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Short Communication

Determination of polyanionic macromolecules by size-exclusion chromatography

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ABSTRACT

This report presents an extension of a method developed for determination of dextran sulfate in rat serum. The drug is a negatively charged polysaccharide with a molecular mass of 8000. It is fractionated by molecular size and separated from serum components by high-performance size-exclusion chromatography. Sensitive detection is achieved by the post-column complexation of the analyte with 1,9-dimethylmethylene blue (DMMB). A metachromatic complex is formed; the absorbance maximum of the complex is shifted from that of the free dye. Various glycosaminoglycans and other macromolecular polyanions interact with DMMB. Several can be determined using the chromatographic conditions developed for dextran sulfate. The method provides a simple procedure for quantitation of these compounds. Compared to spectrophotometric assays, less sample preparation is required, selectivity is enhanced, and molecular mass information is provided. With modification of eluent composition, dye concentration, and detection wavelength, the method can be validated for determination of additional compounds.

INTRODUCTION

A chromatographic method developed for the measurement of dextran sulfate [1] can be used for the determination of other polyanionic macromolecules. The method consists of a size-exclusion chromatographic (SEC) separation with post-column dye complexation. The cationic dye 1,9-dimethylmethylene blue (DMMB) is used; it forms metachromatic complexes with polyanions. Compounds known or expected to react with such dyes were used to determine the suitability of the method.

Metachromasia was originally defined as the staining of certain tissue components in a color different from that of the dye used [2]. When a complex is formed between a metachromatic dye and a polyelectrolyte of opposite charge, the absorbance maximum is shifted from that of the free dye. This shift may be to a higher or lower wavelength [3]. Assays using dyes have been published for heparin [4,5] and other sulfated glycosaminoglycans such as chondroitin sulfates and hyaluronic acid [6], keratan sulfate [7], dermatan sulfate and heparan sulfate [8], as well as alginates [9] and cartilage proteoglycan [10]. Most methods are spectrophotometric, and extensive sample preparation is required to remove interfering compounds. Positive interferences reduce the specificity of the spectrophotometric

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analyses. Negative interferences inhibit the complexation of the analyte with the dye.

Combining size-exclusion chromatography with post-column dye complexation offers advantages over spectrophotometric methods. Dextran sulfate in serum does not react with DMMB. Addition of trichloroacetic acid or organic solvents precipitates the serum proteins; however, dextran sulfate is also precipitated. Injection of untreated serum onto the SEC column resolves dextran sulfate from the interfering compounds. Also heparin in plasma can be analyzed chromatographically with filtration as the only sample preparation step. The ruggedness of the method for direct injection of serum has been validated [1]. The separation of the analyte from positive interferences improves the selectivity. The high absorptivity of the metachromatic complex allows detection of very low concentrations of compounds which typically have no useful chromophore. Because the addition and mixing of the dye solution with the sample are automated, the precision of the chromatographic method is superior to spectrophotometric analyses. The use of SEC for the separation allows a molecular mass distribution to be obtained. Calibration of the chromatographic column is required. This may be performed by laser light scattering analysis of the sample of interest or by the use of molecular mass standards.

EXPERIMENTAL

Materials and reagents

Chondroitin sulfate, fucoidin, hyaluronic acid, pentosan polysulfate, and polygalacturonic acid were purchased from Aldrich (Milwaukee, WI, USA). All compounds except fucoidin were purchased as the sodium salt. Chondroitin sulfate was approximately 70% chondroitin sulfate A; the balance was chondroitin sulfate C. Fucoidin was crude, from *Fucus vesiculosus*. Polygalacturonic acid (sodium polypectate) was 85–90% pure. The purities of hyaluronic acid and pentosan polysulfate were not specified by the supplier. Heparin was purchased as a solution of 5000 USP (United States Pharmacopeia) units/ml from LyphoMed (Melrose Park, IL, USA) and as a solid from Aldrich. Carrageenan, type κ, was obtained from FMC Corporation (Princeton, NJ, USA); bone gelatin was obtained from Rousselot (Paris, France). Sodium docusate (dioctyl sodium sulfosuccinate) was taken from Colace capsules (MeadJohnson and Company, Evansville, IN, USA). All other chemicals were reagent grade and were purchased from Fisher Scientific (Pittsburgh, PA, USA). Water was purified by reverse osmosis.

Equipment

The instrumentation used has been described previously [1]. Briefly, the chromatographic system consisted of pumps equipped with pulse dampeners for the delivery of mobile phase and dye reagents and an autoinjector equipped with a 10-μl sample loop for sample introduction. The separation was performed on a Chromega diol size-exclusion chromatographic column (30 cm × 7.8 mm I.D., 5 μm particles, 100 Å pore size) preceded by a guard column (4.8 cm × 4.6 mm I.D.) packed with the same material as the analytical column. Column effluent was mixed with the dye solution in a 3.1-μl mixing tee.

