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Determination of hyaluronan and galactosaminoglycan disaccharides by high-performance capillary electrophoresis at the attomole level. Applications to analyses of tissue and cell culture proteoglycans

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Abstract

A rapid, highly sensitive and reproducible HPCE method is described for the determination of all non- and variously sulphated disaccharides present in hyaluronan and vertebrate chondroitin sulphates and dermatan sulphates. Following chondroitinase digestion of glycosaminoglycans or proteoglycans, the non-, di- and trisulphated Δ-disaccharides are completely separated and readily determined within 14 min on a fused-silica capillary in 15 m*M* sodium dihydrogen orthophosphate, pH 3.00, using reversed polarity at 20 kV and detection at 232 nm. The determination of the various Δ-disaccharides derived from either glucuronic or iduronic acid and the presence of glucuronic and iduronic clustered structures in dermatan sulphate can also easily be made, using digests with chondroitinase AC or B. A linear detector response was obtained for the entire interval tested (up to 10 mg/l of Δ-disaccharides). Concentrations as small as 32, 65, 100 and 250 pmol/l (22, 38, 50 and 98 ng/l) of tri-, di- and nonsulphated Δ-disaccharides, respectively, can be reliably detected.

1. Introduction

Hyaluronan (HA) and the galactosaminoglycans (GalAGs), chondroitin sulphate (CS) and dermatan sulphate (DS), are components of connective tissues and they are susceptible to digestion with chondroitinase. GalAGs are covalently bound to a protein core, forming proteoglycans (PGs), whereas HA is a polysaccharide not found to be an integral constituent of PG molecules. HA, however, forms large aggregates with the monomeric PG [1]. The GalAGs and HA are composed of repeating disaccharide

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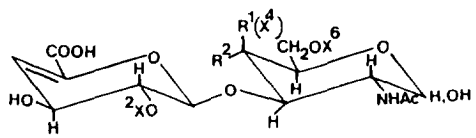
units which generally contain a uronic acid glycosidically linked to hexosamine. The sulphated GalAGs are highly charged polymers and their physiological characteristics depend to a large extent on the sulphate position [2–3]. HA is non-sulphated and difficult to separate from CS or DS of low sulphate content. The latter GalAGs of vertebrate origin are mainly sulphated at C-4 and C-6 of the galactosamine moiety and in some cases at C-2 of the uronic acid. Various types of di- and trisulphated disaccharide units may be formed by combining of these sulphation positions.

Analysis of the glycosaminoglycan (GAGs) disaccharides depends on the susceptibility of GAGs to various enzymes with or without simultaneous digestion with chondro-4- or -6-sulphatases [4–8], or to chemical degradation by nitrous acid [9]. The structure of disaccharides obtained after chondroitinase digestion is demonstrated in Fig. 1. Analyses of some of these disaccharides using modern separation and highly sensitive detection techniques have been performed by HPLC [10–21] and capillary zone electrophoresis [22–24].

Although the HPLC methods have been widely and successfully used to analyse GAGs disaccharides, capillary electrophoresis is a powerful technique which may provide extremely high resolution and sensitivity. During recent years HPCE has been used for the analysis and structural characterisation of various types of glycoconjugates (for an excellent review see Ref. 25). The analysis of GalAGs and HA-derived Δ-disaccharides by HPCE facilitated the detection of fmol levels of these polysaccharide constituents. However, the separation between 2 - acetamido - 2 - deoxy - 3 - O - (4 - deoxy - α - L - *threo* - hex - 4 - enopyranosyluronic acid) - D - glucose (Δdi-nonS_{HA}) and 2 - acetamido - 2 - deoxy - 3 - O - (4 - deoxy - α - L - *threo* - hex - 4 - enopyranosyluronic acid) - D - galactose (Δdi-nonS_{CS}), 2 - acetamido - 2 - deoxy - 3 - O - (4 - deoxy - α - L - *threo* - hex - 4 - enopyranosyluronic acid) - 4 - O - sulpho - D - galactose (Δdi-mono4S) and 2 - acetamido - 2 - deoxy - 3 - O - (4 - deoxy - α - L - *threo* - hex - 4 - enopyranosyluronic acid) - 6 - O - sulpho - D - galactose (Δdi-mono6S), as well as between the three types of disulphated disaccharides is incomplete, and various operating buffers are needed to determine each one of these Δ-disaccharides. HPCE analytical protocols using normal polarity have been previously reported by Ampofo et al. [24], Al-Hakim and Linhardt [22] and Carney and Osborne [23]. By these protocols the fractionation of the variously sulphated Δ-disaccharides has been achieved using multiple buffer systems, resulting in poor peak symmetry and peak tailing. The reversed polarity system used by Pervin et al. [26] improved the resolution and peak symmetry and especially the separation between disulphated Δ-disaccharides. However, the later protocol a period as long as 50–60 min was required for a complete electrophoregram, a fact which reduces the sensitivity of the method. Δ-disaccharides derived from iduronic acid (IdoA)-containing disaccharides have not been determined directly by any of the previous HPCE methods. Such Δ-disaccharides can easily be analysed using separate digestions with chondroitinases ABC, AC or B. Chondroitinase ABC cleaves the $1\beta \rightarrow 4$ glycosidic bonds between the galactosamine and the uronic acids, either GlcA



