Carbohydrate Research, 247 (1993) 263–278
Elsevier Science Publishers B.V., Amsterdam

"Fast moving" and "slow moving" heparins, dermatan sulfate, and chondroitin sulfate: qualitative and quantitative analysis by agarose-gel electrophoresis *

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(Received December 7th, 1992; accepted April 5th, 1993)

ABSTRACT

Heparin from beef intestinal mucosa, dermatan sulfate from beef intestinal mucosa, and chondroitin sulfate from bovine trachea were extracted and purified, and their structures and physico-chemical properties were evaluated by different techniques (disaccharide patterns by specific enzymatic cleavage, relative molecular mass by high-performance size-exclusion chromatography, sulfate-to-carboxyl ratio by potentiometric determination). Heparin was fractionated into "slow moving" and "fast moving" fractions by selective precipitation as the barium salt at different temperatures. The "fast moving" and "slow moving" components of heparin, dermatan sulfate, and chondroitin sulfate were utilized to run calibration curves in agarose-gel electrophoresis. Mixtures containing different amounts of these glycosaminoglycans were made and separated by agarose-gel electrophoresis, and these were analyzed quantitatively. For analysis of relative amounts, the area of each individual component of mixtures, obtained by photodensitometric readings, was divided by the sum of the areas of all glycosaminoglycans and expressed as a percentage. For analysis of absolute amounts, the area under the curve for each

* Abbreviations used. GAGs: Glycosaminoglycans. CS: Chondroitin sulfate. DS: Dermatan sulfate. Hep: Heparin. FM: Fast moving heparin. SM: Slow moving heparin. HS: Heparan sulfate. HPSEC: high-performance size-exclusion chromatography. M_r: Relative molecular mass. SAX-HPLC: Strong anion exchange-high performance liquid chromatography.

ΔDi-0S: 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose. ΔDi-4S: 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-4-*O*-sulfo-D-galactose. ΔDi-6S: 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-galactose. ΔDi-2,6diS: 2-acetamido-2-deoxy-3-*O*-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-galactose. ΔDi-2,4diS: 2-acetamido-2-deoxy-3-*O*-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-4-*O*-sulfo-D-galactose. ΔDi-4,6diS: 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-4,6-di-*O*-sulfo-D-galactose. ΔDi-2,4,6,TriS: 2-acetamido-2-deoxy-3-*O*-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-4,6-di-*O*-sulfo-D-galactose.

ΔDiH-0S: 2-acetamido-2-deoxy-4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose. ΔDiH-NS: 2-deoxy-4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-2-sulfoamino-glucose. ΔDiH-6S: 2-acetamido-2-deoxy-4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-glucose. ΔDiH-2,NdiS: 2-deoxy-4-*O*-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-2-sulfoamino-glucose. ΔDiH-N,6diS: 2-deoxy-4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-2-sulfoamino-D-glucose. ΔDiH-TriS: 2-deoxy-4-*O*-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-2-sulfoamino-D-glucose.

component of mixtures was fitted to specific calibration curves, and the amount of each glycosaminoglycan was calculated in μg. The quantitative procedure performed by analysing absolute amounts was used to obtain an accurate quantitative evaluation of each component in mixtures of glycosaminoglycans utilized for pharmaceutical purposes. A sensitive method was developed for the evaluation of very small amounts (0.2% w/w) of possible glycosaminoglycans as contaminants in preparations of a single species of glycosaminoglycan. This technique requires specific enzymatic degradation by bacterial lyases, separation in agarose-gel electrophoresis, and quantitative analysis by photodensitometric analysis and specific calibration curves.

INTRODUCTION

Glycosaminoglycans (GAGs; heparin and heparan sulfate, dermatan sulfate, and chondroitin sulfates) are sulfated heteropolysaccharides structured as alternating copolymers of uronic acids and amino sugars[1,2]. These polysaccharides are heterogeneous in terms of relative molecular mass (M_r), charge density, physico-chemical properties, and biological activities[3].

Heparin (Hep) and heparan sulfate (HS) have a more heterogeneous structure due to variously sulfated regions distributed along the chain. They are composed of variously nonsulfated and/or 2-*O*-linked sulfated β-D-glucuronic and α-L-iduronic acids α-(1 → 4)-linked to *N*-acetyl-α-D-glucosamine (6-sulfate) or *N*-sulpho-α-D-glucosamine (3-sulfate and/or 6-sulfate)[1]. The quantitative differences of these monosaccharide units and the heterogeneity of disaccharide units and oligosaccharide blocks are responsable for the physico-chemical (charge density, flexibility of the chain, specific oligosaccharides sequences), biological (protein affinity, cell interactions), and pharmacological (anticoagulant, antithrombotic, and thrombolytic activities) properties of Hep and HS. A high proportion (> 80%) of *N*-sulfoglucosamine is present in Hep; hence, this polysaccharide is heavily *O*-sulfated (a sulfate-to-carboxyl ratio > 2) and shows a high iduronic to glucuronic acid ratio[4]. Conversely, HS shows a lower proportion of *N*-sulfoglucosamine units and, therefore, a lower iduronic acid content and lower overall degree of *O*-sulfation[5] (a sulfate-to-carboxyl ratio < 1). Hep has relatively high amounts of pentasaccharide sites with high affinity fo Antithrombin III. Relatively strong anticoagulant activity is associated with fractions of HS, depending on the cells and tissues from which this GAG is extracted and purified. HS from the vascular endothelium and cultured endothelial cells has variable amounts of *N*-sulfoglucosamine 3-sulfate[6].

Polysaccharide chains of dermatan sulfate (DS) consist of a prevailing disaccharide unit [(1 → 4)-*O*(α-L-idopyranosyluronic acid)-(1 → 3)-*O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl 4-sulfate)][2], and other variously sulfated disaccharide units in different amounts are responsable for the biological and pharmacological properties of this GAG[7].

