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Disaccharide mapping of chondroitin sulfate of different origins by high-performance capillary electrophoresis and high-performance liquid chromatography

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Abstract

An HPCE method is described for the determination of disaccharides present in chondroitin sulfate/dermatan sulfate of different origins. Following chondroitinase digestion, nonsulfated, monosulfated and disulfated Δ-disaccharides, are separated and readily determined within 60 min on an uncoated fused-silica capillary using normal polarity at 20 kV and detection at 230 nm. Comparison was made by separation of these disaccharides in strong-anion exchange-HPLC. The system was applied to the analysis of chondroitin sulfate samples obtained from bovine, porcine and chicken tracheas, from shark cartilage, and of dermatan sulfate from porcine skin. The results indicate the existence of particular electropherographic as well as chromatographic patterns for each sample in the study, with some similitudes and differences.
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1. Introduction

Chondroitin sulfates (CSs) are heteropolysaccharides widely spread in the animal kingdom. They are part of connective and structural tissues and so they are mainly found in soft cartilage and articular connections. These macromolecules are composed of alternate sequences of uronic acid and differently sulfated residues of *N*-acetyl-D-galactosamine linked by β(1 → 3) bonds (Jackson, Busch, & Cardin, 1991). The regular disaccharide sequence of CS A, chondroitin-4-sulfate, is constituted by [(1 → 4)-*O*-(D-glucopyranosyluronic acid)-(1 → 3)-*O*-(2-*N*-acetamido-2-deoxy-D-galactopyranosyl-4-sulfate)]. CS C, chondroitin-6-sulfate, is mainly composed of a disaccharide unit [(1 → 4)-*O*-(D-glucopyranosyluronic acid)-(1 → 3)-*O*-(2-*N*-acetamido-2-deoxy-D-galactopyranosyl-6-sulfate)]. Polysaccharide chains of dermatan sulfate (DS) (chondroitin sulfate B) consist of a prevailing disaccharide unit [(1 → 4)-*O*-(idopyranosyluronic acid)-(1 → 3)-*O*-(2-acetamido-2-deoxy-D-galactopyranosyl-4-sulfate)]. Disaccharides with a different number and position of sulfate groups can be located, in different percentages, inside the polysaccharide chains, such as the nonsulfated or disulfated disaccharides in which two sulfate groups are *O*-linked in position 2 of D-glucuronic acid and 6 of *N*-acetyl-D-galactosamine (disaccharide D) or in position 4 and 6 of *N*-acetyl-D-galactosamine (disaccharide E). These heterogeneous structures, in terms of percentage of variously sulfated disaccharides, degree of sulfation, molecular mass, relative amounts of iduronic acid and glucuronate depending on the tissue of origin, are responsible for different and more specialized functions of these glycosaminoglycans (Jackson et al., 1991).

In the last few years, knowledge on the structure, biosynthesis, and physiological function of CS has accumulated. Various species of CS can be recognized by the structure of the repeating disaccharide units. Unsaturated disaccharides generated by the action of bacterial lyases (Beaty & Mello, 1987; Imanari, Toida, Koshiishi, & Toyoda, 1996; Linhardt, Galliher, & Cooney, 1986) are separated with various high-performance liquid chromatography (HPLC) modes (for review see Imanari et al. 1996) and,

Abbreviations: HPCE, high-performance capillary electrophoresis; SAX-HPLC, strong-anion exchange high-performance liquid chromatography; CS, chondroitin sulfate; DS, dermatan sulfate; M_W, weight average molecular weight.

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more recently, high-performance capillary electrophoresis (HPCE) (for review see Mao, Thanawiroon, & Linhardt, 2002). However, our understanding of the distribution of these unit-disaccharides within the respective CS species is apparently limited. Furthermore, no HPCE method was developed to analyze disaccharide units of CS from various sources. In this paper a method for analyzing unsaturated disaccharides derived from CS, and DS, by normal polarity HPCE, was devised and compared with strong-anion exchange HPLC (SAX-HPLC).

2. Materials and methods

2.1. Materials

CS samples from beef trachea (BTCS) having M_W of approx. 30,700, porcine trachea (PTCS) having an M_W of approx. 19,900, and chicken trachea (CTCS) having an M_W of 21,400 were extracted and purified according to Volpi, 1994 and Volpi, Fregni, and Venturelli (1995). Shark cartilage CS (SCCS) having an M_W greater than 50,000 was donated by IBSA (Institut Biochimique SA, Lugano, Switzerland). DS from porcine skin (PSDS) was prepared as previously reported (Volpi et al., 1995). DS was further purified by sequential precipitation with increasing volumes of acetone, by treatment with nitrous acid at low pH and selective precipitation with copper acetate and acetone. This preparation of DS had an M_W of approx. 43,800.

Chondroitin ABC lyase from *Proteus vulgaris* [E.C. 4.2.2.4] was from Sigma. All the other reagents were analytical grade.

