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Carbohydrate Polymers 41 (2000) 37–45

Carbohydrate Polymers

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^{1}H and ^{13}C nuclear magnetic resonance identification and characterization of components of chondroitin sulfates of various origin

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Received 10 November 1998; received in revised form 23 February 1999; accepted 11 May 1999

Abstract

Three natural chondroitin sulfates (CSs), from porcine and bovine trachea, and from shark cartilage, were studied using a variety of NMR techniques (DQS, TOCSY, NOESY, HMQC). A good ^{1}H and ^{13}C characterization of the major components, chondroitin 4-sulfate (CS4) and chondroitin 6-sulfate (CS6), was obtained and a number of signals coming from chondroitin 2,6-disulfate (CS2,6) (present only in shark CS) was identified. The study of a chemically desulfated CS was necessary in order to understand the difficulties encountered in detecting signals from the chondroitin non-sulfate (CS0) component of porcine and bovine CSs.

The singular pattern of UC-4 and NC-1 signals was recognized and explained in terms of a diad model. The "excess of multiplicity" affecting mainly these two signals was attributed to the differences in the conformation of the N 1:4 U glycosidic bond. Further support to this hypothesis comes from the comparison of the NOESY spectra of the three CSs. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: ^{1}H NMR; ^{13}C NMR; Chondroitin; Glycosaminoglycans

1. Introduction

Chondroitin sulfate (CS), formed of alternating *N*-acetyl-D-galactosamine β 1:4 and D-glucuronate β 1:3, is one of the linear polyanions known as glycosaminoglycans (GAGs) (Beaty & Mello, 1987). They are ubiquitous components of all connective tissue extracellular matrices, where they serve a number of functions, mainly covalently attached to proteins in the form of proteoglycans (Ruoslathi, 1988; Kjellen & Lindahl, 1991). Due to the presence of sulfate groups in different amounts, and located in various positions (2 and 3 of uronic acid, Sugahara et al. (1996a), and 4 and 6 of *N*-acetyl-galactosamine residues), CSs represent a heterogeneous family of polysaccharides, mainly because of the degree of sulfatation. Also, in terms of molecular mass, presence of iduronic acid in greater amounts more than glucuronate, depends on the origin of the tissue, its age, and the species (Kuettner, Schleyerbach, Peyron & Hascall, 1992).

CSs are also employed as chondroprotective and anti-rheumatic drugs, with application in the therapy of tibiofibular osteoarthritis of the knee and in the articular cartilage osteoarthritis (Kuettner et al., 1992). Due to the large heterogeneity of various CSs, an accurate evaluation of their structures and properties, molecular mass, charge density and positions of sulfate groups, is of paramount importance mainly for pharmacological applications.

A number of ^{1}H NMR studies on sulfated oligosaccharide fragments generated from natural CSs have been reported. Characterized structures through 1- and 2D ^{1}H NMR techniques range from tri- to octasaccharides (Scott, Chen & Brass, 1992; Sugahara, Masuda, Harada, Yamashina, de Waard & Vliegenthart, 1991; Sugahara, Shigeno, Masuda, Fujii, Kurosaka & Takeda, 1994; Chai, Kogelberg & Lawson, 1996; Sugahara, Tanaka & Yamada, 1996b; Kitagawa et al., 1997). A few ^{1}H and, sometimes, ^{13}C 1D NMR studies on natural CSs are present in the literature (Naggi, 1993; Bociek, Darke, Welti & Rees, 1980; Scott, Heatley & Wood, 1995), and only one report on the use of 2D homo- and heterocorrelated NMR techniques is known to us (Holme & Perlin, 1989). Inspection of these papers reveal

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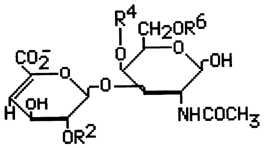
GAGs: glycosaminoglycans; CS: chondroitin sulfate; CS0: chondroitin non-sulfate; CS4: chondroitin 4-sulfate; CS6: chondroitin 6-sulfate; CS2, 6: chondroitin 2, 6-disulfate; U: GlcA: glucuronate; N: GalNAc: *N*-acetyl-galactosamine; U2S: glucuronate 2-sulfate; N4S: *N*-acetyl-galactosamine 4-sulfate; N6S: *N*-acetyl-galactosamine 6-sulfate; DQS: Double-Quantum correlation Spectroscopy; NOESY: Nuclear Overhauser Enhancement SpectroscopY; TOCSY: TOtal Correlation SpectroscopY; HMQC: Heteronuclear Multiple-Quantum Coherence spectroscopy

PII: S0144-8617(99)00075-2

Table 1
Physicochemical properties and structure (percentage of unsaturated disaccharides as weight percent, sulfate to carboxyl ratio, and molecular mass = M_r) of the chondroitin sulfate (CS) from bovine trachea (bt), porcine trachea (pt) and shark cartilage (sc)



	R^2	R^4	R^6	CSbt	CSpt	CSsc
ΔDi-0s	H	H	H	7.3 ± 0.4	6.1 ± 0.3	2.2 ± 0.1
ΔDi-6s	H	H	SO_3^-	40.6 ± 2.0	25.6 ± 1.5	49.0 ± 2.4
ΔDi-4s	H	SO_3^-	H	51.4 ± 3.1	66.3 ± 3.3	27.7 ± 1.1
ΔDi-2,6dis	SO_3^-	H	SO_3^-	0.7 ± 0.0	1.9 ± 0.1	16.1 ± 0.8
ΔDi-4,6dis	H	SO_3^-	SO_3^-	0.0	0.0	3.0 ± 0.1
ΔDi-2,4dis	SO_3^-	SO_3^-	H	0.0	0.0	0.0
ΔDi-2,4,6tris	SO_3^-	SO_3^-	SO_3^-	0.0	0.0	1.9 ± 0.1
SO_3^-/COO^-				0.93 ± 0.05	0.96 ± 0.05	1.21 ± 0.06
4s/6s ratio				1.24 ± 0.06	2.41 ± 0.12	0.47 ± 0.02
M_r (kDa)				23.7 ± 1.6	18.1 ± 1.4	> 50.0

evidences that some signals of the proton spectra are only collectively assigned and only the major component of CSs, usually CS4 and CS6, are considered.