Chondroitin sulfate A (CS A, chondroitin 4-sulfate) is composed of [(1 → 4)-*O*-(β-D-glucopyranosyluronic acid)-(1 → 3)-*O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl 4-sulfate)], and chondrotin sulfate C (CS C, chondroitin 6-sulfate) is mainly composed of a disaccharide unit [(1 → 4)-*O*-(β-D-glucopyranosyluronic acid)-(1 → 3)-*O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl 6-sulfate)][8]. Disaccha-

rides with a different number and position of sulfate groups can be located in different percentages inside the polysaccharide chain, such as the nonsulfated disaccharide or the disulfated disaccharide in which two sulfate groups can be *O*-linked at position 2 of β-D-glucuronic acid and 6 of *N*-acetyl-α-D-galactosamine (disaccharide D) or at positions 4 and 6 of *N*-acetyl-α-D-galactosamine (disaccharide E)[9].

Electrophoretic techniques are powerful tools for the separation and quantitative analysis of GAGs. This is important for evaluating the amounts of GAGs in mixtures utilized for pharmacological purposes and for quantifying the concentration of heteropolysaccharides possibly present as pollutants of "purified" preparations of GAGs. Paper[10], cellulose acetate[11–14], polyacrylamide[15,16], agarose-gel[17], and capillary[18] electrophoresis are analytical techniques used for qualitative and quantitative analysis of GAG mixtures extracted and purified from organs, tissues, and cells, and in preparations for clinical and pharmacological uses.

In this paper, we report the extraction and purification of Hep, SM, FM, DS, and CSs, and the analytical techniques used to evaluate their physico-chemical properties and structures. Moreover, calibration curves of purified GAGs were performed in agarose-gel electrophoresis. Mixtures containing different amounts of these GAGs were made and separated. Quantitative evaluation of each GAG component in mixtures was made by analysis of relative amounts (without considering calibration curves) and absolute amounts (using specific calibration curves). Small amounts (0.2% w/w) of contaminant GAGs were detected in preparations of Hep, DS, and CSs by specific enzymatic degradation and agarose-gel electrophoresis separation, and by quantitative analysis utilizing specific calibration curves.

EXPERIMENTAL

Extraction and purification of heparin.—Beef intestinal mucosa was ground and treated with proteolytic enzymes at 65°C for 12 h in a reaction vessel fitted with a thermostatic bath, stirrer, and thermometer. After heating to 100°C for 30 min, the product was filtered on a Diatomite filter (Dicalite, High Performance Filter Aids), and the solution containing polysaccharides was percolated through a column of strong anion-exchange resin (HO^- form; Purolite A860, batch 1/88 from Purolite International). Peptides, nucleic acids, and GAGs with low charge density (CS, DS, HS) were eluted with 0.7–1.5 M NaCl. Hep was eluted with 2.0–3.0 M NaCl, and the recovered solution was added to 1.0–1.5 vol of acetone[19]. Any DS present was removed by selective precipitation as its copper salt, as reported elsewhere[20].

The SM and FM components of Hep were purified as their barium salts at different temperatures, as previously reported[21]. Purified beef-intestinal mucosa Hep was dissolved in water, and barium acetate (5%) was slowly added with stirring (the pH of the solution was adjusted to 6.0–7.0). After heating to 50–70°C, the solution was left at room temperature (20–25°C) for 24 h.

The precipitate obtained was solubilized in water and converted into the sodium salt by a cation-exchange resin (Na^+ form; Amberlite IR-120, Rohm & Haas). The crude SM sodium salt was collected by precipitation with 2.0 vol of acetone and dried. The supernatant solution was maintained at 5°C for 24 h and the precipitate was collected by centrifugation at 5°C. The FM barium salt was purified as reported for SM.

Extraction and purification of dermatan sulfate.—DS extraction and purification was performed as reported by Volpi[20]. Beef intestinal mucosa was treated as described for heparin. Peptides, nucleic acids, and GAGs with low charge density (HS) were eluted from a column of strong anion-exchange resin (HO^- form; Purolite A860, batch 1/88) with 0.7–1.5 M NaCl. DS was eluted with 1.7–1.8 M NaCl, and the recovered solution was added to 0.3–0.7 vol of acetone. The precipitate was discarded and 1.0–1.5 vol of acetone were added to the filtrate. DS was purified (elimination of CS and Hep) by selective precipitation with copper acetate and acetone. DS copper salt was converted into DS sodium salt by a cation exchange resin (Na^+ form; Chelex 100, Bio-Rad). The crude DS sodium salt was collected by precipitation with 1.0–1.5 vol of acetone and dried.

Extraction and purification of chondroitin sulfate.—Bovine trachea was treated as reported for heparin. The solution containing polysaccharides was percolated through a column of a strong anion-exchange resin (HO^- form; Purolite A860, batch 1/88). Peptides and nucleic acids were eluted with 0.7–1.5 M NaCl, and CS was eluted with 1.7–1.8 M NaCl; the recovered solution was added to 1.0–1.5 vol of acetone. The CS was percolated through columns of strong cation-exchange resin (Na^+ form; Amberlite IR-120, strongly acidic polystyrene gel-type resin) and converted into CS sodium salt.

Determination of relative molecular masses.—Equipment: HPLC by Jasco (pump, model 880 PU; system controller, model 801 SC; ternary gradient unit, model 880-02; injector, Rheodyne equipped with a 100-μL loop). UV detector (Jasco, model 875 UV). The mobile phase was composed of 125 mM Na_2SO_4 and 2 mM NaH_2PO_4 adjusted to pH 6.0 with 0.1 M NaOH. The flow rate was 0.9 mL/min with a back pressure of 25 kg/cm^2.

GAGs with different M_r were solubilized in the mobile phase at a concentration of 5 mg/mL; 10 μL (50 μg) were injected for HPLC.

Columns: Protein Pak 125 and 300 assembled in series (Waters, 84601 and T72711). Protein Pak 125 (7.8 mm × 30 cm) has the following M_r exclusion ranges: native globular, 2 000–80 000; and random coil, 1 000–30 000. Protein Pak 300 (7.5 mm × 30 cm) has the following M_r ranges: native globular, 10 000–400 000; and random coil, 2 000–150 000.