$R^1 = H, R^2 = OH$ $R^1 = OH, R^2 = H$

1: $X^2, X^4, X^6 = H$

2: $X^2,X^4,X^6 = H$
3: $X^4,X^6 = H, X^2 = SO_3H$
4: $X^2,X^6 = H, X^4 = SO_3H$
5: $X^2,X^4 = H, X^6 = SO_3H$
6: $X^4 = H, X^2,X^6 = SO_3H$
7: $X^6 = H, X^2,X^4 = SO_3H$
8: $X^2 = H, X^4,X^6 = SO_3H$
9: $X^2,X^4,X^6 = SO_3H$

Fig. 1. Structures of the non-sulphated and sulphated Δ-disaccharides of hyaluronan, (1) and chondroitin sulphate and/or dermatan sulphate, (2 through 9), produced by chondroitinase digestion.

or IdoA, whereas chondroitinases AC cleave only those to GlcA and chondroitinase B those to IdoA [27].

In this paper we describe an HPCE method of reversed polarity, by which the various disaccharide types present in pure or cell-secreted HA, CS, DS and in PGs can be determined as their Δ-disaccharide derivatives following digestions with chondroitinases. The separation and determination are performed within 14 min with high sensitivity and accuracy.

2. Experimental

2.1. Chemicals

CSA from whale cartilage, CSC from shark cartilage, CSB from porcine skin and HA from human umbilical cord were obtained from the Sigma Chemical Co. (St. Louis, MO, USA). CS-E was isolated from squid cranial cartilage [28,29]. The nonsulphated CS (chondroitin), monosulphated CS and oversulphated CS fractions of GAGs from squid skin were prepared as previously described by Karamanos et al. [30–33]. Shark fin cartilage, CS, was kindly provided by Dr. Atti-La Dahlgren (Institute for Socio-Medical Research, Areuse, Switzerland). Standard preparations of Δdi-nonS_{HA}, Δdi-nonS_{CS}, Δdi-mono4S, Δdi-mono6S, 2 - acetamido - 2 - deoxy - 3 - O - (4 - deoxy - 2 - O - sulpho - α - L - *threo* - hex - 4 - enopyranosyluronic acid) - D - glucose (Δdi - mono2S), 2 - acetamido - 2 - deoxy - 3 - O - (4 - deoxy - 2 - O - sulpho - α - L - *threo* - hex - 4 - enopyranosyluronic acid) - 4 - O - sulpho - D - galactose [Δdi-(2,4)diS or Δdi-diS_B], 2 - acetamido - 2 - deoxy - 3 - O - (4 - deoxy - 2 - O - sulpho - α - L - *threo* - hex - 4 - enopyranosyluronic acid) - 6 - O - sulpho - D - galactose [Δdi-(2,6)diS or Δdi-diS_D], 2 - acetamido - 2 - deoxy - 3 - O - (4 - deoxy - α - L - *threo* - hex - 4 - enopyranosyluronic acid) - (4,6) - di - O - sulpho - D - galactose [Δdi-(4,6)diS or Δdi-diS_E] and 2 - acetamido - 2 - deoxy - 3 - O - (4 - deoxy - 2 - 0 - sulpho - α - L - *threo* - hex - 4 - enopyranosyluronic acid) - (4,6) - di - O - sulpho - D - galactose [Δdi-(2,4,6)triS or Δdi-triS] were purchased from the Seikagaku Kogyo Co. (Tokyo, Japan). Chondroitinases AC, ABC and B were also obtained from Seikagaku. Membrane filters (0.2 μm) were purchased from Millipore (Waters, Milford, MA, USA). All other chemicals used were of analytical reagent grade.

