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Size-exclusion chromatographic determination of dextran sulfate in rat serum

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ABSTRACT

A sensitive and selective method for the determination of dextran sulfate in rat serum has been developed. The analysis is suitable for quantitation of the drug and for monitoring molecular mass changes occurring during biotransformation. Dextran sulfate is resolved from higher molecular mass serum components by high-performance aqueous size-exclusion chromatography. The method has been validated for the direct injection of serum. Sensitive detection is achieved by post-column reaction of the polyanionic drug with the dye 1,9-dimethylmethylene blue. Components of serum which inhibit complex formation are separated chromatographically from dextran sulfate. Absorbance of the metachromatic complex is monitored at 525 nm.

INTRODUCTION

Dextran sodium sulfate is a polyanionic polysaccharide with antiviral activity [1]. It is a potent inhibitor of human immunodeficiency virus type 1 (HIV-1) *in vitro* [2–4]. The drug is a D-glucose polymer with $\alpha(1\rightarrow6)$-glycosidic linkages. Clinical trials for treatment of AIDS and AIDS-related complex [5,6] have been conducted using material with a relative molecular mass of *ca.* 8000 and containing up to three sulfate groups per monomer.

Development of the drug has been hampered by difficulties in developing a suitable method of analysis [7,8]. Previously published analytical methods require radiolabeled drug [9,10] or bioassays [8,11]. While radiolabeling provides excellent sensitivity, many methods lack specificity. Existing methods were not suitable for determination of dextran sulfate in serum; a competitive binding assay was then developed for monitoring the drug in clinical trials [8,12]. The method uses radiolabeled drug and provides excellent sensitivity, but fragments and metabolites may not be distinguished from the intact drug. Sample preparation is cumbersome, and the analysis time is lengthy.

The method presented here, which was developed for the determination of dextran sulfate in rat serum, offers many advantages over existing assays. The equipment and reagents are commonly available in most laboratories; radiolabeled drug is not required; sample preparation is minimal, and the analysis time is fairly short. High-performance size-exclusion chromatography (SEC) is used to fractionate the sample by molecular size. This resolves the drug from many serum components and reduces sample preparation. Changes in the molecular mass of dextran sulfate which occur during biotransformation [13] can be monitored by SEC. Molecular mass and molecular mass distribution can be deter-

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mined on a column calibrated with appropriate standards.

Sensitive and selective detection is achieved by the post-column complexation of dextran sulfate with 1,9-dimethylmethylene blue (DMMB). Many polyelectrolytes interact with DMMB and similar dyes to form a metachromatic complex [14]. The absorbance maximum of the complex is shifted from that of the free dye. Compounds with no useful chromophore are readily detected at visible wavelengths. The phenomenon is well known [15–18], but the mechanism is not clearly understood [19–22]. Dextran sulfate complexed with DMMB has a detection sensitivity comparable to radiolabeling. Metachromasia has formed the basis for spectrophotometric analysis of glycosaminoglycans, carbohydrate polymers which usually contain sulfate groups. Sample preparation for spectrophotometric methods is generally complicated; components of biological fluids interfere with the metachromatic interaction. Dextran sulfate in serum does not react with DMMB. Size-exclusion chromatography separates the drug from serum components which inhibit the reaction.

EXPERIMENTAL

Materials and reagents

Dextran sulfate, sodium salt, was donated by Ueno Fine Chemicals Industry (Sanda, Hyogo, Japan). 1,9-Dimethylmethylene blue, dye content *ca.* 80%, was purchased from Aldrich (Milwaukee, WI, USA). All other chemicals were reagent grade and were purchased from Fisher Scientific (Pittsburgh, PA, USA). Water was purified by reverse osmosis.