This study examines different natural CSs, prepared from porcine trachea, bovine trachea and shark cartilage, by ^1H and ^{13}C NMR spectroscopy. Both the homo- and inverse-detected (more sensitive than the traditional ^{13}C-detected) heteronuclear correlated techniques are employed. The aim of this research is, firstly, the NMR characterization of natural CSs of various origins, as far as both their major and minor components are concerned. A second purpose is to evaluate the feasibility of a quantitative analysis of CSs from ^1H and ^{13}C NMR spectra. The principal conclusion is that the CS4, CS6 and CS2,6 components of natural CSs can be easily recognized and assigned, whereas serious problems arise with the CS0 and other minor components due to their unfavorable spectral properties. The singular behavior of interglycosidic NC-1 and UC-4 carbon signals of CSs is for the first time reported and explained in terms of a diad model.

2. Experimental

CS from bovine trachea, porcine trachea and shark cartilage was obtained from IBSA, Institut Biochimique SA, Lugano, Switzerland, and also prepared as previously reported (Volpi, 1994). Totally desulfated CS was prepared according to Nagasawa, Inoue and Kamata (1977) by treating bovine trachea CS pyridine salt in 5% methanol in dimethylsulfoxide at 80°C for 6 h. After dialysis against bidistilled water for 24 h at 4°C, the product was lyophilized.

The peak molecular mass and polydispersity of CS samples were determined by high-performance size-exclusion chromatography according to Volpi and Bolognani (1993). The percentage of the constitutive disaccharides and the sulfate-to-carboxyl ratio was evaluated by degradation with chondroitinase ABC, and separation of unsaturated disaccharides by strong-anion exchange chromatography (SAX-HPLC) (Volpi, 1994). The possible presence of iduronic acid was determined by degradation with chondroitinase ABC and chondroitinase ACII and separation of unsaturated disaccharides as described above.

The ^1H and ^{13}C NMR spectra of CSs were recorded by a Bruker AMX400 WB spectrometer operating at 400.13 and 100.61 MHz, respectively. The CS samples were lyophilized three times previously with D_2O, and finally prepared by dissolving 9 (bovine and porcine CS samples, and totally desulfated CS) and 22 mg (CS from shark) in 0.6 ml of D_2O at a high level of deuteration (99.997%). The spectra were recorded at temperature of 33°C and pH 6.5, unless specified. At this temperature, the residual signal of HOD does not hide the proton signals from the sample and resolution problems, mainly arising in shark cartilage CS (due to the formation of bubbles at higher temperatures), are minimized. ^1H and ^{13}C chemical shifts (δ, ppm) are quoted with respect to external sodium 4,4-dimethyl-4-silapentane-1-sulfonate (0.0 ppm).

DQS (Mareci & Freeman, 1983) parameters: spectral width $(f_2) = 4$ ppm, 2048 complex point; spectral width $(f_1) = 8$ ppm, 512 t_1 increments with 32 scans per t_1 value; relaxation delay = 0.2 s, 18–70 ms evolution time, 135 degree read pulse.

NOESY–TPPI (Bodenhausen, Kogle & Ernst, 1984) parameters: spectral width $(f_2) = 6.5$ ppm, 2048 complex point; spectral width $(f_1) = 6.5$ ppm, 256–512 t_1 increments with 32–64 scans per t_1 value; relaxation delay = 0.2 s; mixing time = 20–50 ms.