2.2. Capillary electrophoresis

Capillary electrophoresis was performed on a Beckman HPCE instrument (P/ACE system 5000) equipped with a UV detector set at 230 nm. Separation and analysis were carried out on an uncoated fused-silica capillary tube (50 μm I.D., 85 cm total length and 65 cm from the injection point to the detector) at 25 °C. Before each run, the capillary tube was washed with 0.1 M NaOH for 1 min, bidistilled water for 5 min, and then with the operating buffer constituted of disodium hydrogen phosphate (40 mM), sodium tetraborate (10 mM) and SDS (40 mM) buffered at pH 9.0 by the addition of 1 M HCl for 5 min. The operating buffer used was degassed by vacuum filtration through a 0.2 μm membrane filter, followed by agitation in an ultrasonic bath. The samples to be analyzed were injected automatically, using the pressure injection mode, in which the sample is pressurized for 20 s. The injection volume can be calculated with the Poiseuille equation as proposed by the manufacturer, giving an estimated volume of 6 nl per second of injection time. The electrophoresis was performed at 20 kV (about 50 μA) using normal polarity. Peak areas were recorded and calculated using the Beckman software system Gold V810.

2.3. Strong-anion exchange high-performance liquid chromatography

HPLC equipment was from Jasco (pump mod. PU-1580, UV detector mod. UV-1570, Rheodyne injector equipped with a 100 μl loop, software Jasco-Borwin rel. 1.5). The unsaturated disaccharides generated from CS samples treated with chondroitinase ABC were analyzed by strong anion-exchange (SAX)-HPLC separation using a 150 × 4.6-mm stainless-steel column spherisorb 5-SAX (5 μm, trimethylammoniopropyl groups $Si-CH_2-CH_2-CH_2-N^+(CH_3)_3$ in Cl^- form, from Phase Separations Limited, Deeside Industrial Park, Deeside Clwyd, UK) and detection at 232 nm, as reported (Volpi, 1999, 2000). Isocratic separation was performed using 50 mM NaCl pH 4.00 at a flow rate of 1.5 ml/min.

2.4. Enzymatic digestion of chondroitin sulfate samples

About 100 μg of CS samples were treated with 25 mU of chondroitinase ABC in 50 μl of 100 mM Tris/150 mM sodium acetate buffer pH 8.0 at 37 °C for 5 h. The reaction was blocked by boiling the solutions for 1 min.

3. Results

The determination of CSs is based on analyses of Δ-disaccharides produced by digestion with chondroitin lyases, and the separation of unsaturated disaccharides is therefore essential for the analysis of these polyanions. This separation is generally performed by HPLC, an analytical approach greatly used to analyze other similar polysaccharides such as hyaluronic acid, heparan sulfate, heparin (Beaty & Mello, 1987; Imanari et al., 1996). Their unsaturated disaccharides generated by the action of several lyases are normally separated by strong anion-exchange (SAX)-HPLC by using a linear gradient of salt, even if normal-phase and reversed-phase ion-pair HPLC methods are used (Beaty & Mello, 1987; Imanari et al., 1996). Nonsulfated and variously sulfated unsaturated disaccharides from CS of different origins and a sample of DS were effectively separated by SAX-HPLC (Fig. 1 from A–E). A good resolution of the peaks was obtained to quantify the variation in the species-related distribution of the disaccharides (Fig. 1 from A–E and Table 1).

HPCE was able to separate the nonsulfated, monosulfated and disulfated Δ-disaccharides with good resolution (Fig. 1 from A–E), and permitted determination of the relative amounts of the different constitutive disaccharide species of CS of various origins and a sample of DS (Table 1). The charge density expressed as sulfate to carboxyl ratio is also compared.

CS samples from bovine, porcine and chicken are mainly constituted of disaccharides, in different amounts, mono-sulfated in position 4 and 6 of *N*-acetyl-galactosamine, with