2.2. Enzymic degradation of GAGs

Digestions of the GAGs with chondroitinase AC or ABC and chondro-4- and 6-sulphatases were performed at 37°C for 90 min in 50 m*M* Tris–HCl, pH 7.5 (4–6), using 0.01 units per 10 μg of uronic acid and 10–100 μl of solution. Digestion with chondroitinase B was performed at 37°C for 60 min in 50–100 μl of 50 m*M* Tris–HCl, pH 8.0, using 0.01 units per 40 μg of uronic acid [27]. The digestions were terminated by heating in a boiling water bath for 1 min, whereafter the mixtures were centrifuged in a Microfuge at 10 000 *g* for 5 min. Aliquots were then taken for direct HPCE analysis of non-, mono-, di- and trisulphated Δ-disaccharides.

2.3. High-performance capillary electrophoresis

Capillary electrophoresis was performed on a Beckman HPCE instrument (P/AGE system 5510) equipped with a diode array detector with a window of 100 × 800 μm set at 232 nm for detection of eluted peaks and at 200 to 600 nm for recording the spectrum of these peaks. Separation and analysis were carried out on an uncoated fused-silica capillary tube (75 μm I.D., 55 cm total length and 50 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, and then with the operating buffer (various sodium orthophosphate buffers at pH ranging from 2.55 to 5.00) for 4 min. The samples to be analysed were injected automatically, using the pressure injection mode, in which the sample is pressurised for 10 s. The injection volume can be calculated with the Poiseuille equation as proposed by the manufacturer (Beckman), giving an estimated volume of

6 nl per second of injection time. For optimal separation, the electrophoresis was performed at 20 kV using 15 m*M* sodium orthophosphate buffer at pH 3.00 and reversing the electrodes so that the constituents to be analysed would migrate from the negative (cathode) to the positive (anode) electrode by electrophoretic mobility (EM) and against the electroosmotic flow (EOF) of the buffer. Peak heights and areas were recorded and evaluated using the Beckman software system Gold V810. Quantitation of Δ-disaccharide contents in samples was performed using an external standard mixture of commercially available disaccharides, diluted with the chondroitinase digestion buffer to a concentration similar to that of the samples. The operating buffer used was degassed by vacuum filtration through a 0.2 μm membrane filter, followed by agitation in an ultrasonic bath.

2.4. Linearity and detection sensitivity

In order to test the linearity of the detector response, precisely known amounts of all Δ-disaccharides were dissolved in chondroitinase digestion buffer to give stock solutions of 10 mg/l and 10 ng/l each. Standard Δ-disaccharide solutions of 0.1 1.0, 10, 50 and 100 ng/l and 0.5, 10, 25, 50 and 100 μg/l were then prepared by dilutions of the stock solutions. A specified injection time of 10 s was used for the analysis, corresponding to an injection volume of 60 nl. The calibration graphs were constructed by plotting the peak heights of Δ-disaccharide signals against their concentration. The detection limits were estimated as the quantity of the Δ-disaccharides producing a signal of the peak height twice the baseline noise.

2.5. Other analytical assays

Total uronic acid was determined by the carbazole method of Bitter and Muir [34] and the IdoA/GlcA ratios obtained by comparing different digests were verified chromatographically, using the HPLC procedure described by Karamanos et al. [35].