TOCSY–TPPI (Bax & Davis, 1985) parameters: spectral

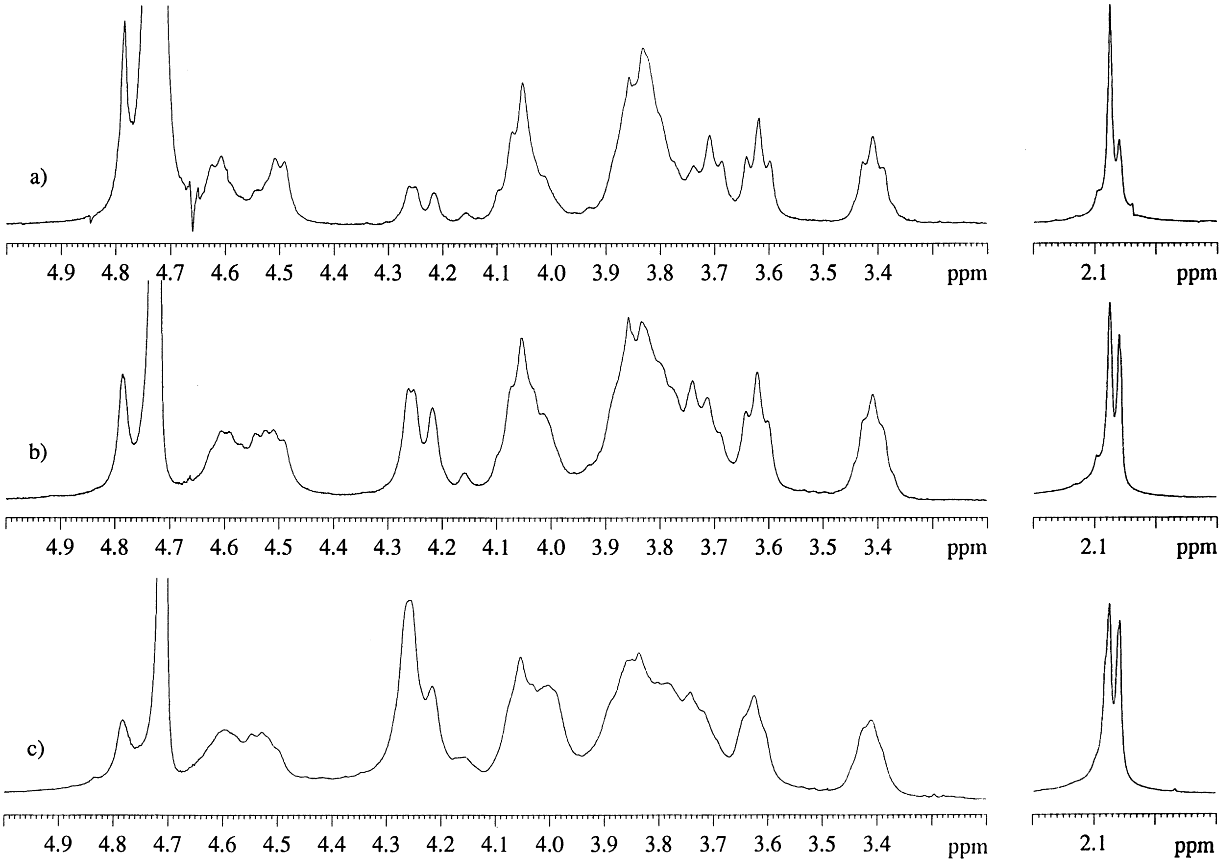


Fig. 1. ^{1}H NMR spectra of (a) porcine, (b) bovine and (c) shark CSs.

width (f_2) = 4 ppm, 2048 complex point; spectral width (f_1) = 4 ppm, 256 t_1 increments with 32 scans per t_1 value; relaxation delay = 0.2 s, spin-lock field of 4 kHz applied for 40–140 ms.

HMQC (Bax, Griffey & Hawkins, 1983) parameters: spectral width (f_2) = 8 ppm, 1024 complex points; spectral width (f_1) = 104 ppm, 512 t_1 increments with 128 scans per t_1 value, relaxation and evolution delays = 0.5 s and 3.44 ms, respectively. The HDO signal was presaturated during the relaxation delay.

Table 2
^{1}H NMR data of CSs from porcine trachea, bovine trachea, shark cartilage and of chemically desulfated CS (GlcA, glucuronic acid; GalNac, *N*-acetyl-galactosamine; CS4, chondroitin 4-sulfate; CS6, chondroitin 6-sulfate; CS2,6, chondroitin 2,6-disulfate; CS0, chondroitin non-sulfate)

GlcA	UH-1	UH-2	UH-3	UH-4[a]	UH-5	
CS4	4.50/1	3.41/2	3.62	3.80/2	3.69/70	
CS6	4.53/4	3.39/40	3.62	3.77/9	3.72/3	
CS2,6	4.75	4.16	3.85	3.83	3.78	
Desulfated CS	4.61	3.42	3.68	3.84	4.00	
GalNac	NH-1	NH-2	NH-3	NH-4	NH-5	NH-6
CS4	4.71	4.08/9	4.04/6	4.78/9	3.87	3.83
CS6	4.58/9	4.06/7	3.88/9	4.22	4.01	4.26
CS2,6	4.60	[b]	3.99	4.26	[b]	4.26
CS0		4.02[a]	3.85	4.16		
Desulfated CS	4.56	4.04	3.88	4.13	3.74	3.78/82

[a] See text.
[b] In the region 4.05–3.93 ppm.

3. Results

Table 1 illustrates the percentage of unsaturated disaccharides for the three CSs after chondroitinase ABC degradation. Moreover, molecular mass, sulfate to carboxyl and 4-/6-sulfate ratios are reported. Different batches of the CS from various origins were analyzed to calculate the standard deviation for the numeric values that express the polysaccharide properties. No iduronic acid was found after comparing the unsaturated disaccharides obtained by chondroitinase ABC and AC treatments. Significant differences were found for the molecular mass (Table 1) of the three CS, as shark CS has a higher M_r than the other two. Pig and beef trachea CS samples have about the same charge density whilst a significant difference was calculated for the 4-/6-sulfate ratio value, double for the former polysaccharide (Table 1). CS from shark cartilage shows a greater sulfate to carboxyl ratio due to the large amount of disulfated disaccharides, in particular, the disaccharide 2,6-disulfate, and a