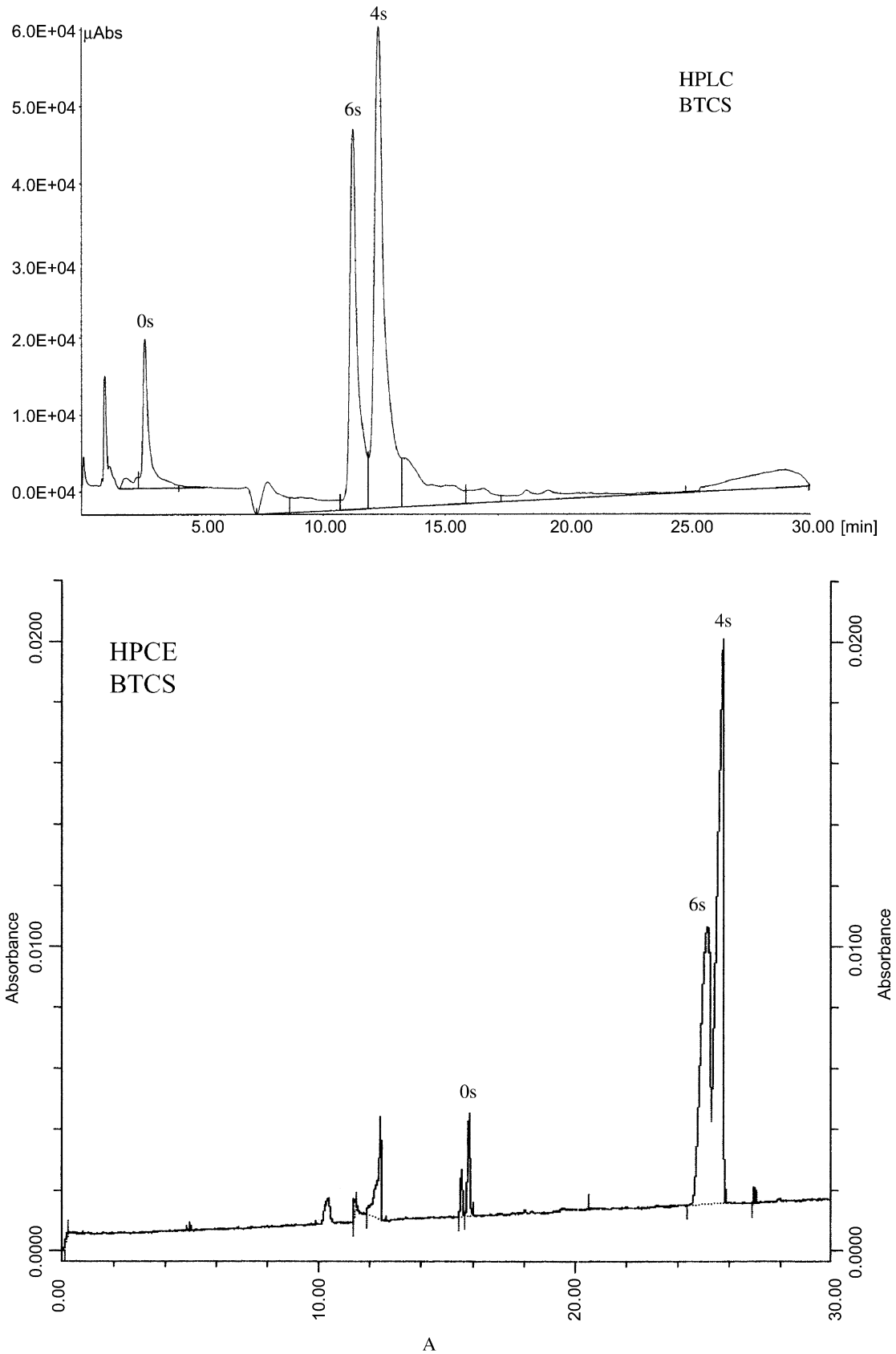
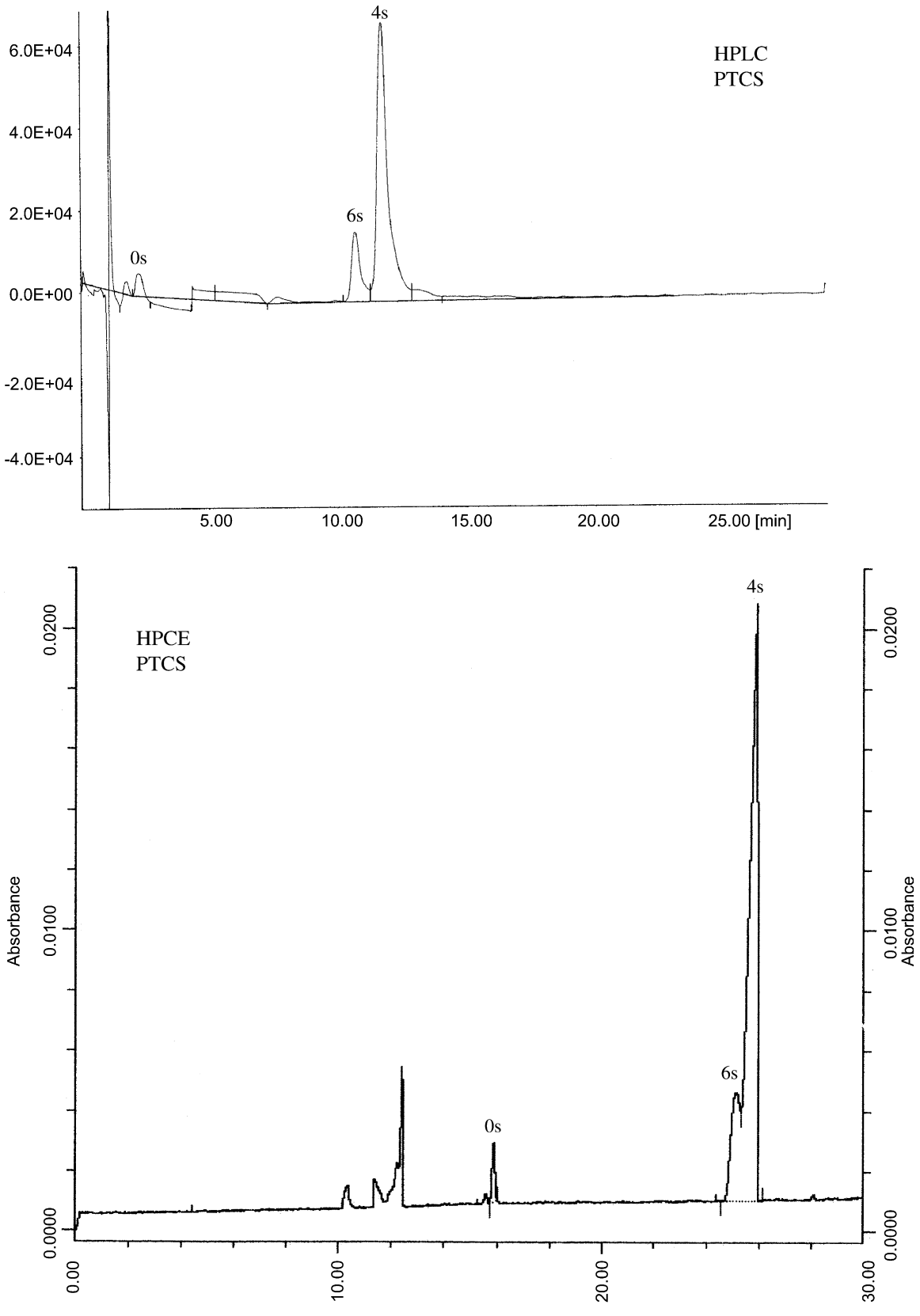
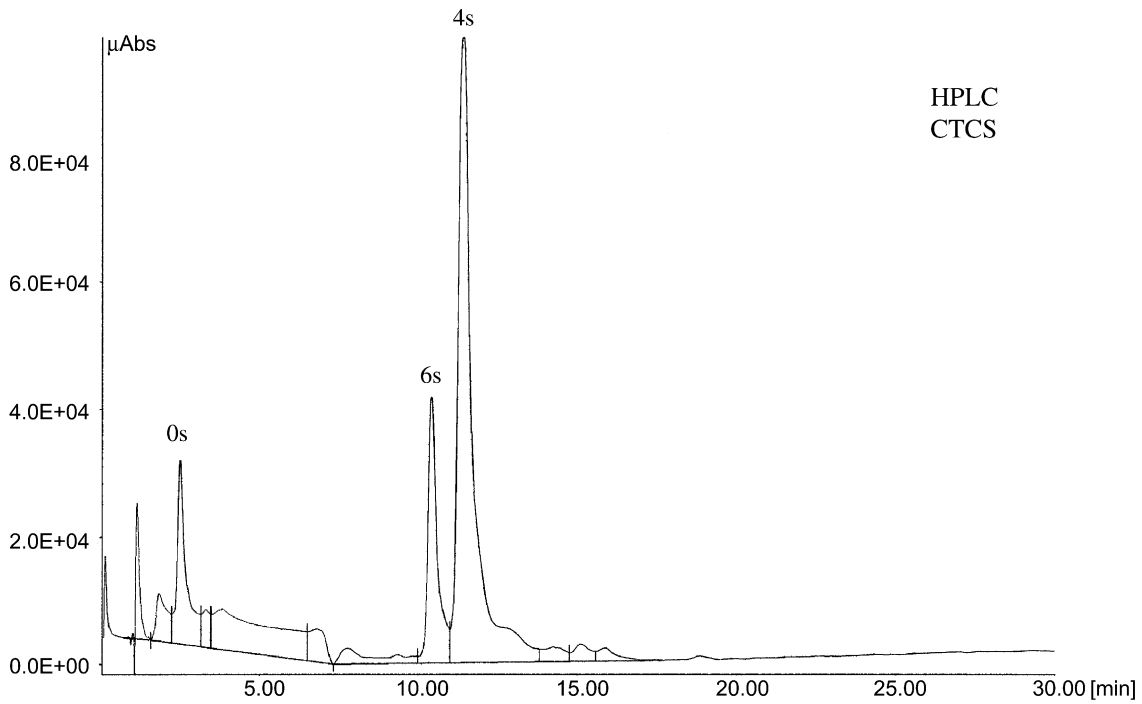