2.6. Applications

Analysis of GAGs secreted by cultured cells

We tested the applicability and sensitivity of the proposed HPCE method by using it to analyse the extracellular medium of two human malignant mesothelioma cell lines secreting GAGs, the quantitation of which can be used as an important test in the diagnosis of this tumour [36–37]. In brief, the medium was clarified by centrifugation at 3000 *g* for 15 min and the macromolecules secreted into the medium were precipitated with 4 volumes of ethanol. The GAGs were then liberated from their PGs with papain digestion [38] and purified by a two-step precipitation procedure [39]. The first precipitation was obtained with 1% (w/v) cetyl pyridinium chloride and the second by 90% (v/v) ethanol, containing 2.5% (w/v) sodium acetate. Two sublines were tested, one with the epithelial phenotype obtained through culture in 10% (v/v) human AB serum, and one with a fibroblast-like morphology, obtained in 10% (v/v) fetal calf serum [40]. The isolated GaGs were degraded with a mixture containing chondroitinases ABC and AC and chondro-4- and -6-sulphatases. The chondroitinase-resistant GAGs were then precipitated with 4 volumes of 90% (v/v) ethanol containing 2.5% (w/v) sodium acetate. The supernatant, containing the Δ-disaccharides from the chondroitinase-susceptible GAGs, was dried, dissolved in distilled water and taken for the HPCE analysis.

2.7. Analysis of PGs and GAGs isolated from tissues

To test the applicability of the HPCE method described, PGs were extracted from squid skin, corresponding to 95% of the total content of PGs in this tissue, and analysed. In brief, powdered tissues (5 mg) were extracted with 5 ml of 4 *M* guanidine hydrochloride–50 m*M* sodium acetate, pH 5.8, containing proteinase inhibitors [31–32] and the extract was precipitated twice with 4 volumes of ethanol. The precipitates were dissolved in distilled water and aliquots were taken for digestion with both chondroitinases

ABC and AC. To these digests, 4 volumes of ethanol were added and, after centrifugation at 10 000 *g* for 10 min, the supernatant was analysed for Δ-disaccharides.

Another aliquot of the PG preparation was digested with papain [38] and the liberated GAGs were separated by chromatography on DEAE-Sephacel eluted with a linear NaCl gradient (0.1 to 1.0 *M*), as previously described [32]. The fractions that were positive with the borate–carbazole reaction [34] were pooled to give three different GAG preparations: nonsulphated CS (chondroitin) eluted with 0.2 *M*, monosulphated CS eluted with 0.6 *M* and oversulphated CS, obtained with 0.9 *M* NaCl. The isolated GAGs were then digested with both chondroitinases ABC and AC and analysed by HPCE.

3. Results and discussion

3.1. Resolution of Δ-disaccharides

The determination of HA is based on an analysis of Δdi-nonS_{HA} produced by digestion with chondroitinases ABC and/or AC, and the separation of Δdi-nonS_{CS} and Δdi-nonS_{HA} is therefore essential for the HA analysis. This separation is preferably performed by HPLC, using a primary amino column eluted with sodium dihydrogen orthophosphate at pH 2.55 [16,19]. The HPLC separation requires that the pH of the eluent be kept slightly below the pK_a (3.1–3.2) of the carboxyl groups, giving a separation based on ion suppression, the separation being obtained only in a narrow pH interval. On the other hand, the HPLC separation of oversulphated Δ-disaccharides on an amino column requires a higher pH (pH 5.0) and salt concentration. Using a pH of 2.55 in the HPCE buffer and concentrations of the phosphate buffer varying between 15 and 50 m*M*, the nonsulphated Δ-disaccharides were not observed within 30 min in either normal or reversed-polarity electrophoresis. At this pH, however, oversulphated and monosulphated Δ-disaccharides were completely resolved within 15 min, using reversed polarity (Fig. 2A).

These anionic sulphated Δ-disaccharides will show only limited interactions with the similarly charged silanol groups of the capillary wall, and their migration is thus mainly influenced by the EM and the EOF. At this low pH, the EM of sulphated Δ-disaccharides is much larger than the EOF which, with reversed polarity, is directed against the migration of the carbohydrates. The nonsulphated Δ-disaccharides, however, are anionic only at about 20% of the time, which means that the active charge per disaccharide is only about 0.2. This is obviously not sufficient to overwhelm the EOF. Both of these factors, EOF and EM, are comparatively low and of similar magnitude, since these substances were detected neither with normal nor with reversed polarity within a 30-minute period.