The M_r was determined by a calibration curve plotted with GAG standards whose M_r values were evaluated by the analytical ultracentrifuge according to Nieduszinski[22]. The third grade polynomial regression was performed by a Macintosh computer program.

Determination of sulfate-to-carboxyl ratio.—Sulfate and carboxyl groups were determined by potentiometric titration[23], with 0.1 M NaOH in water–DMF, of the acid-form GAGs obtained by removing the inorganic cations and possible impurity anions through strong ion-exchange resins (Amberlite IRA-400, HO^- form; and Amberlite IR-120, H^+ form). The sulfate-to-carboxyl ratio was also determined by enzymatic degradation after HPLC separation of the disaccharides. The ratio was calculated by considering the percentage and the presence of carboxyl and sulfate groups for each disaccharide.

Specific optical rotation.—Specific optical rotations were determined (Perkin–Elmer polarimeter 243B) on 10-mL samples at 25°C and at a concentration of 5% (w/v) of GAGs in water.

Determination of constituent disaccharides by cleavage with heparinase I, II, and III.—Samples (200 μg; 20 mg/mL in H_2O) of native Hep or its SM and FM components were incubated with 18.5 unit of Heparinase I (EC 4.2.2.7; Sigma H-7505; 12 000 unit/mg of protein; one unit forms 1.0 nmol of unsaturated uronic acid per min at pH 7.0, 37°C), 0.5 unit of Heparinase II (no assigned EC number; Sigma, H-6512; 147 unit/mg of protein; one unit forms 0.1 μmol of unsaturated uronic acid per hour at pH 7.0, 25°C); and 0.5 unit of Heparinase III (EC 4.2.2.8; Sigma, H-8801; 215 unit/mg of protein; one unit forms 0.1 μmol of unsaturated uronic acid per hour at pH 7.0, 25°C) in 100 mM acetate buffer, pH 7.0, in the presence of 2.5 mmol of calcium acetate. The reactions were stopped, after 24 h at 37°C, by boiling for 1 min.

The constituent disaccharides were determined by strong anion exchange(SAX)-HPLC[19,24]. Isocratic separation from 0 to 5 min with 0.2 M NaCl, pH 4.00; linear gradient separation from 5 to 60 min with 100% 0.2 M NaCl, pH 4.00, to 100% 1.2 M NaCl, pH 4.00. Flow: 1.4 mL/min. UV wavelength: 232 nm. AUFS: 0.100. Injection: 20 μL (4 μg of enzymatically degraded Heps).

Separation of nonsulfated and variously sulfated disaccharides was performed according to the standards and retention times of Seikagaku Kogyo Co.[25] (Fig. 1).

Determination of constituent disaccharides by cleavage with chondroitinase ABC.—Samples (100 μg; 10 mg/mL in H_2O) of native DS or CS were incubated with 625 munit of chondroitinase ABC (EC 4.2.2.7; Seikagaku Kogyo 100320; one unit will cleave DS or CS to form 1.0 μmol of unsaturated uronic acid per min at pH 8.0 and 37°C) in 50 mM pH 8.0 Tris · HCl buffer. The reactions were stopped, after 3 h of incubation at 37°C, by boiling for 1 min. The disaccharide units were determined by SAX-HPLC as reported above (Fig. 2).

Agarose-gel electrophoresis.–CSs, SM component of heparin, FM component of heparin, and DS were analyzed by agarose-gel electrophoresis in barium acetate–1,2-diaminopropane[17]. Agarose (Bio-Rad, Electrophoresis purity reagent, Standard low-mr) solution (0.50%) in 0.04 M barium acetate–acetic acid buffer (pH 5.8) was prepared by heating (100°C). After cooling to 60°C, the solution was layered (ca. 2-mm thickness) on glass plates (5 × 7 cm) and cooled (4°C); 5 μL of each sample were deposited by micropipette.

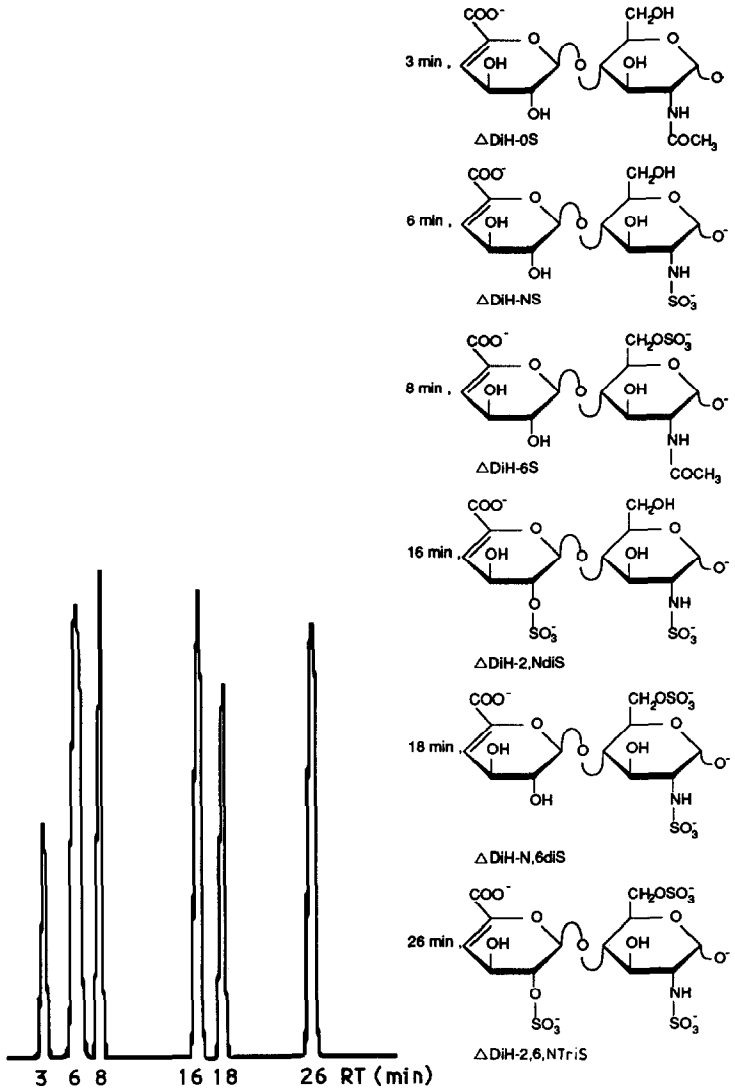


Fig. 1. SAX-HPLC separation and structural formulas of Hep (native, FM and SM components) standard unsaturated disaccharides.