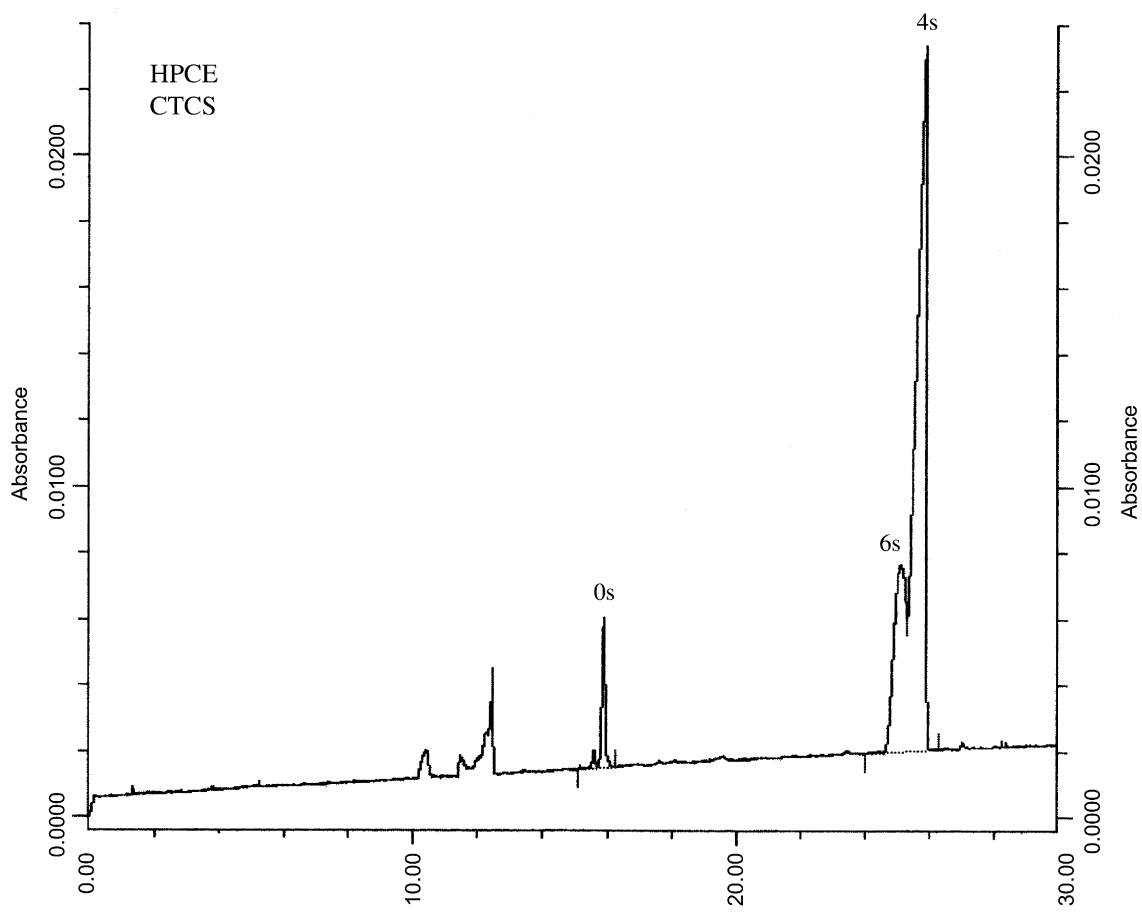
Fig. 1. SAX-HPLC chromatograms and HPCE electrophoregrams of (A) bovine trachea chondroitin sulfate (BTCS), (B) porcine trachea chondroitin sulfate (PTCS), (C) chicken trachea chondroitin sulfate (CTCS), (D) shark cartilage chondroitin sulfate (SCCS), and (E) porcine skin dermatan sulfate (PSDS). 0S, ΔHexA–GalNAc; 6S, ΔHexA–GalNAc (6-OSO_3); 4S, ΔHexA–GalNAc (4-OSO_3); 2,6diS, ΔHexA (2-OSO_3)–GalNAc (6-OSO_3); 2,4diS, ΔHexA (2-OSO_3)–GalNAc (4-OSO_3); 4,6diS, ΔHexA–GalNAc (4-OSO_3, 6-OSO_3). For the structure of unsaturated disaccharides see Table 1.