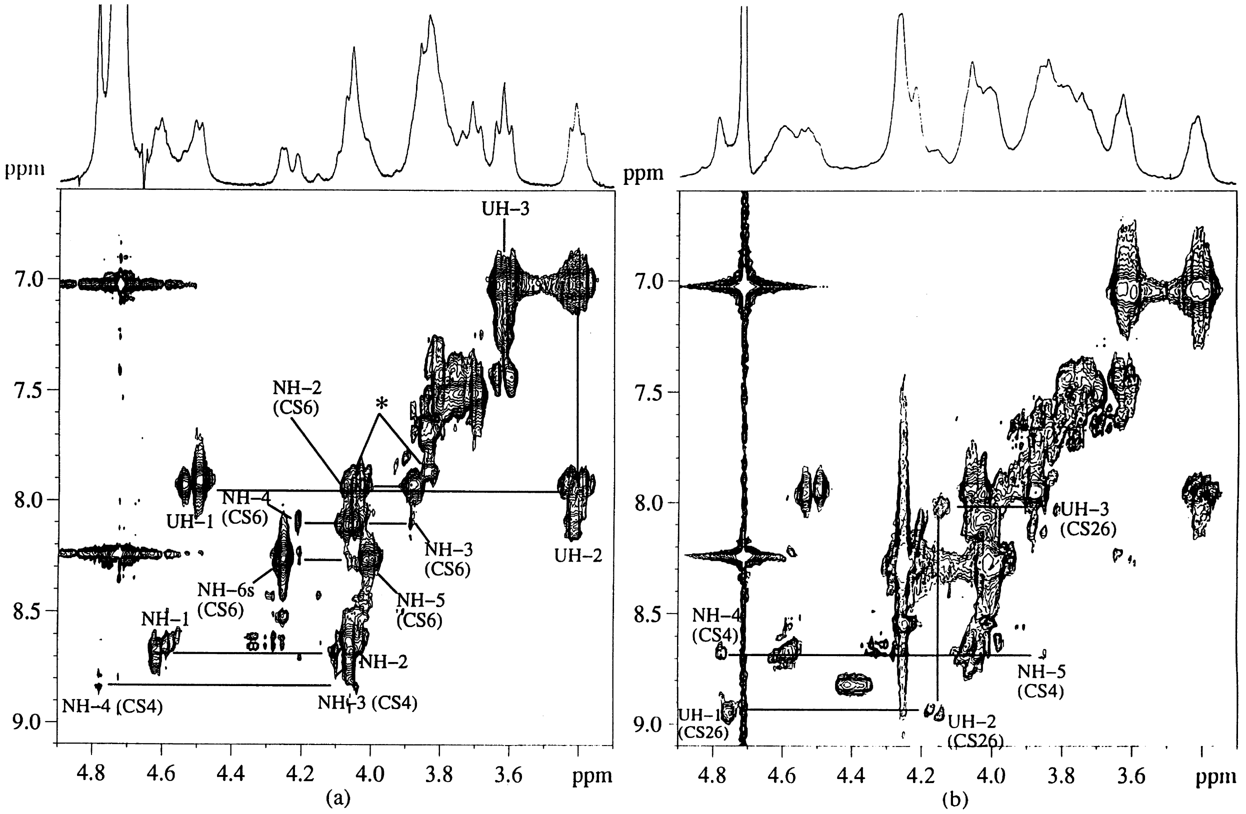


Fig. 2. DQS spectra of (a) porcine and (b) shark CSs obtained with an evolution delay of 18 ms. Only the significant new correlations are labelled in (b). The asterisked correlation in (a) is probably due to the CS0 component.

4-/6-sulfate ratio lower than 0.5 due to the greater percentage of the disaccharide 6-sulfate.

3.1. Analysis of proton spectra

The ^{1}H spectra of CSs from pig, beef and shark are illustrated in Fig. 1. The ^{1}H NMR data of the three various CS samples are reported in Table 2. All proton signals are found in two spectral regions, one between 2.0 and 2.1 ppm, in which acetamido methyl signals are present, the other between 3 and 5 ppm, where all the other signals are found.

Observation of the former region of the three CS spectra shows that only two acetamido methyl signals are observed even in the presence of a third component in a high percentage, similar to CS2,6 in CS from shark. The analysis of the proton spectral region in the range 3–5 ppm is more useful for the characterization of the three different CSs as far as both major and minor components are concerned.

The DQS (Fig. 2(a)) and TOCSY (Fig. 3(a)) spectra of porcine CS enable the assignment of minor CS6, as well as major CS4 proton signals, to be made. Passing from porcine to bovine and shark CS ^{1}H NMR spectra, changes in the shapes of almost all signals in the 3–5 ppm region (Fig. 1), correlated to the different sulfate distribution, are observed. The DQS and TOCSY spectra of bovine CS are similar to those of porcine CS, apart from the relative intensities of some correlations. It is to be underlined that UH-4s are localized with difficulty from 2D spectra; they are found in a range of chemical shifts (3.82–3.77 ppm), and their fine structure is hardly distinguishable.

New cross peaks are found in shark CS spectra (Figs. 2(b) and 3(b)). Correlation between a proton at 4.75 ppm and a proton at 4.16 ppm (the shape of which, in the ^{1}H NMR spectrum, is different from that of the low signal at 4.16 ppm found in porcine and bovine spectra), and between a proton at 4.16 ppm and a proton at 3.85 ppm are found in shark DQS spectrum. These three signals come from UH-1, UH-2 and UH-3 belonging to the U2S unit according to the substituent effect expected for sulfatation at position 2 (Bossennec, Petitou & Perly, 1990). Also, the TOCSY spectrum of shark CS (Fig. 3(b)) confirms that these three protons belong to the same molecular framework and allows us to localize UH-4 and UH-5 (see also NOESY results). These assignments compare well with those recently reported for oligosaccharides containing (2-sulfo)-glucuronic acid residues (Chai, Lawson, Gradwell & Kogelberg, 1998). Signals from the N6S part of an U2S-N6S unit are found, with the exception of NH-1 at 4.59 ppm, in two regions from 4.28 to 4.23 ppm and from 4.05 to 3.93 ppm. In the first region, NH-6 and NH-4 signals are superimposed on the NH-6 signal of the U–N6S unit (justifying the relative enhancement in the ^{1}H NMR spectrum of this signal with respect to that at 4.22 ppm, passing from bovine to