Chromatographic conditions

The mobile phase consisted of purified water containing 0.025 *M* monobasic potassium phosphate, 0.025 *M* dibasic potassium phosphate, and 0.05 *M* potassium chloride; the pH was approximately 6.8 and the ionic strength was 0.15 *M*. A flow-rate of 1.0 ml/min was used. The sample detection wavelength was 525 nm. The dye solution consisted of 18.5 μg/ml of 1,9-dimethylmethylene blue in purified water; the flow-rate was 0.5 ml/min.

Sample solutions

All compounds were dissolved in mobile phase. A concentration of 0.2 mg/ml was used for all samples for comparison of the chromatographic responses.

RESULTS AND DISCUSSION

The method was previously validated for determination of dextran sulfate in rat serum [1]. It is also suitable for determination of dextran sulfate in tissue samples. Linearity of response

TABLE I

PRECISION OF ANALYSIS: PEAK AREAS OF REPLICATE INJECTIONS OF HEPARIN STANDARDS

Injection No.	Heparin concentration			
	0.2 mg/ml		0.14 mg/ml	
	Day 1	Day 2	Day 1	Day 2
1	7916.3	8059.9	5817.7	5846.5
2	7791.4	7947.7	5861.7	5818.1
3	7737.9	8004.7	5696.7	5707.2
4	7904.5	7879.8	5762.6	5755.5
Mean	7837.5	7973.0	5784.7	5781.8
R.S.D. (%)	1.1	1.0	1.2	1.1

for the determination of heparin was determined. Nine samples ranging from 1 to 200 USP units/ml were analyzed. Concentrations of 100 and 200 USP units/ml were beyond the linear range. A line was fitted to the peak heights of the remaining seven samples, which ranged from 1 to 50 USP units/ml. The slope was 3.01 with a standard error of 0.27; the intercept was 9.7 with a standard error of 5.9. At the 95% confidence level the y-intercept was equivalent to zero. The standard error of the regression was 11.4 and the correlation coefficient was 0.981. Benzyl alcohol used as a preservative did not interfere in the determination of heparin. Precision determined for heparin was comparable to that previously reported for dextran sulfate. See Table I.

The following compounds gave a good chromatographic response: chondroitin sulfate (Fig. 1), fucoidin (Fig. 2), heparin (Fig. 3), pentosan polysulfate (Fig. 4), and polygalacturonic acid. Carrageenan, sodium docusate and hyaluronic acid (Fig. 5) were only weakly metachromatic. They produced a smaller shift in absorbance of the dye; a detection wavelength of 525 nm was not appropriate for these compounds. Gelatin and sodium docusate did not form a stable complex with the dye. When dye was added to solutions of these compounds, the color changed from blue to violet, then returned to blue. Table II shows the relative sizes of peaks seen in chromatograms monitored at 525 nm. The chromatograms are shown at approximately equal

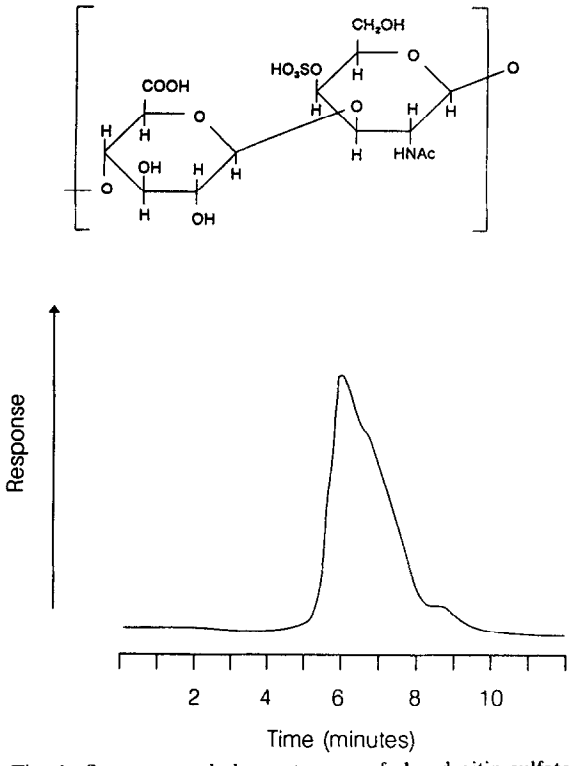

Fig. 1. Structure and chromatogram of chondroitin sulfate.

