ml/min (pressure 400 p.s.i.); the detector was set at 206 nm, at 0.1 absorbance and 100% transmittance a.u.f.s.; the recorder chart speed was 10 mm/min; 20 $\mu$l of the heparin solutions were injected at a concentration of 10%.

### *Calculations*

Chromatographic data obtained from the elution curve [absorbance expressed as peak height in millimetres (every 0.5 ml)] and elution volume (in millilitres) were used for calculating the average molecular weights [weight average molecular weight ($M_w$), number average molecular weight ($M_n$), *z* average molecular weight ($M_z$) and the polydispersivity ($Q$)]; molecular weight distributions ($MWD$) expressed as the cumulative weight fraction $MWD$ and as the weight fraction frequency distribution

TABLE I

CHARACTERISTICS OF HEPARINS AND CALCULATED AVERAGE MOLECULAR WEIGHTS

| *Sample No.* | *Lot No.* | $M_w$ | $M_n$ | $M_z$ | $Q$ |
|---|---|---|---|---|---|
| 1 | 483.183.6001 | 11,463 | 9649 | 13,375 | 1.19 |
| 2 | 3836001 | 10,702 | 9186 | 12,292 | 1.17 |
| 3 | 714/2836001 | 11,408 | 9444 | 13,372 | 1.21 |
| 4 | 1905901 | 10,781 | 9534 | 12,257 | 1.13 |
| 5 | G38 | 13,393 | 11,293 | 15,413 | 1.19 |
| 6 | 02-14 | 5215 | 4389 | 6160 | 1.19 |
| 7 | 02-31A | 6008 | 4884 | 7270 | 1.23 |
| 8 | GAGPS | 4129 | 3668 | 4676 | 1.13 |
| 9 | 10172; CP 081083 | 7517 | 4823 | 10,055 | 1.54 |
| 10 | 089187 | 10,377 | 8667 | 12,079 | 1.20 |
| 11 | Standard 2 | 13,079 | 12,409 | 13,820 | 1.05 |
| 12 | Standard 3 | 10,269 | 9712 | 10,957 | 1.06 |
| 13 | Standard 5 | 4502 | 4275 | 4783 | 1.05 |
| 14 | Mixture of heparins | | | | |

were calculated according to Yau *et al.*[16]. The mixture of tritiated 1, 4, 8, 12 and 16 heparin oligosaccharides with defined chain length was run under the same conditions and was used for calibration.

## RESULTS

Table I lists the calculated average molecular weights of the heparins analysed. Fig. 1 shows the chromatogram of heparin samples 6, 9 and 10; Fig. 2 the cumulative weight fraction molecular weight distribution and Fig. 3 the weight fraction fre-

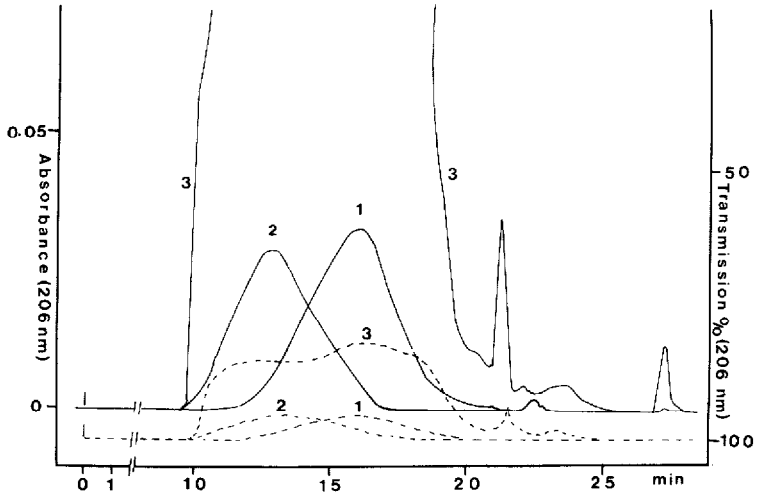


Fig. 1. Chromatograms of heparins. 1 = Sample 6; 2 = sample 3; 3 = sample 9. ———, Absorbance; – – –, transmission (%).

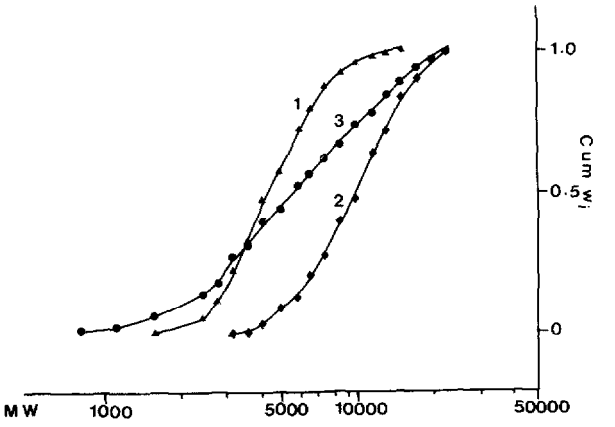


Fig. 2. Cumulative weight fraction distribution for samples as in Fig. 1.