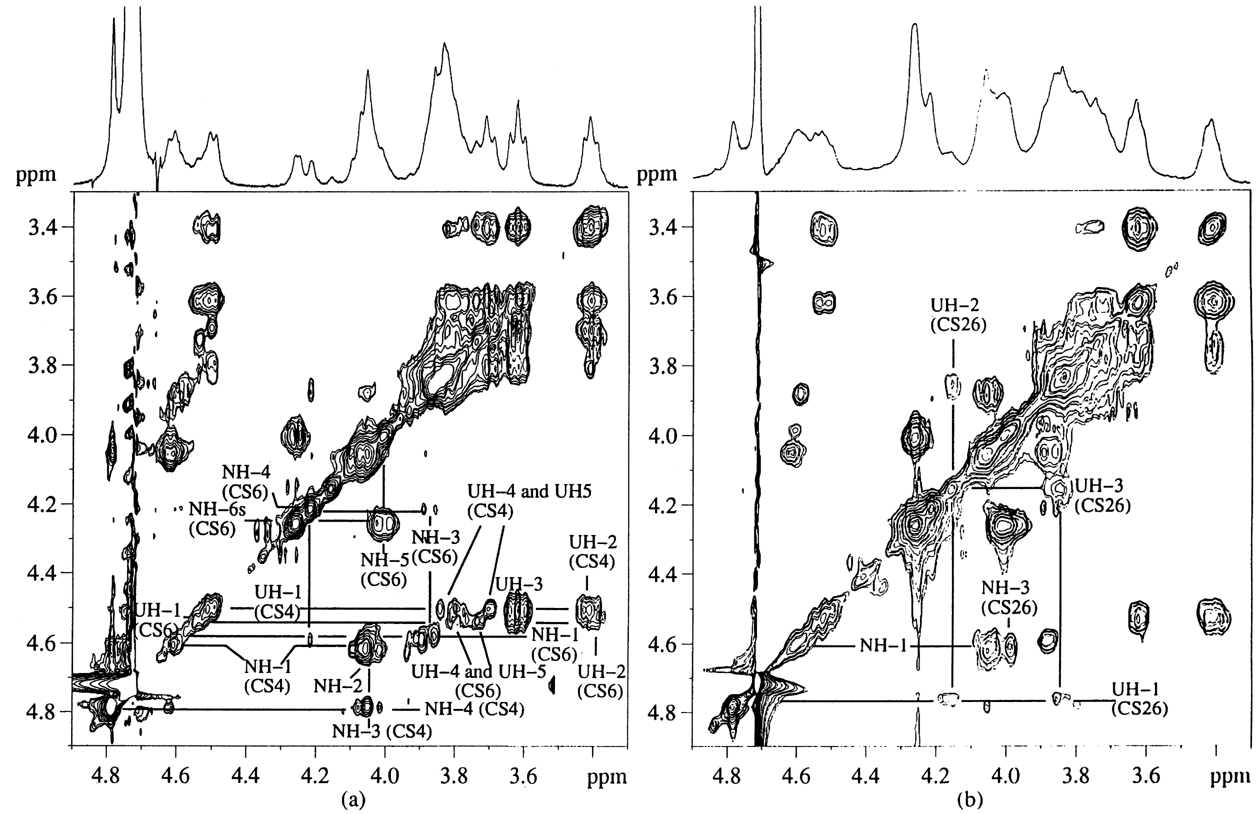


Fig. 3. TOCSY spectra of (a) porcine and (b) shark CSs obtained with a spin-lock field applied for 70 and 40 ms, respectively. Only the significant new correlations are labelled in (b). The TOCSY spectrum of shark CS obtained with 70 ms spin-lock does not show the cross-peak between UH-1 and UH-2 of CS2,6.

shark CS). In the second region, NH-2, NH-5 and NH-3 are found.

No signals clearly assignable to the CS0 component are derived from 1D and 2D proton spectra of porcine and bovine CSs, apart from the low proton signal at 4.16 ppm, attributable to NH-4 of CS0 on the basis of its shape and spectral position.

To better understand why CS0 signals are only found with

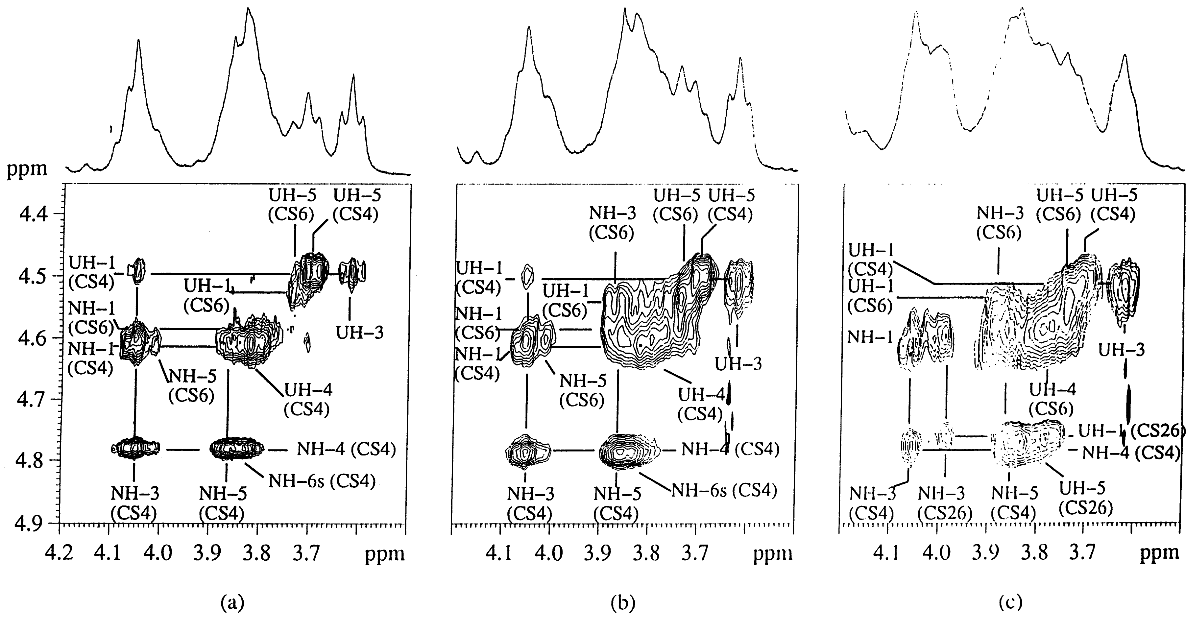


Fig. 4. Partial NOESY spectra of (a) porcine, (b) bovine and (c) shark CSs.