The first run was performed in 0.04 M barium acetate buffer (pH 5.8) for 30 min at 120 V (ca. 60 mA) per plate; the second run was in 0.05 M 1,2-diaminopropane (buffered at pH 3.0 with acetic acid) for 60 min at 120 V (ca. 50 mA) per plate. After migration, the plate was soaked in Cetavlon (0.1% solution) for ca. 3 h, dried, and then stained with Toluidine Blue (0.2% in 40:9:1 EtOH–water–acetic acid) for 30 min. After bleaching with 50:49:1 ethanol–water–acetic acid, quantitative analysis of GAGs was performed by a photodensitometer (Minidensit Seac) at 583 nm, and calibration curves were obtained by measuring the absorbance of

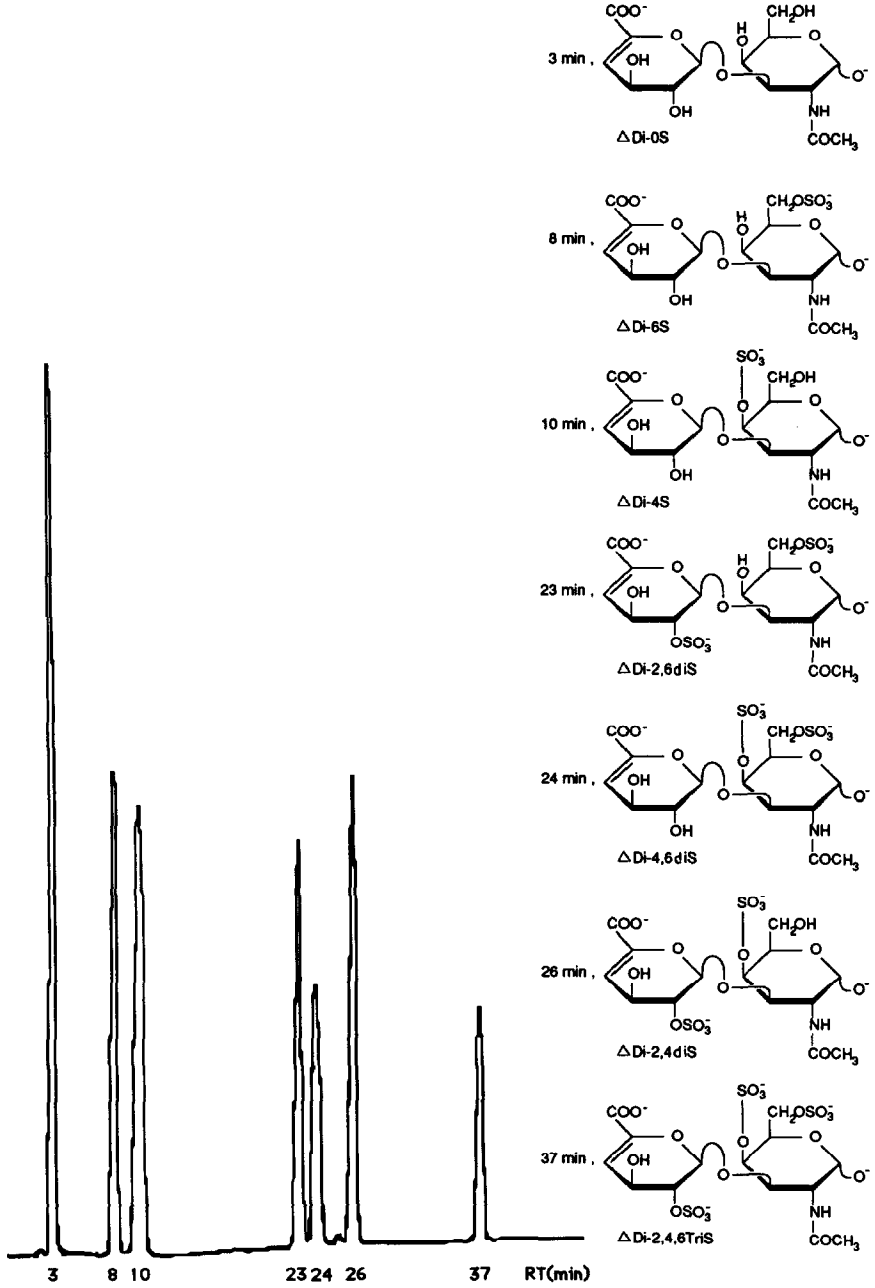


Fig. 2. SAX-HPLC separation and structural formulas of CS and DS standard unsaturated disaccharides.

increasing concentrations (1–6 μg; 1 mg/mL of GAGs in water) of CSs, FM, SM, and DS (Fig. 3).