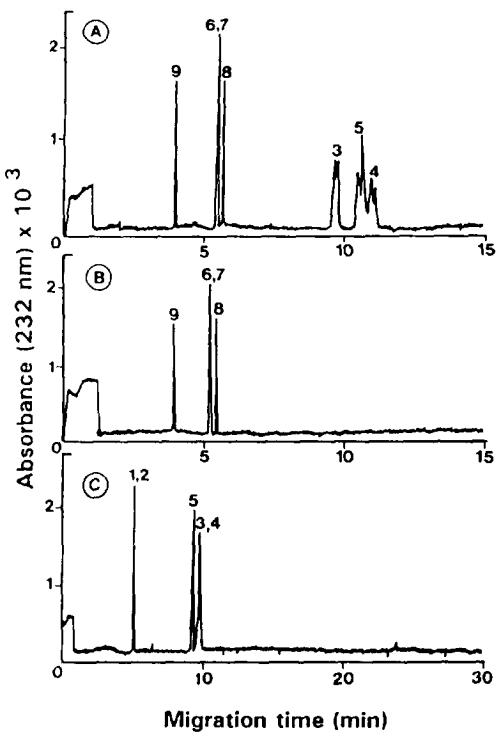


Fig. 2. HPCE profiles of non-sulphated and variously sulphated Δ-disaccharides obtained (A) at pH 2.55, using reversed polarity and (B, C) at pH 5.00, using reversed and normal polarity, respectively. Peaks as indicated in Fig. 1.

Increasing the pH from 2.55 to 5.00 increases both the EOF and the EM. The magnitude of the increase in EM depends on the number of active charges and has very different effects on migration times (Fig. 2A and B). At pH 5.0, the EMs of di- and trisulphated Δ-disaccharides were larger than the EOF, allowing the separation of these structures by reversed polarity, the various types of disulphated Δ-disaccharides migrating similarly (Fig. 2B). The EMs of non- and monosulphated Δ-disaccharides, however, were insufficient to overcome the counter-current EOF, and normal polarity was required to migrate them. Here the separation obtained depended mainly on the number of sulphate groups, with only a poor separation of the different monosulphated types (Fig. 2C).

Better separations were obtained when the pH values between those referred to above were used. At pH 3.00, all Δ-disaccharides tested showed baseline separation (Fig. 3A and B). The double peaks obtained for Δdi-mono4S and Δdi-nonS are well explained by the anomeric forms of the hexosamines present in the reducing terminal of these disaccharides. An increase in the concentration of the operating orthophosphate buffer, pH 3.00, from 15 to 25 and 50 m*M* caused only small differences in the migration times of over- and monosulphated Δ-disaccharides. This pH at a concentration of 15 m*M* was found to be optimal as an operating buffer, since it gave the lowest current value (42 mA) that ensures the best performance of the instrument. At pH 3.50, the separation between non-, mono- and oversulphated Δ-disaccharides was still sufficient, but the resolution between Δdi-(2,4)diS and Δdi-(2,6)diS, as well as between Δdi-mono4S and Δdi-mono6S was somewhat affected. Baseline separation between Δdi-nonS_{CS} and Δdi-nonS_{HA} was obtained at both of these pHs, but they eluted close to each other at pH 3.50. To ensure complete separation with higher injection volumes, a pH of 3.0 seemed preferable also for analysis of the nonsulphated Δ-disaccharides. Repeated injections gave retention times with a standard deviation smaller than 1%.