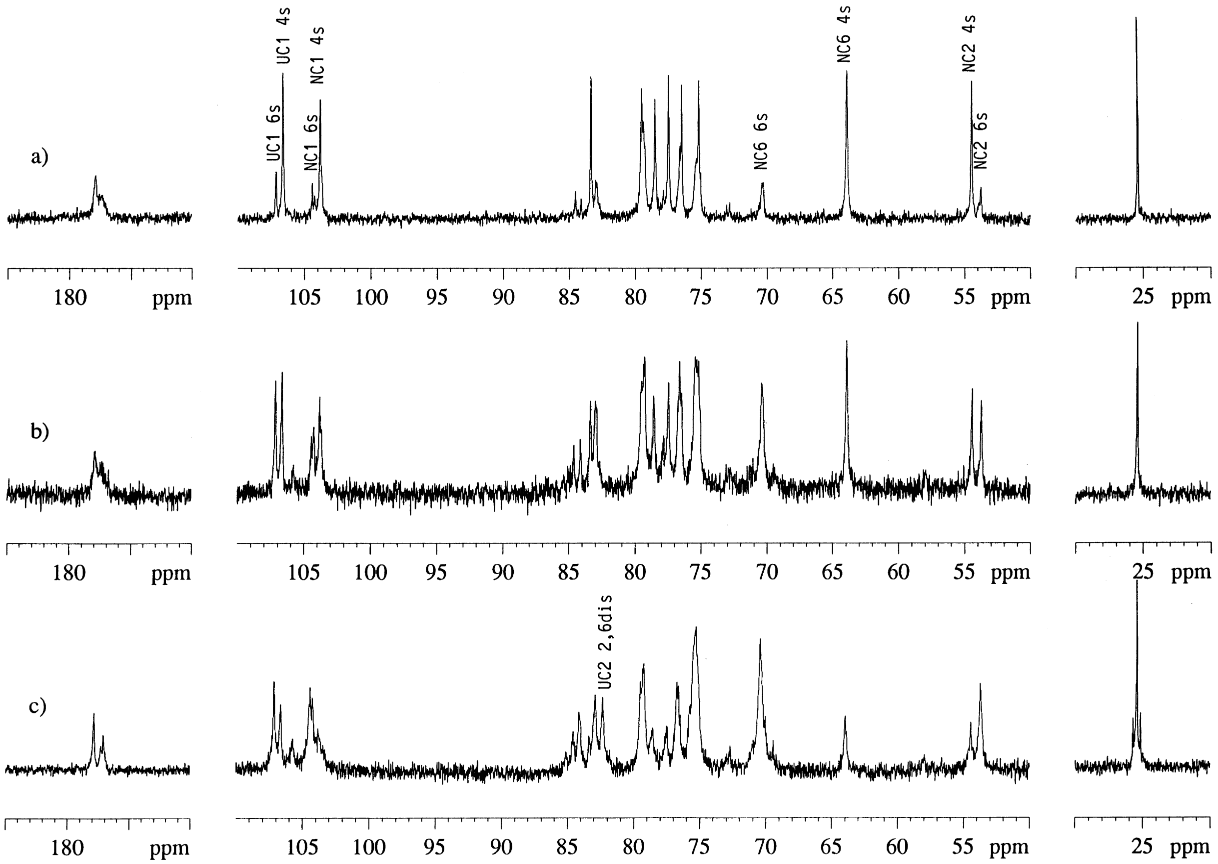


Fig. 5. ^{13}C NMR spectra of (a) porcine, (b) bovine and (c) shark CSs.

great difficulty in the CS samples examined, an NMR analysis of a totally desulfated CS was undertaken following the procedure used for the three natural CSs (Table 2). From ^{1}H NNR data it is clear that only the NH-4 of CS0, slightly shielded with respect to that of CS6, is likely to appear as distinguished in the spectra of natural CSs. The correlation between the protons at 4.02 and 3.85 ppm, found in the DQS spectra of porcine and bovine CSs, is now attributable to NH-2 and NH-3 (and/or to UH-4 and UH-5) of the CS0 component.

Table 3
^{13}C NMR data of CSs from porcine trachea, bovine trachea, shark cartilage and of chemically desulfated CS (GlcA, glucuronic acid; GalNac, *N*-acetylgalactosamine; CS4, chondroitin 4-sulfate; CS6, chondroitin 6-sulfate; CS2,6, chondroitin 2,6-disulfate; CS0, chondroitin non-sulfate)

GlcA	UC-1	UC-2	UC-3	UC-4[a]	UC-5	
CS4	106.6/7	75.2/3	76.5	83.4	79.6	
CS6	107.2	75.3	76.6/8	83.0/84.2	79.3	
CS2,6	105.8	82.4	75.8			
Desulfated CS	107.2	74.9	76.6	82.8[a]	76.6	
GalNac	NC-1	NC-2	NC-3	NC-4	NC-5	NC-6
CS4	103.8	54.4/5	78.5/6	79.4	77.5	63.9
CS6	104.3/5	53.7/8	82.9/83.0	70.3/5	75.3/4	70.4/5
CS0				77.9		
Desulfated CS	104.2	53.9	82.9[a]	70.5	77.8	63.7

[a] See text.