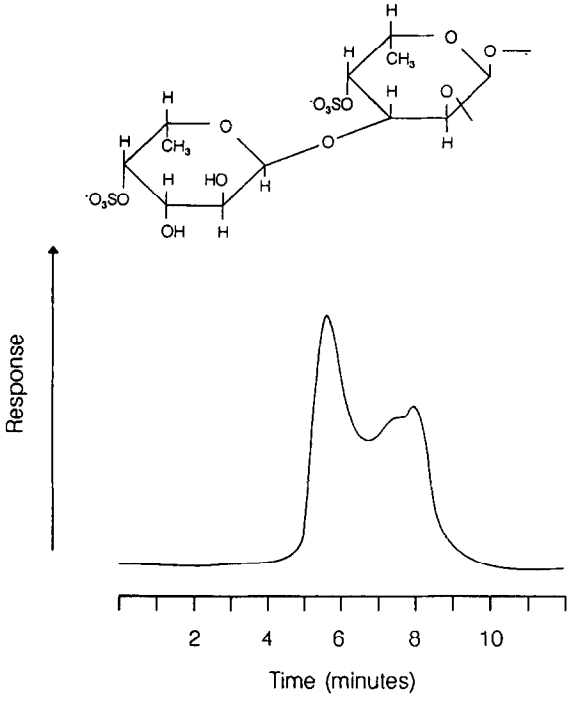

Fig. 2. Structure and chromatogram of fucoidin.

TABLE II

CHROMATOGRAPHIC RESPONSES OF 0.2 mg/ml SOLUTIONS AT 525 nm

Analyte	Peak area
Carrageenan	500
Chondroitin sulfate	18 000
Fucoidin	19 000
Gelatin	N.D.[a]
Heparin[b]	7000
Hyaluronic acid	600
Pentosan polysulfate	12 000
Polygalacturonic acid	10 000
Sodium docusate	N.D.[a]

[a] Not detected.

[b] 0.2 mg/ml is approximately 30 units/ml based on 140–170 units/mg.

sizes. The chromatogram of hyaluronic acid (Fig. 5) appears noisy due to the expanded y-scale used to display the small peak.

Components of serum from male Sprague-Dawley rats eluted between 4.5 to 6 min and did not interfere in the determination of dextran

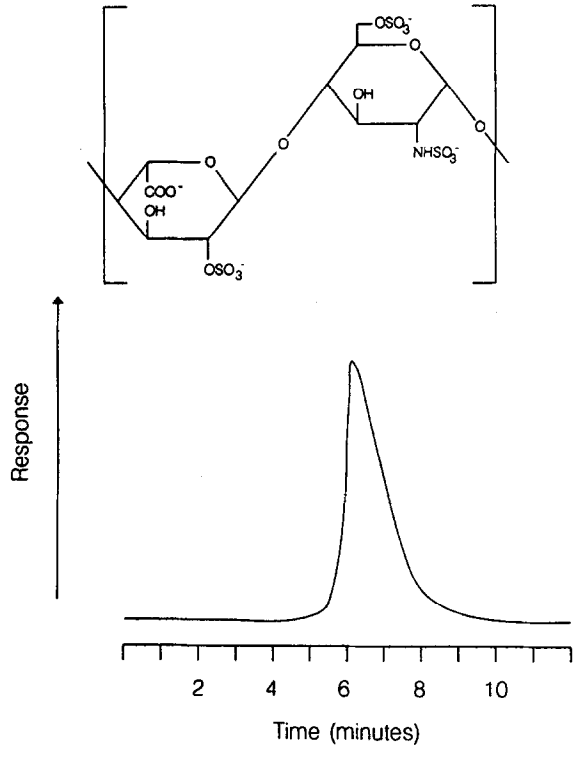

Fig. 3. Structure and chromatogram of heparin.

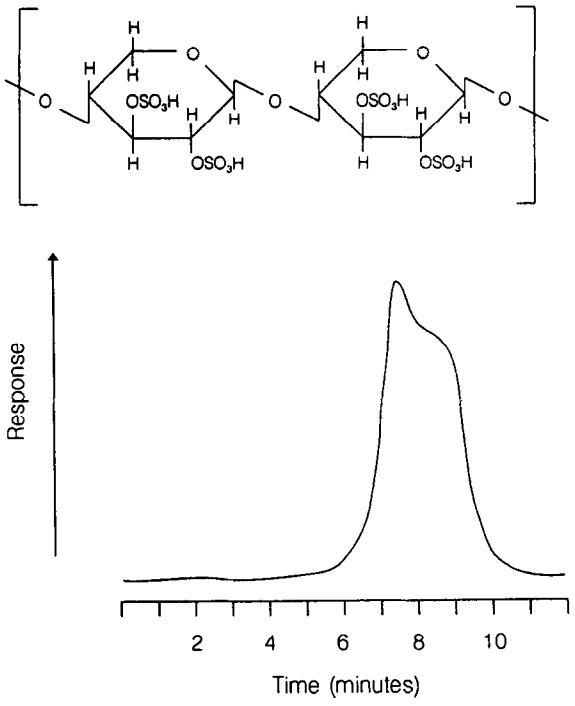

Fig. 4. Structure and chromatogram of pentosan polysulfate.

sulfate [1]. The serum components will not interfere in the determination of compounds with molecular masses (M_r) below approximately 120 000.