Five mixtures of purified GAGs (SM from 2 to 10%, FM from 25 to 70%, DS

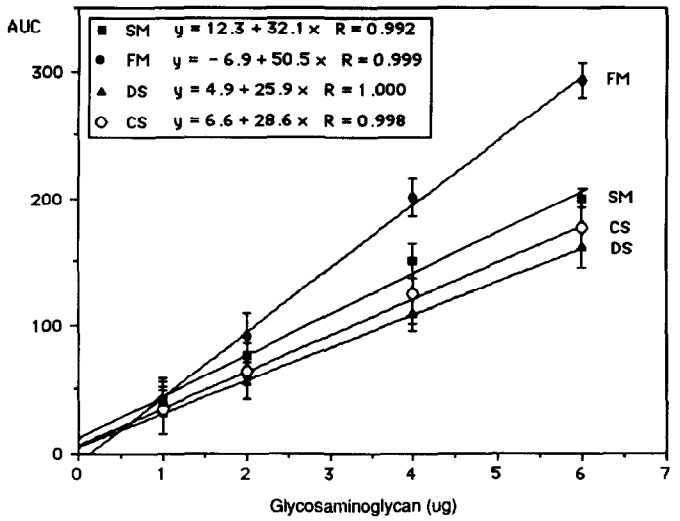


Fig. 3. Calibration curves of SM and FM heparin fractions, DS, and CS performed by separation in agarose-gel electrophoresis and photodensitometric analysis. For each GAG, at least six repeated tests were carried out. The equations and the correlation coefficients for each GAG are reported. AUC: area under the curve evaluated by a photodensitometer at 583 nm.

from 20 to 60%, and CSs from 2.5 to 15%) were prepared. The amounts of heteropolysaccharide in mixtures were chosen to resemble pharmaceutical GAG preparations, such as "Sulodexide" (Hep, 50 IU/mg; DS 25–60% and CS 3–15%; Sigma H-1642) and "Mesoglycan" (Hep, 50–70 IU/mg; DS 20–35% and CS 2–7%; Sigma H-0519). After separation by agarose-gel electrophoresis (also in this case, for each mixture, a minimum of six tests were repeated), the amounts of the single components of mixtures were evaluated by analysis of relative and absolute amounts. Analysis of the relative amounts of each component was performed by photodensitometric scanning at 583 nm. The area under the curve of each GAG in the mixtures was divided by the sum of the areas of all GAGs and expressed as a percentage. Analysis of absolute concentrations was obtained by fitting the area of each heteropolysaccharide on the calibration curves of the specific GAG and by calculating the absolute amount of each component in μg.

Agarose-gel separations of single purified GAGs for the calibration curves or mixtures were performed in different experiments due to the limited number of samples that can be separated in a single electrophoresis run. To exclude different processing of calibration curves and mixtures, repeated runs were performed for each purified GAG or mixture (from 1 to 6 μg for the curves and for each mixture containing a different percentage of GAGs).

Small amounts of Hep and/or HS as contaminants in DS and CS preparations were detected by enzymatic degradation by chondroitinase ABC (EC 4.2.2.4) and agarose-gel electrophoresis: 500 μg of DS or CS (500 μg/10 μL of distilled water) were treated with 0.5 unit of Chondroitinase ABC (Sigma, 0.34 unit/mg of

protein) in 40 μL of 50 mM Tris · HCl buffer, pH 8.0. After incubation at 37°C for 3 h and boiling for 1 min, 5 μL of the solution were deposited on the agarose plate as reported, and quantified by the specific calibration curves.

Small amounts of DS and/or CS as contaminants in Hep preparations were detected by enzymatic degradation by Heparinase I (EC 4.2.2.7) and agarose-gel electrophoresis: 500 μg of Hep (500 μg/10 μL of distilled water) were treated with 25 unit of Heparinase I (Sigma, 12 000 unit/mg of protein) in 40 μL of 100 mM acetate buffer, pH 7.0 in the presence of 2.5 μmol of calcium acetate. After incubation at 37°C for 24 h and boiling for 1 min, 5 μL of the solution were deposited on the agarose plate as reported, and quantified by specific calibration curves.

RESULTS AND DISCUSSION

The physico-chemical properties and the structures of extracted and purified Hep (from bovine intestinal mucosa), FM, and SM heparin fractions obtained by precipitation as barium salts from bovine mucosa Hep, DS (from bovine intestinal mucosa), and CS (from bovine trachea) were evaluated by different analytical approaches. The M_r evaluated by HPSEC[26], the sulfate-to-carboxyl ratio determined by titrimetric determination and specific enzymatic cleavage, and the optical rotation are shown in Table I.

The Hep chains synthesized as a proteoglycan and attached to a peptide core composed essentially of alternating serine and glycine residues are much more extended (M_r = 60 000–100 000)[27] than the chains of commercially available preparations[28]. The newly synthesized chains are degraded by an endo-β-D-glucuronidase to fragments similar in size to the commercially available polysaccharide[29]. Under our experimental conditions, Hep extracted and purified from bovine intestinal mucosa has M_r = 11 600, about the same order of size as commercial Hep (M_r = 10 00–14 000)[30] (Fig. 4). The bivalent cationic salt–high M_r Hep chains can be fractionated at room temperature and divided from the bivalent cationic salt–low M_r Hep chains that are precipitated at low temperatures or in a mixed methanol–water solvent. The SM and FM sodium salt fractions, obtained by removing the bivalent cations with strong cation-exchange resins, were

TABLE I

Physico-chemical characteristics of purified Hep (from beef intestinal mucosa), SM and FM fractions, DS (from beef intestinal mucosa), and CS (from bovine trachea)

	Hep	FM	SM	DS	CS
Peak M_r	11,600	7,920	14,900	25,580	26,140
Sulfate-to-carboxyl ratio (titrimetric)	2.29	2.10	ND [a]	1.12	0.99
Sulfate-to-carboxyl ratio (enzymatic)	2.37	2.12	2.66	1.09	0.97
Specific rotation	+50°	+50°	+50°	−55°	−20°

[a] Not determined.

analyzed for physico-chemical properties (Table I) and disaccharide pattern (Table II). According to Takahashi[21], the SM fraction of Hep presents a higher M_r (14 900) (Fig. 4) and sulfate-to-carboxyl ratio (2.66) than the unfractionated Hep (M_r = 11 600; and ca. 2.35). On the contrary, FM has a lower M_r (7920) (Fig. 4) and sulfate-to-carboxyl ratio (2.12) than unfractionated Hep. The different charge density for different Heps is shown by the presence of variously nonsulfated and sulfated disaccharides (Table II). FM exibits greater amounts of nonsulfated, monosulfated, and disulfated disaccharides and a lower percentage of trisulfated disaccharides than unfractionated Hep. SM shows a very high percentage of trisulfated disaccharide (71.2%) and a low concentration of nonsulfated and mono- and di-sulfated disaccharides as compared to Hep (Table II). Purified FM and SM components of heparin were utilized to plot calibration curves for quantitative analysis in agarose-gel electrophoresis.