B

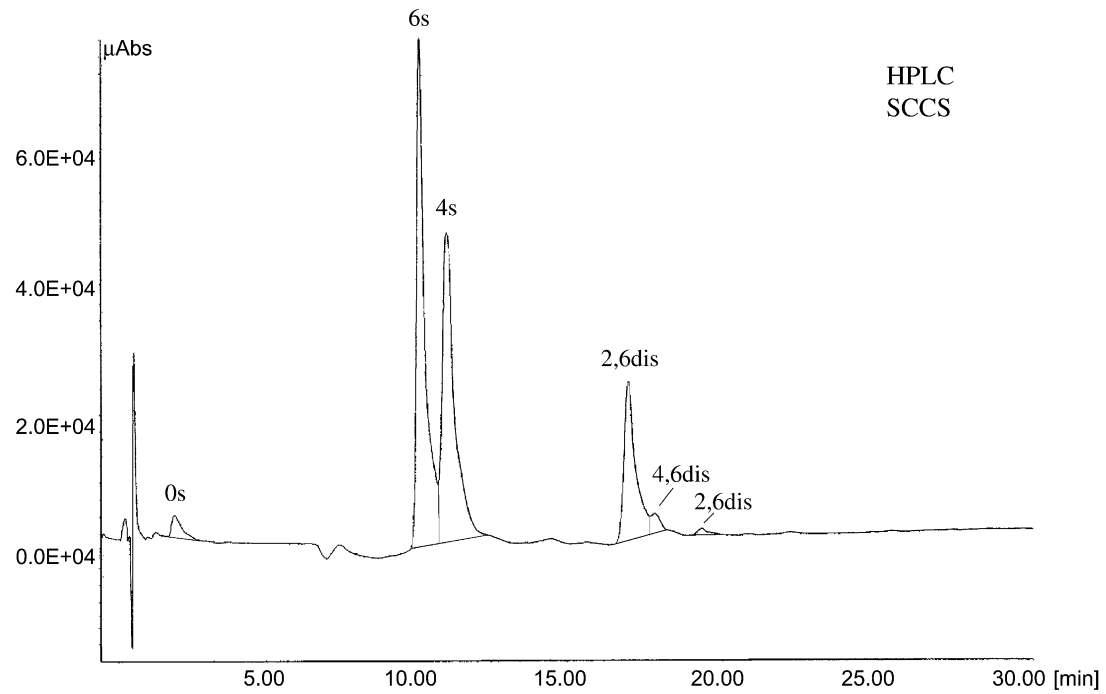
Fig.1 (*continued*)