Most of the compounds analyzed were not highly purified or fractionated. The broad peaks show that they are polydisperse. One measure of polydispersity is the ratio of weight average to number average molecular mass, generally denoted by M_w/M_n. This index of polydispersity may be calculated by using the method of digitization of the chromatogram [11]. Dextran sulfate had a peak width at the baseline of 3.4 min and an M_w/M_n ratio of 1.9. Materials with a narrow molecular mass distribution have values close to unity. A dextran sulfate sample was fractionated and had a peak width of 1.9 min and an M_w/M_n ratio of 1.1. In comparison, fucoidin had a total peak width of 4.5 min and an M_w/M_n ratio of 2.5. The presence of multiple peaks in this and other samples indicates that they are mixtures of discrete molecular mass populations. Since the materials examined are polyelectrolytes, the peak shapes and retention volumes are affected

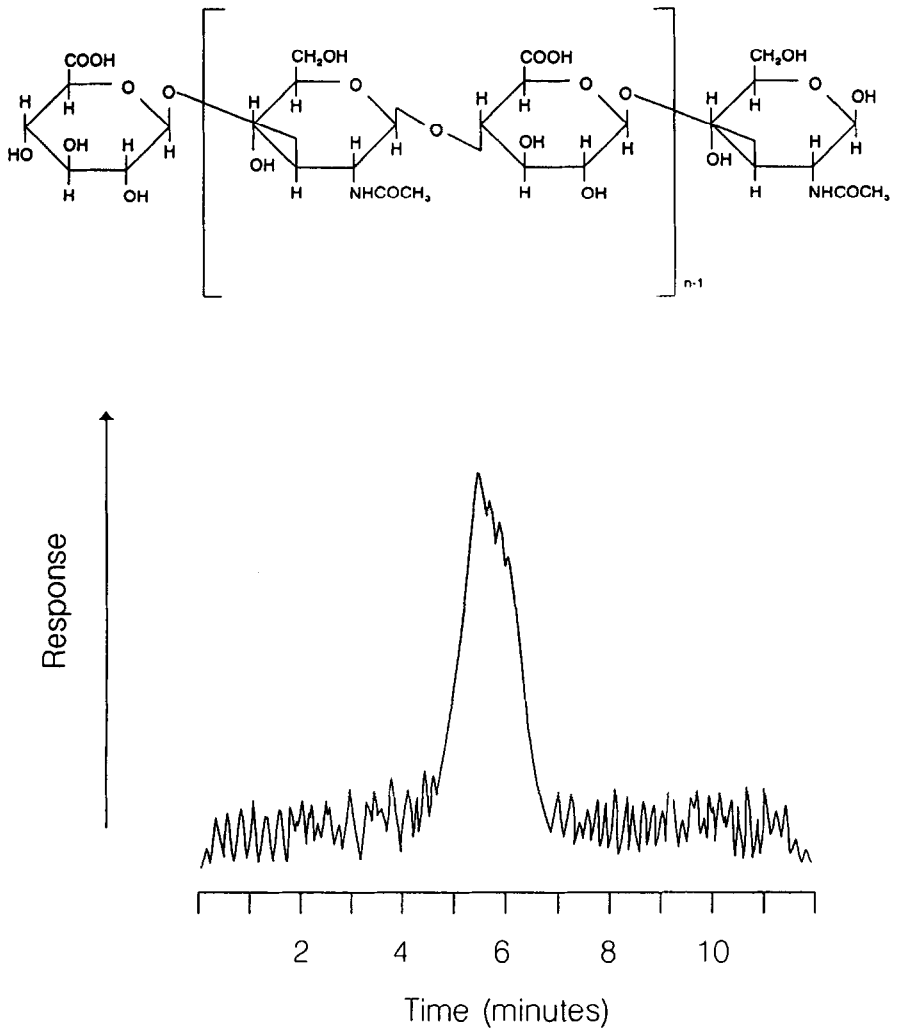

Fig. 5. Structure and chromatogram of hyaluronic acid.

by the ionic strength of the mobile phase. Accurate molecular masses cannot be determined by reference to the column calibration performed for dextran sulfate.

CONCLUSIONS

The method described is suitable for quantitation and molecular mass determination of a variety of polyanions. This class of compounds includes endogenous glycosaminoglycans and drugs used as thrombolytics, antivirals and angiogenesis inhibitors. With optimization of pH, ionic strength and dye concentration, the method can be validated for the quantitation of specific compounds in body fluids and tissues. The advantages of combining a size-exclusion chromatographic separation with the post-column dye reaction include increased selectivity, simplified sample preparation, improved precision, and molecular mass information.

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