DS extracted and purified from bovine intestinal mucosa has an M_r or ca. 25 580 (Fig. 4), a sulfate-to-carboxyl ratio of ca. 1.10 (Table I) and a disaccharide pattern containing ca. 86% of unsaturated disaccharide (with a sulfate group *O*-linked in position 4 of α-*N*-acetyl-D-galactosamine) and ca. 8% of disaccharide B (with a sulfate group *O*-linked at position 2 of α-L-iduronic acid and at position 4 of α-*N*-acetyl-D-galactosamine) (Table III). The physico-chemical characteristics of CSs purified from bovine trachea are shown in Table I. This CS is a mixture of CS A (56%) and CS C (38%) (Table III).

SM, FM, DS, and CSs were separated on discontinuous agarose-gel electrophoresis (Fig. 5). SM has the lowest electrophoretic mobility (2.8×10^{-4} cm/V/min). FM shows a higher mobility (6.5×10^{-4} cm/V/min) and higher "dispersivity" of the spot, probably due to the greater molecular weight and charge-density heterogeneity of the chains. The higher heterogeneity of FM chains can be also seen in HPSEC profiles, in comparison with that of native Hep and SM (Fig. 4). The same large spot is evident for depolymerized Hep with an M_r of ca. 2 000–5 000[19]. The electrophoretic mobility of Heps is caused by different M_r and charge density. DS has a mobility of ca. 8.6×10^{-4} cm/V/min and CS of ca. 9.1×10^{-4} cm/V/min. These two GAGs have about the same M_r (25 580 and 26 140) and charge density (1.12 and 0.99) but different mobility, probably due to the flexibility of their chains and/or different affinity towards barium cations by different uronic acids[1,22].

The calibration curves for increasing concentration (1–6 μg) of purified GAGs are reported in Fig. 3, with their associated linear regression equations and the correlation coefficients. For this technique and under our experimental conditions, the coefficient of variation was always < 15. Commercially available pharmaceutical preparations of GAGs also consist of mixtures of SM, FM, DS, and CS A and C with different relative amounts ("Sulodexide" and "Mesoglycan"). Agarose-gel electrophoresis is one of the main analytical techniques used to perform quantitative analysis of mixtures of GAGs for pharmaceutical purposes. It allows the separation of the four components of a mixture (FM, SM, DS, and CSs) in a single

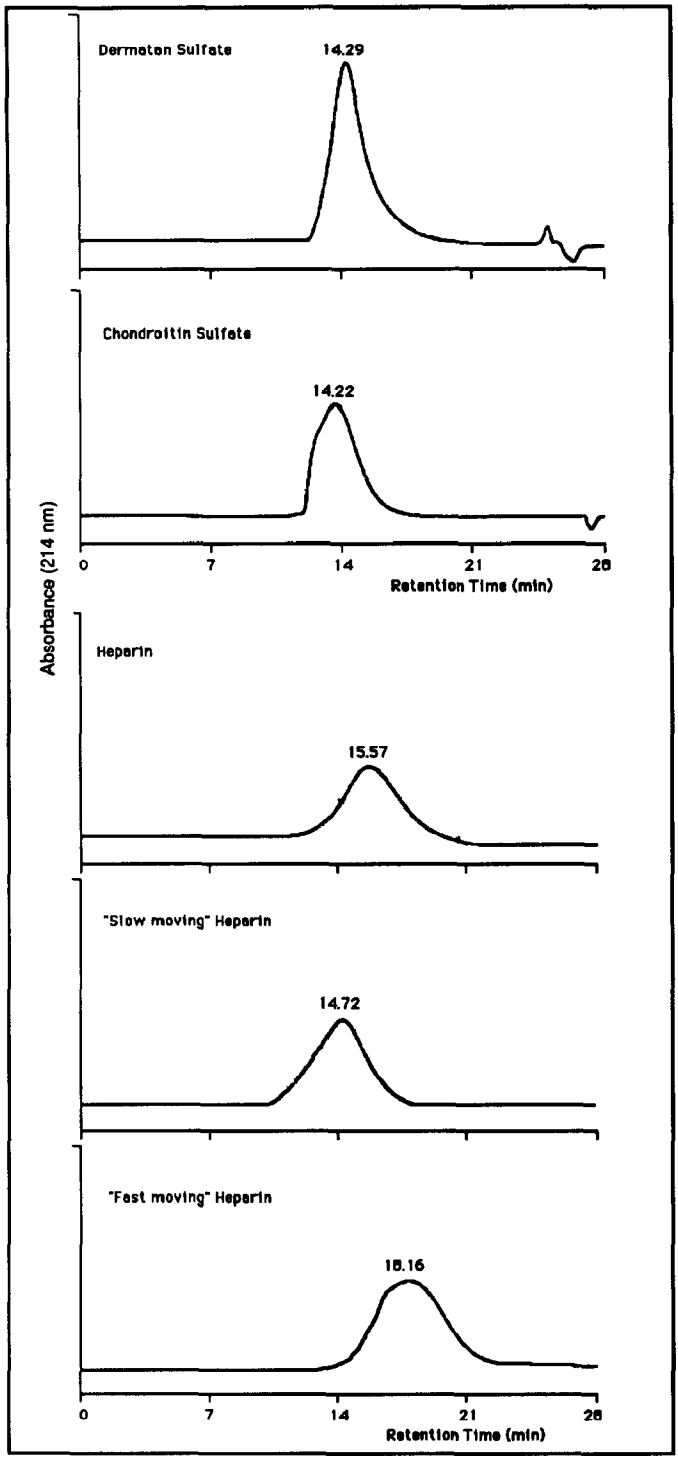
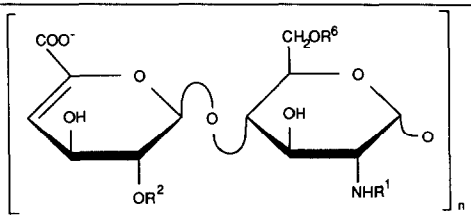


Fig. 4. High-performance size-exclusion chromatography profiles of purified Hep, SM and FM fractions, DS, and CS.