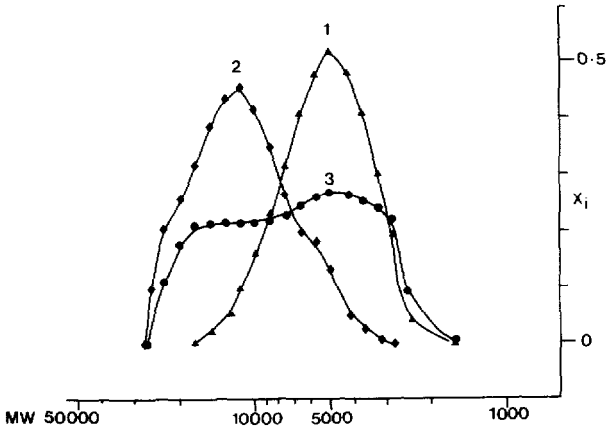


Fig. 3. Weight fraction frequency distribution for samples as in Fig. 1.

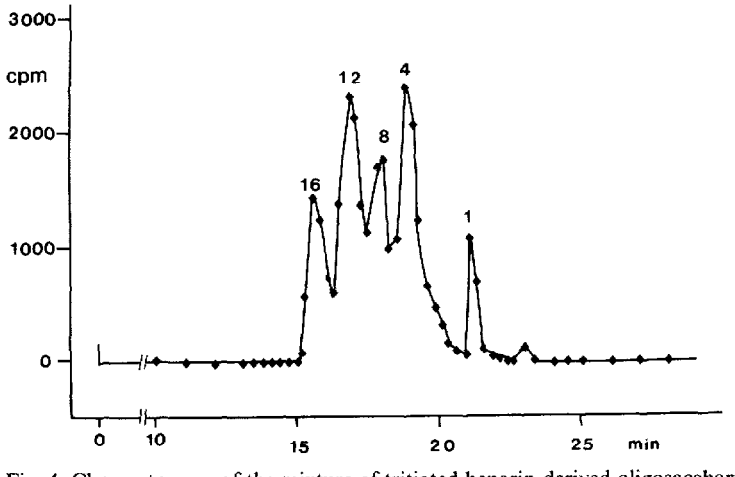


Fig. 4. Chromatogram of the mixture of tritiated heparin-derived oligosaccharides. Peak numbers denote the number of monosaccharide units per chain. As can be seen from the elution curves, the heparin oligosaccharides are separated precisely by this technique and appear as a single peak.

quency distribution of the heparin samples. The elution curve of the tritiated heparin oligosaccharides is shown in Fig. 4. The reproducibility of the method was 2.5% (coefficient of variation, $n = 5$).

## DISCUSSION

The method described for the characterization of the molecular weight of heparins and some other polysulphated mucopolysaccharides is based on the use of highly purified heparin oligosaccharides as calibration standards. Thus this procedure overcomes the disadvantage of previously described techniques, which adopted fractionated heparins[14] or dextrans[15] for calibration. The fractionated heparins, for their part, were not defined exactly in the molecular weight range; the dextrans differ substantially from heparin in their structure and chromatographic behaviour. These considerations have to be taken into account when these substances are employed for the calibration of heparins. This problem can be solved by using heparin oligosaccharides with defined chain length and known molecular weight to standardize the determination of the molecular weight of heparins, as in the method described here.

The TSK 3000 microparticulate stationary phase[17] was found to be the best for separating the molecular weight fractions of the heparin preparation. Other chromatographic packing materials such as modified dextrans[18], silica gel[15], μBondagel[14] and silica gels modified with $C_{18}$, amino or diol groups (Merck, Darmstadt, G.F.R.), (data not shown) did not differentiate adequately the molecular weight of the heparin samples.

We also decided to determine heparin with UV detection at a wavelength of 206 nm, which is very close to the maximum absorption at 204 nm. Previously described detections were carried out at higher wavelengths[18] or with differential refraction[13,14]. UV detection at higher wavelengths is non-specific for heparin and UV detection at 206 nm is much more sensitive than the differential refraction method. Additionally, it has to be considered that the absorbance of heparin preparations at 206 nm is considerably influenced by sulphamido groups. Thus, this method also gives valuable information on the sulphate substitution of a heparin preparation. This is confirmed by the results for heparin samples 8 and 9, which are polysulphated mucopolysaccharides with high anti-factor Xa activity.

Several methods for the determination of the molecular weight of heparins have been reported, such as gel filtration[19], viscosimetry[20] and ultracentrifugation[21]. Electrophoretic techniques have also been used for the fractionation, identification and quantitation of heparins and acidic mucopolysaccharides[22–24]. Cellulose acetate, agarose and polyacrylamide gels have been adopted as supports with barium acetate[23], barbital[24] and ampholines[22]. Molecular weight seems to play an important role in the electrophoretic mobility of heparins[23,24], but molecular weight distributions have not been measured with these techniques. The electrophoretic migration is expected to be caused more by modifications of the number and type of negatively charged functional groups. All of these methods are time consuming and determine primarily the mean molecular weight of heparins. The application of liquid chromatography facilitates the additional determination of the molecular weight distribution and in a sense also the sulphate substitution. This is of special interest because

some new heparin preparations are polysulphated mucopolysaccharides. Thus, an exact, sensitive and rapid method for the characterization of the molecular weight, the molecular weight distribution and the degree of sulphation is needed.

The results presented here indicate that our procedure is adequate for the analysis and characterization of newly developed heparins and other related mucopolysaccharides. Further, the method offers the possibility of adoption for preparative purposes with heparin samples with defined molecular weight ranges.

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