NOESY experiments (partially reported in Fig. 4) were carried out on the three CSs under study with a mixing time of 50 ms for porcine and bovine CSs, and with 20 ms for shark CSs to avoid spin-diffusion (Bossennec et al., 1990). Under these conditions a number of significant intra- and inter-residue NOE cross-peaks were detected. Apart from confirming the prevalence of the $^4C_1$ conformation for both U and N units (Scott et al., 1995), the NOE cross peaks between UH-2s and UH-4s allow us to localize the spectral position of UH-4s.

Interesting inter-residue cross-peaks between UH-1s and NH-3s, and NH-1s and UH-4s are found, confirming that these protons are prevalently on the same side of the glycosidic bond in porcine CS. In the case of bovine CS, NOE cross-peaks have a pattern very similar to that observed in porcine CS, but NH-1 of N6S clearly shows a NOE cross-peak with both UH-4 and UH-5, the second one is lower than the first, indicating a distortion of interglycosidic bond in CS6 with respect to CS4.

The NOESY spectrum of shark CS displays, in addition to the cross-peaks of CS4 and CS6 already present in the NOESY spectrum of bovine CS, some distinguishable cross-peaks deriving from the CS2,6 component: between UH-1 at 4.75 and a proton at 3.99 ppm (assigned to NH-3), and between UH-1 and a proton at 3.78 ppm (assigned to UH-5).

3.2. Analysis of carbon-13 data

The ^{13}C NMR spectra of the three natural CSs, together with carbon chemical shifts, are reported in Fig. 5. ^{13}C chemical shifts are reported in Table 3. All signals are found in the region 50–110 ppm except those of carbonyl (around 178 ppm with a broad right shoulder) and acetamido methyl carbons (all at 25.5 ppm). Passing from porcine to bovine CS, significant changes are seen in the ^{13}C NMR spectra. In particular, inspection of the 50–72 and 100–110 ppm regions reveal that the relative amount of CS4 and CS6 changes in favor of the latter.

The lines in the region 50–110 ppm were assigned through inverse-detected heteronuclear multiple-quantum coherence. All the highest signals and some of the lowest were attributed. It was observed that, independent of the natural source, the chemical shifts of a given carbon in the two major components (CS4 and CS6) were found in very narrow regions as is summarized in Table 3.

The heteronuclear correlations permitted also a better localization of some CS4 proton signals (NH-5 and NH-2 result to be slightly downfield from NH-6 and NH-3, respectively). In particular, the HMQC spectrum of beef CS shows that the signal at 82.9 ppm comes from NC-3 of CS6 and that NC-5 of CS6 is found at 75.5 ppm.

The HMQC study of the totally desulfated CS showed that the low signal present at 77.9 ppm in porcine and bovine CS ^{13}C NMR spectra can probably be attributed to NC-5 of CS0 (Table 3), whereas signals at 84.2 and 84.7 ppm cannot be assigned to CS0.

The new strong signal present at 82.4 ppm in the ^{13}C NMR spectrum of shark CS correlates in the HMQC spectrum with UH-2 of U2S at 4.16 ppm and it is thus assigned to UC-2 of U2S. No clear correlations involving UH-1 at 4.75 and UH-3 at 3.85 ppm were found in the HMQC spectrum of shark CS, but the shielding effect expected for the directly bonded carbons (similar to that observed for UC-1 and UC-3 in 2-sulfated iduronate residues of dermatan sulfate, Holme and Perlin (1989)) makes the attribution of the two signals at 105.8 and 75.8 ppm to UC-1 and UC-3 of CS2,6, respectively, probable. These assignments agree with the ^{13}C NMR data reported for oligosaccharides containing (2-sulfo)-glucuronic acid residues (Chai et al., 1998) (a systematic shift, due to the different spectral reference, has to be taken into account).

4. Discussion

Some remarks on the NMR data reported are worth noting. From Tables 2 and 3 it is apparent that both the proton and carbon chemical shifts of the same component in CS of different sources have a good reproducibility and only minor shifts are observed in the three different samples.

The ^{1}H NMR signals, which readily permit an identification of N4S, N6S and U2S components are—(a) the NH-4 signals, relatively sharp and without fine structure, at 4.78/9 and 4.22 ppm, for N4S and N6S, respectively; (b) the NH-6s of N6S signal, a doublet at 4.26 ppm; this signal is only a little higher than that at 4.22 ppm; (c) the presence of a U2S component which can be suspected if the relative intensity of the signal at 4.26 ppm is enhanced with respect to that at 4.22 ppm. The two signals of UH-1 and UH-2 of U2S at 4.75 and 4.16 ppm must also be present in this case, but they are partially hidden in the monodimensional ^{1}H NMR spectrum. A confirmation of their presence should come from the corresponding correlations in DQS and TOCSY spectra (see Figs. 2 and 3).

The non-sulfated N component is very difficult to detect, as its signals are hidden under those of the major components, with the exception of NH-4, at 4.16 ppm. A correlation between NH-2 (and/or UH-5) at 4.02 ppm, and NH-3 (and/or UH-4) at 3.85 ppm, has also to be detected in the DQS spectra.

In the case of a hypothetical CS containing both U2S and non-sulfated N units, the signal of UH-2 of U2S can be distinguished from that of NH-4 of N, both at 4.16 ppm, from the cross-peaks in the DQS spectrum.

Other components, with abundance lower than 3%, were not identified, presumably because their signals are hidden under the major ones.