TABLE II

Disaccharide composition of purified Hep, and SM and FM fractions

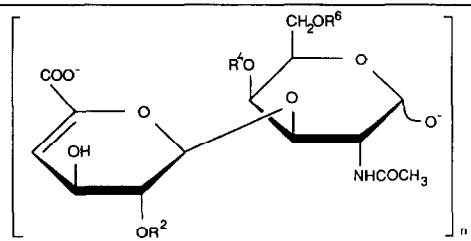


	R^1	R^2	R^6	Hep	FM	SM
ΔDiH-0S	$COCH_3$	H	H	6.3	8.9	2.0
ΔDiH-NS	SO_3^-	H	H	3.8	5.5	0.9
ΔDiH-6S	$COCH_3$	H	SO_3^-	2.4	2.5	0.2
ΔDiH-2,NdiS	SO_3^-	SO_3^-	H	19.2	22.9	15.3
ΔDiH-N,6diS	SO_3^-	H	SO_3^-	12.5	22.4	10.3
ΔDiH-2,6,NTriS	SO_3^-	SO_3^-	SO_3^-	55.8	37.7	71.2

run, and photodensitometric scanning provides quantitative analysis. For a more accurate determination of the relative amounts of GAGs in mixtures, quantitative analysis can be performed utilizing calibration curves. The extracted and purified GAGs were used to prepare five mixtures with different percentages of GAGs. This procedure is required to evaluate the accuracy of quantitative analysis (and percentage error) of GAGs in mixtures, not as a single purified component. After separation by agarose-gel electrophoresis, quantitative analysis was performed both with and without reference to specific calibration curves (absolute and relative measurements, respectively). The percentage error between these two

TABLE III

Disaccharide composition of purified DS and CS



	R^2	R^4	R^6	DS	CS
ΔDi-0S	H	H	H	1.0	4.4
ΔDi-6S	H	H	SO_3^-	3.0	37.9
ΔDi-4S	H	SO_3^-	H	86.0	56.0
ΔDi-2,6diS	SO_3^-	H	SO_3^-	0.5	0.8
ΔDi-4,6diS	H	SO_3^-	SO_3^-	1.3	0.8
ΔDi-2,4diS	SO_3^-	SO_3^-	H	8.0	0.0
ΔDi-2,4,6TriS	SO_3^-	SO_3^-	SO_3^-	0.2	0.0

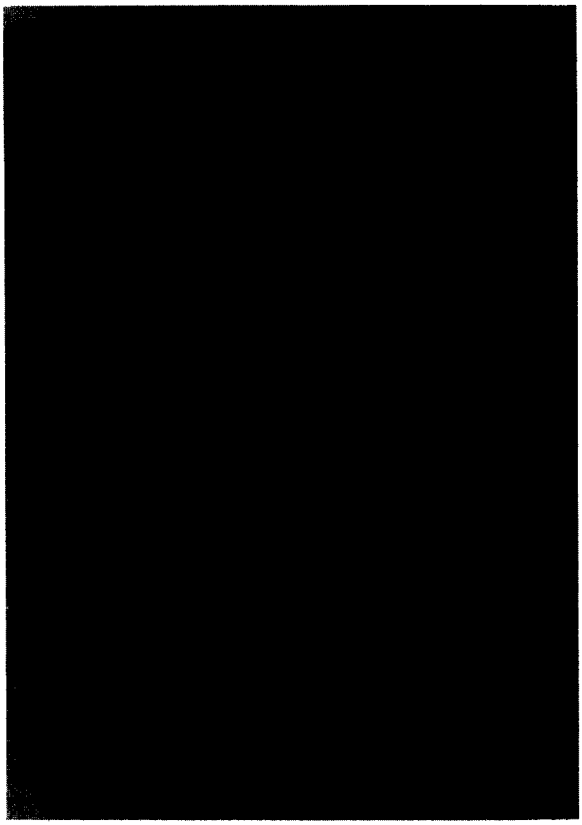


Fig. 5. Agarose-gel electrophoretic separation of SM and FM heparin fractions, DS, and CS. From left to right: SM heparin, FM heparin, a mixture of the four GAGs, DS, and CS.

procedures for quantitative analysis of GAGs mixtures (i.e., for analysis of relative and absolute amounts) is reported in Fig. 6. The percentage errors for quantitative analysis of SM, FM, DS, and CSs mixtures were always higher for the analysis of relative amounts than for absolute amounts obtained by using the calibration curves for each GAG (Fig. 6).