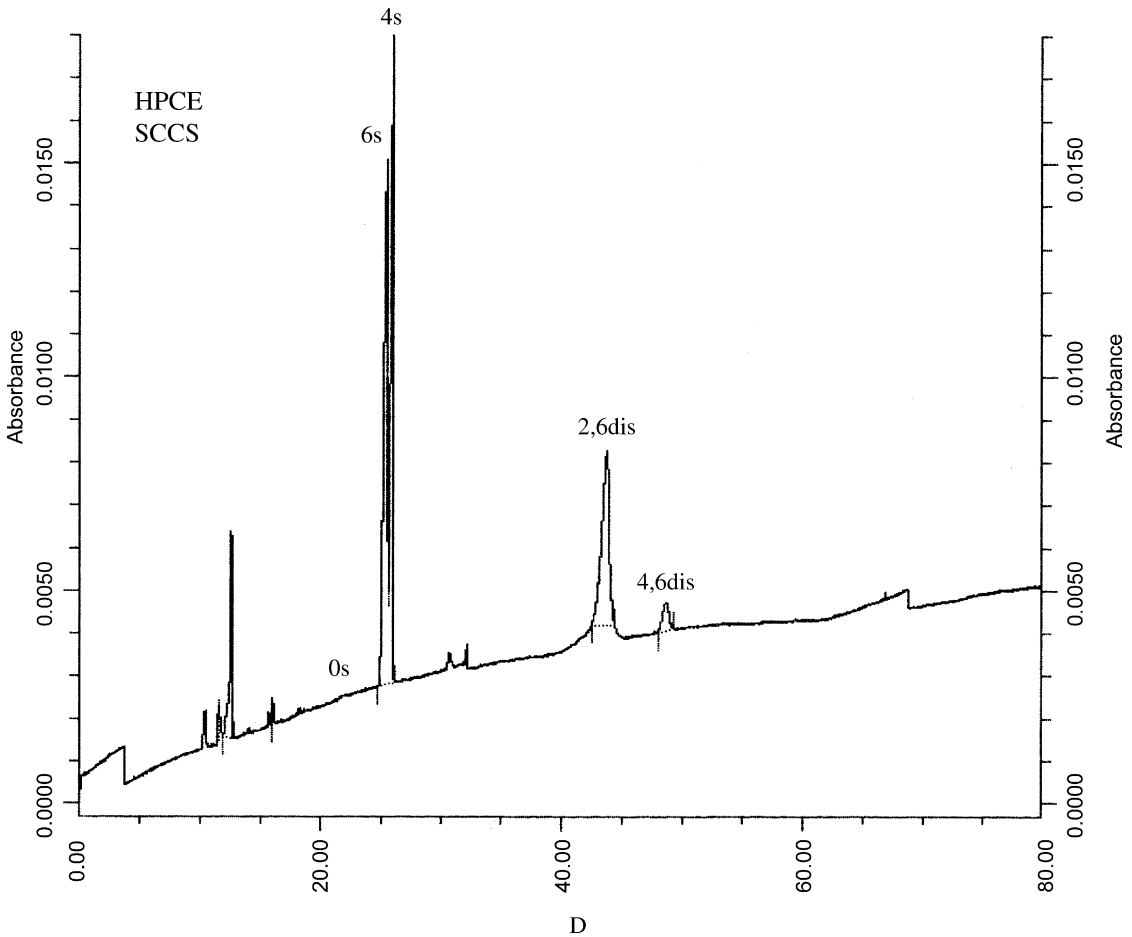




C

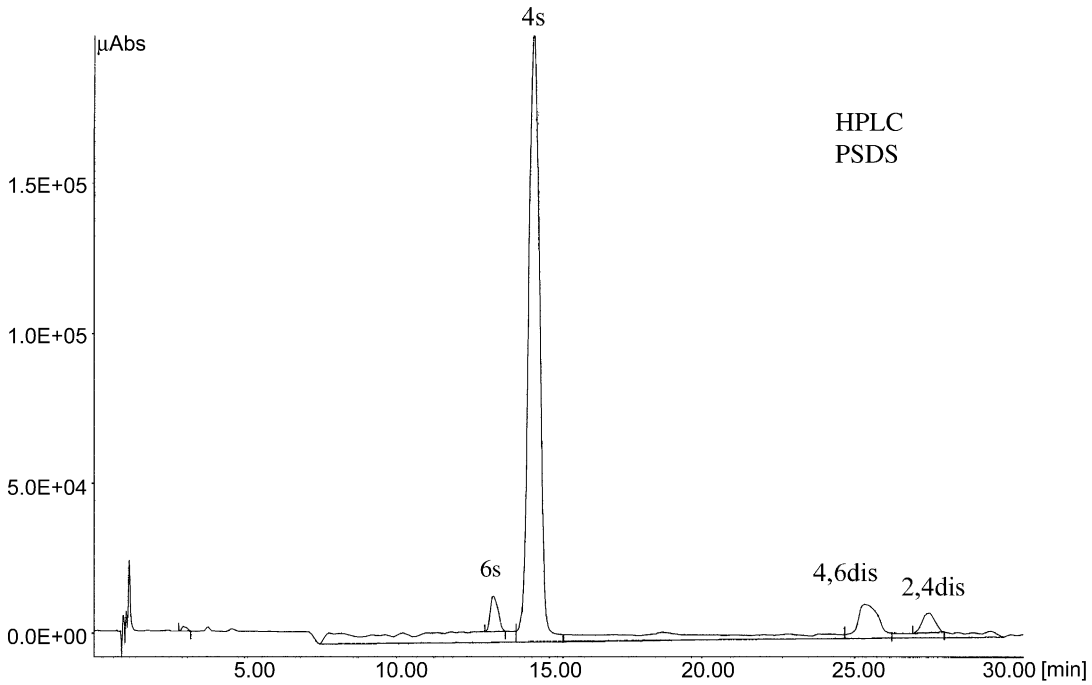
Fig.1 (*continued*)