Equipment

The chromatographic system consisted of an Altex Model 110A pump (Beckman, Fullerton, CA, USA) equipped with a pulse dampener for delivery of the mobile phase and a Model 725 autoinjector (Micromeritics, Norcross, GA, USA) with a 10-μl sample loop for sample introduction. The separation was performed on a Chromega diol (ES Industries, Berlin, NJ, USA) 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., ES Industries, Berlin, NJ, USA). A water jacket on the analytical column was thermostatted at 40°C. Column effluent passed through a 3.1-μl mixing tee (Upchurch Scientific, Oak Harbor, WA, USA), where the dye solution was added by a Model 6000A pump (Waters Associates, Milford, MA, USA) with the high sensitivity accessory in use. A Schoeffel Spectroflow Monitor SF770 detector (Applied Biosystems, Foster City, CA, USA) with a tungsten lamp and a Model 450 Data System/Controller (Beckman Instruments) were used to monitor the separation and collect data. A schematic diagram of the HPLC system is shown in Fig. 1. Centrifugation of blood samples was performed in a Model CL centrifuge (International Equipment, Needham Heights, MA, USA) in 1.5 ml conical polypropylene tubes (Fisher Scientific, Pittsburgh, PA, USA).

Chromatographic conditions

The mobile phase consisted of purified water containing 0.025 *M* potassium phosphate, monobasic, 0.025 *M* potassium phosphate, dibasic, 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. 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.

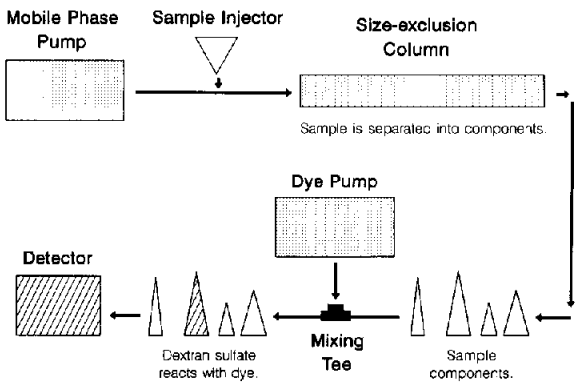

Fig. 1. Schematic diagram of chromatographic system.

Standard solutions

Dextran sulfate was dissolved in mobile phase. A concentration of 0.2 mg/ml was used for most experiments.

Sample preparation

Blood was collected from the lateral tail vein of male Sprague–Dawley rats (Ace Animals, Boyertown, PA, USA). Samples were allowed to clot for 30 min at room temperature and were centrifuged at *ca.* 400 *g* for 20 min to separate serum as the supernatant. Serum was filtered through 0.45 μm porosity nylon filters (Alltech Associates, Deerfield, IL, USA) to remove solids which tended to clog the sample injector.

RESULTS AND DISCUSSION

Metachromasia

The drug–dye complex absorbs strongly at 525 nm, as shown in Fig. 2; free dye has very little absorbance at that wavelength. The complexation reaction is rapid, and the complex is stable during the time interval between formation and measurement. Metachromatic complexes tend to aggregate and precipitate. Their absorbance decreases with time; sample handling accelerates the process. In a flowing system the time interval between complex formation and measurement is accurately fixed. Variability in sample handling is reduced in the automated system, and precision is improved.

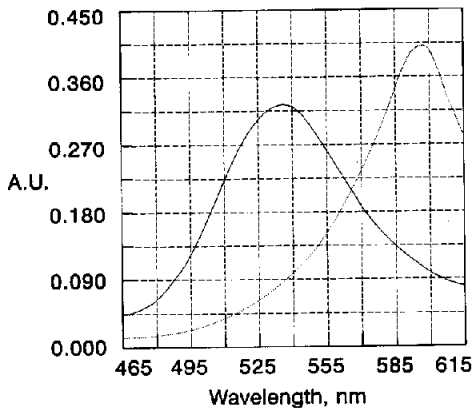

Fig. 2. Metachromatic interaction between dextran sulfate (DS) and 1,9-dimethylmethylene blue (DMMB). (———) DS–DMMB complex; (------) DMMB dye alone.