An estimate of the acetylation percentage can be obtained from the ^{1}H NMR spectrum after moving the water signal sufficiently downfield by lowering the sample temperature (14°C in our case). The integral corresponding to the 4.85–3.30 ppm region should be four times that of the methyl signals in the case of complete acetylation, as is our case.

An indication of the U2S percentage can be obtained from the area of UH-2 signals at 3.4 ppm. This should be 1 when the integral of the region 4.85–3.30 ppm is set to 12, and less when U2S units are present. This integral is found to be around 0.80 in shark CS, indicating that about 20% of CS2S is present, whereas its value is very close to 1 in porcine and bovine CS samples.

When carbon chemical shifts are considered (Table 3), the most reliable indication of the presence of CS2,6 is the carbon signal of U2S UC-2 at 82.4 ppm, even though a good differentiation of the CS4, CS6 and CS2,6 components could be derived from UC-1 signals if, as already discussed, the assignment of the signal at 105.8 ppm to UC-1 of CS2,6 was certain.

Two interconnected aspects of the ^{13}C NMR study deserve some comments. The first is that at least two critical regions, i.e. regions with very loose correlations, are found in the HMQC spectra: between 105 and 103 ppm (NC-1), and between 85 and 82 ppm (UC-4). Difficulties with the detection of UC-4 and NC-1 signals have also been reported by other researchers (Holme & Perlin, 1989).

The second aspect deals with the changes in the appearance of the carbon spectra of the three CSs under study in

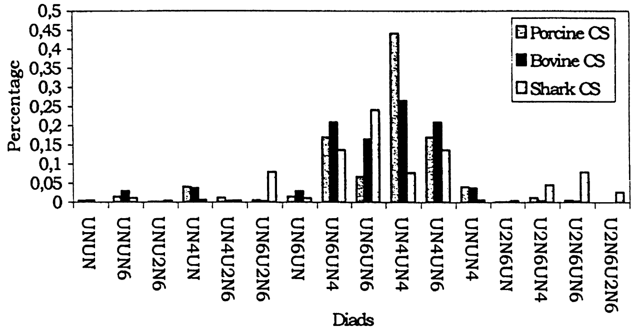


Fig. 6. Statistical diad distribution derived from the data in Table 1.

these two regions (Fig. 5). It is apparent that NC-1 and UC-4 signals suffer from an "excess of multiplicity". This signal dispersion is the likely cause of the difficulties in the detection of the corresponding heterocorrelations. This "excess of multiplicity", hardly attributable to the presence of CSs with different degrees of polymerization (because in this case we should expect broad rather than multiple signals), is, in our opinion, the symptom of long-range effects, very common and documented in synthetic polymers such as polymethacrylate (Dong, Hill, O'Donnell & Whittaker, 1994), polythiophene (Mucci & Schenetti, 1995) and others (Matsuzaki, Uryu & Asakura, 1996).

The experimental pattern of the ^{13}C NMR spectra can be explained on the basis of a diad model. Starting from the two major monomeric units of the CS polysaccharide, (U-N4S) and (U-N6S), four different diads (U–N4S)– (U–N4S), (U–N4S)– (U–N6S), (U–N6S)– (U–N4S), and (U–N6S)– (U–N6S) can be generated. As a consequence, four signals for each carbon and proton should be observed. In the case of three or more monomeric starting units, the number of diads increases and the evaluation of the diad distribution can be supported by a statistical approach. The results are reported in Fig. 6. What we see is an intensity pattern that closely resembles that found in the UC-4 and NC-1 regions of the three ^{13}C NMR spectra. In this comparison it is to be taken into account that, when present, the signals of NC-3 of N6S and of UC-2 of U2S are found in the 85–82 ppm region together with UC-4 signals.

In porcine CS, the most populated diad should be (U–N4S)– (U–N4S), whereas the remaining three should be present at lower percentages. In bovine CS, the amount of the three minor diads increases and, consequently, the ^{13}C NMR signals change their relative intensities. The greater number of signals displayed in the ^{13}C NMR spectrum of shark CS is due to the presence of new diads containing the U2S–N6S subunit that become significantly populated.

Two questions arise. The first is why this dispersion effect is so evident for UC-4 and NC-1 and not, for instance, for UC-1, NC-6 or NC-3 (which are found only in two different environments, depending on substitution). A possible answer is that UC-4 and NC-1 are involved in the same glycosidic bond and that changes occur at this level in the conformation of the different diads, as the NOESY experiments evidenced. The N–U junction seems to be more conformationally mobile than the U–N interglycosidic bond, probably due to different hydrogen bonding frameworks induced by the differences in substitution.

The second question regards the detection and assignment of the signals from the CS0 component. If the diad model holds, the percentage of diads containing (U–N) units is less than 5%, and the corresponding signals are lost in the noise or hidden under the higher lines of the ^{13}C NMR spectrum.

5. Conclusions

^{1}H and ^{13}C NMR spectra, as well as 2D-NMR experiments (DQS, TOCSY, and HMQC), prove to be particularly effective in the characterization of major and minor components of three natural CSs of different origins. In particular, the CS2,6 component was for the first time distinguished and assigned in shark CS.

The accurate analysis of the ^{1}H NMR spectra readily permits the identification and quantification of N4S, N6S and U2S components directly on natural CSs.

The study of the ^{13}C NMR spectra of the three natural CSs allows us to evidence, for the first time, that an excess of multiplicity affects NC-1 and UC-4 signals. This behavior can be rationalized on the basis of the different diad distribution and it is attributed to the higher conformational mobility of N–U with respect to U–N junctions.

Acknowledgements

Research supported by 60% grants to N.V. and L.S. The authors are indebted to the Centro Interdisciplinare Grandi Strumenti (CIGS), University of Modena, for the use of the Bruker AMX400 WB spectrometer.

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