D

Fig.1 (*continued*)



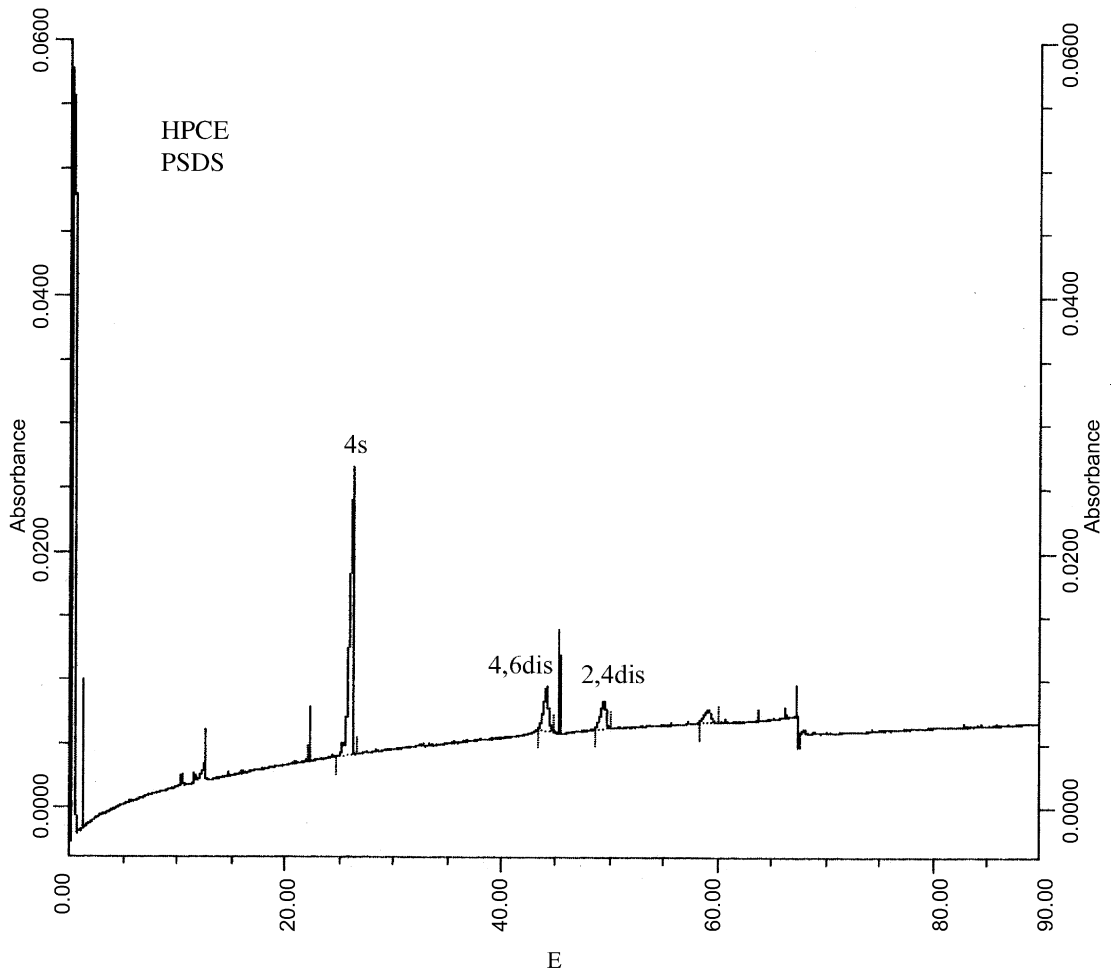
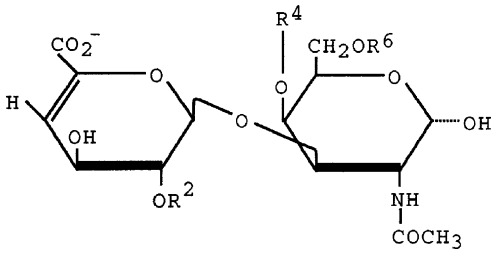


Fig.1 (*continued*)

Table 1
Percentages of unsaturated disaccharides of beef trachea chondroitin sulfate (BTCS), porcine trachea chondroitin sulfate (PTCS), chicken trachea chondroitin sulfate (CTCS), shark cartilage chondroitin sulfate (SCCS), and porcine skin dermatan sulfate (PSDS) evaluated by anion-exchange (A) and HPCE separation (B)



	R^2	R^4	R^6
ΔDi0S, ΔHexA–GalNAc	H	H	H
ΔDi6S, ΔHexA–GalNAc (6-OSO_3)	H	H	SO_3^-
ΔDi4S, ΔHexA–GalNAc (4-OSO_3)	H	SO_3^-	H
ΔDi2,6diS, ΔHexA (2-OSO_3)–GalNAc (6-OSO_3)	SO_3^-	H	SO_3^-
ΔDi2,4diS, ΔHexA (2-OSO_3)–GalNAc (4-OSO_3)	SO_3^-	SO_3^-	H
ΔDi4,6diS, ΔHexA–GalNAc (4-OSO_3, 6-OSO_3)	H	SO_3^-	SO_3^-