TABLE I

EFFECT ON PEAK SIZE OF DIMENSIONS OF TUBING USED TO CONNECT MIXING TEE TO DETECTOR

Tubing I.D. (mm)	Tubing length (cm)	Time[a] (s)	Peak area
0.254	115.3	2.33	14 000
	57.6	1.17	15 000
	28.8	0.58	16 000
0.508	21.6	1.75	12 000
	12.8	1.04	8000
	8.8	0.71	4000
0.762	21.6	3.94	5000
	12.8	2.33	4600
	8.8	1.60	3600

[a] Time is transit time for sample band to travel from mixing tee to detector.

Mixing of the sample and dye in the online mixing tee was not adequate for maximal complex formation. The length and diameter of the tubing connecting the mixing tee to the detector had a pronounced effect on the peak size (Table I). Tubing of 0.254 mm I.D. and 28.8 cm length was chosen for the analysis.

Molecular mass

A molecular mass determination of dextran sulfate was performed by Wyatt Technology Corporation (Santa Barbara, CA, USA). Samples were analyzed using SEC/LLS (size-exclusion chromatography/laser light scattering) on a Wyatt DAWN®F laser light scattering detector with a HeNe laser at 633 nm and a Wyatt/Optilab 903 interferometric refractometer at 633 nm. The chromatographic column was calibrated for molecular mass determination of dextran sulfate. A plot of molecular mass *versus* elution volume is shown in Fig. 3.

Dextran sulfate was administered intravenously to rats (20 mg/kg) through the lateral tail vein. Blood samples taken between five and fifteen min after dosing were analyzed. In agreement with a previous study [13], our data indicate that dex-

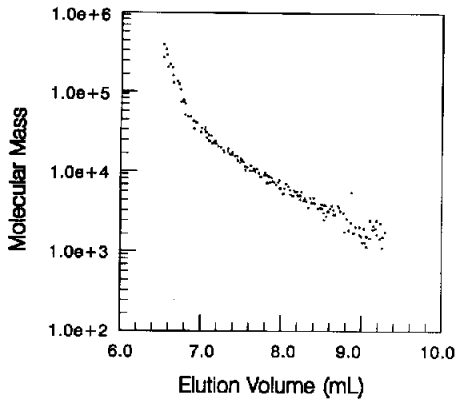

Fig. 3. Molecular mass of dextran sulfate *versus* elution volume.

tran sulfate is metabolized to form products with higher and lower molecular masses (Fig. 4).

Precision

For replicate injections on different days, the relative standard deviation in peak areas is typically less than 2%. Table II shows peak areas of 0.2 mg/ml standards of dextran sulfate dissolved in mobile phase. Use of a pulseless pump is important for delivery of the dye solution. Pump pulsations cause baseline noise which interferes with peak detection and integration.

Accuracy

The concentration of dextran sulfate in serum can be determined accurately relative to a standard of the same material dissolved in mobile phase. No drug is lost during sample preparation. Standard solutions and serum samples spiked with drug were centrifuged and filtered; recovery was 100%. Whole blood spiked in duplicate was prepared and analyzed. A 99.9% recovery of spike (98.1, 101.6%) was found. Another test of accuracy used the linearity data shown below. The equation of the line was used to recalculate the concentrations of selected samples from the original data set; samples near the limits of linearity were excluded. The calculated values were compared to the nominal concentrations. For twelve samples ranging from 10 μg/ml

TABLE II

PRECISION OF ANALYSIS; PEAK AREAS OF REPLICATE INJECTIONS OF DEXTRAN SULFATE STANDARDS

	Sample 1		Sample 2	
	Day 1	Day 2[a]	Day 1	Day 2[a]
	7257.2	7012.2	7005.0	7182.3
	7190.5	7211.4	7091.5	7343.1
	7288.8	7119.4	7207.4	7285.7
	7112.8	7268.7	7114.0	7264.5
Mean	7212.3	7152.9	7104.5	7268.9
R.S.D. (%)	1.1	1.1	1.2	0.9
Mean of four days		7184.7		
R.S.D. of four days (%)		1.0		

[a] One week apart.