The absence of other GAGs as contaminants in the preparations of DS is very important in biological and pharmacological studies. In fact, low amounts of Hep can modify the biological activity of DS as regards HCII. Furthermore, it should be pointed out that small amounts of Hep and other GAGs could aggregate as supramolecular complexes with DS chains, in vitro and in tissues[31]; to date, the biological and pharmacological effects of these complexes have been poorly understood[32]. DS is known to give either self-aggregation or association with other GAGs[33]. On the other hand, several commercial preparations of Hep show different percentages (from < 1–15%) of contaminant DS[34]. The current analytical techniques, such as electrophoretic separations, HPSEC, specific optical rotation, and NMR[34], are not sensitive enough to quantify small amounts of Hep as a

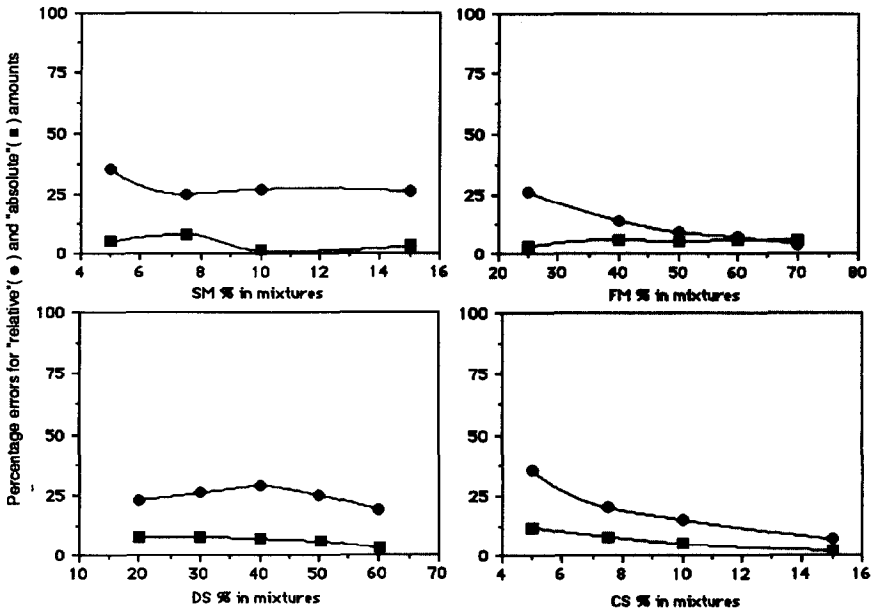


Fig. 6. Comparison of the percentage errors following quantitative measurements of the different amounts of glycosaminoglycans in mixtures, analyzed as relative and absolute amounts after agarose-gel separation.

contaminant in preparations of DS and CS, and small amounts of DS and CS as contaminants in preparations of Hep.

Simple agarose-gel electrophoresis can detect ca. 4% of contaminant GAG in a preparation of Hep or DS. Under our experimental conditions, the lower limit of detection of a heteropolysaccharide is 0.25 μg (Fig. 3), representing ca. 4.2% of a GAG present as contaminant in Hep, DS, or CS preparations.

Thus, we developed a method that requires specific enzymatic degradation of large amounts of DS, CS, or Hep (500 μg) followed by separation and quantification by specific calibration curves of small amounts of contaminant GAGs by agarose-gel electrophoresis and colorimetric detection. This method can detect 0.2% (w/w) of contaminant GAGs.

Small amounts of Hep as contaminant in preparations of DS were quantified by specific degradation of DS with chondroitinase ABC (which depolymerizes CS A and C, and DS). Possible contaminant Hep (SM and FM components) was separated by agarose-gel electrophoresis and quantified by the calibration curves at 583 nm; 0.2% (w/w) of Hep (1 μg in 500 μg of DS) can be detected by this procedure. The DS extracted and purified from beef intestinal mucosa was monitored for possible Hep contamination. The GAGs formed after degradation of DS with chondroitinase ABC were quantified at ca. 2% (w/w) and were found to be similar to FM.

Small amounts of DS and/or CSs as contaminant in preparations of Hep were detected by degradation of Hep with heparinase I (which depolymerizes Hep). Possible contaminants (DS and/or CSs) were separated by agarose-gel electrophoresis and quantified by calibration curves; 0.2% (w/w) of DS and/or CSs (1 μg in 500 μg of Hep) can be recognised. The GAGs formed after degradation of Hep (extracted and purified from beef intestinal mucosa) with heparinase I were quantified at ca. 1.5% (w/w) and were found to be similar to DS.

These results show that Hep extracted and purified from beef intestinal mucosa by conventional procedures[35] contains small amounts (ca. 1.5%) of DS. These results agree with those of Ludwig-Baxter and Perlin[36], who reported that the DS in some pharmaceutical Hep preparations is not substantially different from the "normal" DS of porcine mucosal tissue, as well as other DS. DS extracted and purified from beef intestinal mucosa by conventional procedures[37] shows small amounts (ca. 2%) of contaminant Hep (FM component). The presence of DS as a contaminant in preparations of Hep and FM as contaminant in preparations of DS probably depends on the organs from which these GAGs are extracted and purified: beef intestinal mucosa is rich in DS and Hep. The extraction and purification of heteropolysaccharides from other organs could produce GAG preparations with different contaminants (SM Hep, CSs, and HS).

Under our experimental conditions, CS purified from bovine trachea and used to plot the calibration curve, and in mixtures, does not show appreciable amounts of other GAGs. Small amounts of Hep as contaminant in preparations of CS were detected by degradation of CS with chondroitinase ABC and separation by agarose-gel electrophoresis, and eventually contaminant GAGs were quantified by the calibration curves. No detectable [$< 0.2\%$ (w/w)] contaminant Hep was detected in CS preparations and no DS was detected by conventional agarose-gel electrophoresis. CS was purified from bovine trachea by a strong anion-exchange resin, utilizing a salt molarity (1.7–1.8 M) similar to that used to purify DS. And under our experimental conditions bovine trachea CS does not show the presence of DS.

In this study, we report an accurate technique for quantitative analysis of the percentage of single components in a mixture of GAGs. This method requires separation of GAGs by agarose-gel electrophoresis and quantitative analysis using specific calibration curves. A highly sensitive and specific method for accurately detecting small amounts of heteropolysaccharide contaminant in preparations of GAGs was also developed. It is very important to evaluate accurately the purity of preparations of GAGs utilized for pharmaceutical purposes. Several activities (anticoagulant, antithrombotic, thrombolytic, and antilipemic) could be influenced by other GAG impurities, at the molecular and cellular levels as well as at the biological and pharmacological levels.

ACKNOWLEDGMENT

The author thanks Professor L. Bolognani for his constructive criticism.

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