HexA: hexuronic acid
GalNAc: N-acetyl-galactosamine

	BTCS		PTCS		CTCS		SCCS		PSDS	
	A	B	A	B	A	B	A	B	A	B
ΔDi0S	10.3	7.1	3.9	2.9	12.6	9.0	2.5	0.5	0.3	0.0
ΔDi6S	33.0	35.6	16.5	16.3	18.5	20.6	39.2	41.9	3.4	0.0
ΔDi4S	56.7	57.3	79.6	80.8	68.8	70.4	29.6	30.7	84.2	84.9
ΔDi2,6diS	0.0	0.0	0.0	0.0	0.0	0.0	22.1	22.7	0.0	0.0
ΔDi4,6diS	0.0	0.0	0.0	0.0	0.0	0.0	4.4	4.2	8.9	10.0
ΔDi2,4diS	0.0	0.0	0.0	0.0	0.0	0.0	2.2	0.0	3.1	5.1
SO_3^-/COO^-	0.90	0.93	0.96	0.97	0.87	0.91	1.26	1.26	1.12	1.15

The sulfate to carboxyl ratio is also indicated. The values are the average of three different analyses with a coefficient of variation lower than 10%. For structure of disaccharides see the Scheme above.

a sulfate to carboxyl ratio, respectively, of approx. 0.91, 0.96 and 0.88. These values are mainly due to variable amounts of nonsulfated disaccharide. On the contrary, CS from shark cartilage is more sulfated (charge density of 1.26) due to the presence of a discreet percentage of disulfated disaccharides, in particular the disaccharides D and E. Porcine skin DS has a sulfate to carboxyl ratio of about 1.12–1.15 due to the presence of large amounts of disaccharide 4-sulfate and disulfated disaccharides in position 4 and 6 of *N*-acetyl-galactosamine (disaccharide E), and in position 2 of uronic acid and 4 of *N*-acetyl-galactosamine (disaccharide B) (Table 1).

4. Discussion

In the last few years, CE has proved to be a very attractive alternative separation technique for GAGs and GAG-derived oligosaccharides. CE affords high resolving power and great flexibility in the separation order. CE also has several advantages over a variety of other analytical methods, including an extremely high separation efficiency, on-line detection, simple operation, short analysis time, automated and reproducible analysis, and very low consumption of samples and buffers (Linhardt & Pervin, 1996; Mao et al., 2002). CS/DS Δ-disaccharides produced by the action of lyases are currently separated by normal or reverse polarity CE (Carney & Osborne, 1991; Karamanos, Vanky, Tzanakakis, & Hjerpe, 1995; Pervin, Al-Hakim, & Linhardt, 1994) but no HPCE method was developed to analyze disaccharide units of CS from various sources compared with more common HPLC separation. Nonsulfated, monosulfated and disulfated CS/DS unsaturated disaccharides were resolved by normal polarity CE in a unique separation, and the system was applied to the analysis of CS of various origins, including highly sulfated CS sample and DS, and an excellent agreement with previous compositional analysis was obtained.

HPCE using reverse polarity has been applied to the separation of all known CS/DS unsaturated nonsulfated, mono-, di-, and trisulfated disaccharides (Karamanos et al.,

1995; Pervin et al., 1994). Normal polarity HPCE has also been used to qualitatively separate unsaturated disaccharides derived from CS/DS after treatment with lyases (Al-Hakim & Linhardt, 1991; Carney & Osborne, 1991; Michaelsen, Schroder, & Sorensen, 1993). However, Carney and Osborne (Carney & Osborne, 1991) were unable to separate nonsulfated, monosulfated, disulfated and trisulfated unsaturated CS disaccharides by using normal polarity in a unique electrophoretic migration. Al-Hakim and Linhardt (1991) and Michaelsen et al. (1993) effectively separated nonsulfated and variously sulfated CS/DS unsaturated disaccharides but no comparison with other techniques, in particular the largely used HPLC separation (Imanari et al. 1996), was performed to evaluate the quantitative application of this methodological approach. Furthermore, this study illustrates the disaccharide compositional analysis of CS purified from different sources.

CS exhibits a wide variety of biological functions mainly due to the presence of structural building units that form domain structures that interact with other molecules, such as the regulation of neuronal patterning in the retina (Brittis, Canning, & Silver, 1992), interactions with fibronectin (Barkalow & Schwarzbauer, 1994), neurite outgrowth promoting activity (Nadanaka, Clement, Masayama, Faissner, & Sugahara, 1998), activation of monocyte and B-cell (Rachmilewitz & Tykocinski, 1998), and activation of plasminogen (Sakai et al., 2000). In the therapeutic field, its principal application is the treatment of arthropaties and articular degenerative complications, osteoporosis treatment and hyperlypidemias (Kuettner, Schleyerbach, Peyron, & Hascall, 1991). It is indicated for human as well as for veterinary use. Due to these properties, the sulfation of the constituent disaccharides plays a main role and this considerably changes in quantity and position according to the species. The results indicate the existence of particular electropherographic as well as chromatographic patterns for each sample in the study, with some similitudes and differences. Samples of CS from bovine and porcine tracheas, shark cartilage CS, and a DS sample, can be easily distinguished by the use of the mentioned methodology. However, CS samples obtained from chicken and porcine tracheas show electropherographic profiles with a degree of similarity that makes the identification difficult.

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