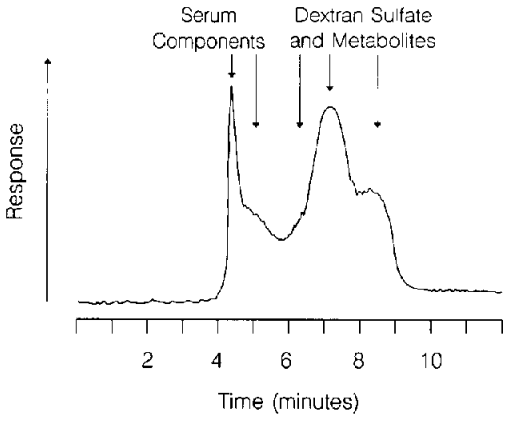

Fig. 4. Chromatogram of rat serum collected fifteen min after intravenous administration of dextran sulfate.

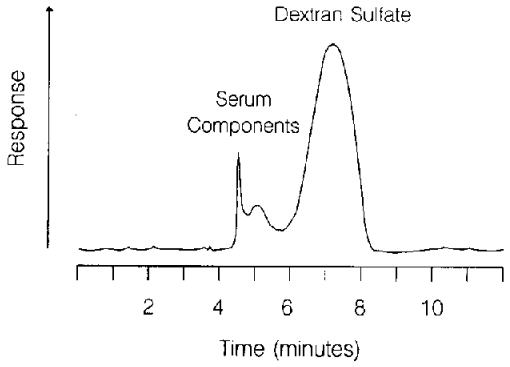

Fig. 5. Chromatogram of rat serum spiked with dextran sulfate (0.4 mg/ml).

to 330 μg/ml, the mean difference between the calculated and actual values was 3 μg/ml. No concentration related bias was seen in a scatter plot of the results (data not shown).

Linearity and limit of detection

The chromatographic response was linear from the quantitation limit to *ca.* 0.4 mg/ml. At higher sample concentrations the peak heights and areas approached a maximum value. Thirteen sample concentrations were used to calculate the line. The slope was 318 with a standard error of 4.6; the intercept was −0.9 with a standard error of 0.87. At the 95% confidence level the *y*-intercept was equivalent to zero. The standard error of the regression was 2.07, and the correlation coefficient was 0.999.

The detection limit, defined as a peak height three times the width of the baseline, was 0.3 μg/ml; the quantitation limit was 8 μg/ml.

Specificity

Dextran sulfate was resolved from peaks arising from components of rat serum (Fig. 5). For a compound to interfere in the analysis, it must have the same molecular size as the drug in the mobile phase and either exhibit metachromasia or absorb light at 525 nm. Constituents of rat blood absorb strongly at ultraviolet wavelengths; most are transparent at 525 nm. Two serum peaks were seen in chromatograms. These materials have higher molecular weights than dextran sulfate and did not interfere in its analysis.

Ruggedness

Retention time and peak shape of dextran sulfate are affected by the ionic strength of the mobile phase. Ionic strength of the mobile phase should be kept constant from batch to batch. As dye coats the lenses of the flow cell, peak areas decrease. The flow cell should be flushed every three to four hours with 1.8 *M* sulfuric acid. Direct injection of serum causes no damage to the analytical column. The small injection volume of 10 μl enables hundreds of samples to be run before the guard column must be replaced. Temperature of the analysis was found to be important in the SEC analysis of carrageenan [23]. However, no change in dextran sulfate retention time or peak shape occurs at temperatures from ambient to 50°C. Control of pH reduces secondary retention mechanisms. In an unbuffered mobile phase injection of 100 to 200 μl of simulated gastric fluid temporarily changed the peak shape and retention time.

CONCLUSIONS

The method described here is suitable for quantitation of dextran sulfate in rat serum. Detection of the metachromatic drug–dye complex enhances the selectivity and sensitivity of the analysis. The sensitivity is comparable to methods involving radiolabeling. The extensive sample preparation required for spectrophotometric analyses is not necessary. The procedure is automated and uses equipment readily available in most pharmaceutical laboratories.

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