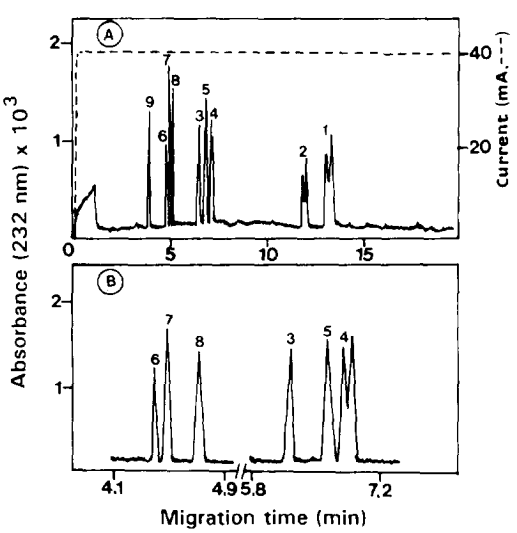


Fig. 3. (A) Typical electrophoregrams obtained at pH 3.00, showing the analysis of nine Δ-disaccharide components (1 to 9). (B) Expanded parts of the same electrophoregram showing the complete separation of individual Δ-disaccharides.

The sensitivity and linearity of the method were tested with the use of standard mixtures at various concentrations. The peak heights and the peak areas obtained were found to be linearly related to the injected amount of each Δ-disaccharide up to the 10 μg/l tested, i.e., through the entire interval tested (Fig. 4). The precision of the method was determined by six repeated determinations of all Δ-disaccharides. When 5 pmol of each disaccharide was measured, the relative standard deviations for the various sulphated disaccharides ranged from 2.6% to 3.2% of the registered value. The baseline noise was as small as 10^{-5} AU. The detection limit of these Δ-disaccharides (molar absorptivity 5500 $M cm^{-1}$), expressed as twice the baseline noise, would then correspond to 22 ng/l (32 pmol/l) for Δdi-triS, 38 ng/l (65 pmol/l) for Δdi-diS, 50 ng/l (100 pmol/l) for Δdi-monoS and 98 ng/l (250 pmol/l) for Δdi-nonS. To obtain an accurate determination of the Δ-disaccharide composition in one injection within a 95% confidence interval, as little as 15–150 fg of the GAG is required with the present injection interval of 10 s. The loading of the injector unit with 5 μl of a sample solution and the triplicate analysis using each 60 nl injection volume would then give a similar

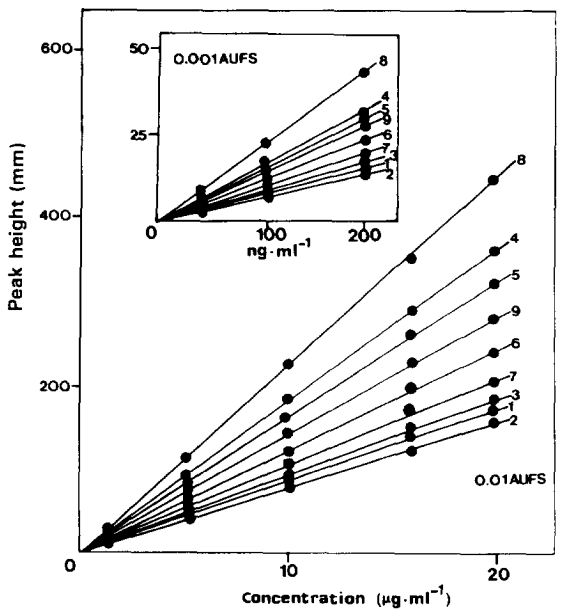


Fig. 4. Calibration curves for the analysis of disaccharides 1 to 9 showing detector response, expressed as peak heights as a function of the concentration of Δ-disaccharides in μg/l. Insert figure shows the calibration curves obtained up to 200 ng/l. Injections of 10 s time interval were performed (injection volume 60 nl). The detector responses ($AU \cdot 10^{-3}$) corresponds to the following equations for the respective disaccharides: 1, $y = 10.9x + 0.1$; 2, $y = 10.3x - 0.1$; 3, $y = 11.7x + 0.4$; 4, $y = 24.3x \pm 0$; 5, $y = 22.0x - 0.2$; 6, $y = 16.1x - 0.1$; 7, $y = 13.7x + 0.2$; 8, $y = 29.7x \pm 0$; 9, $y = 18.9x - 0.2$.

95% accuracy when the sample to be analysed contains approximately 60–200 ng/l of the various Δ-disaccharides. Detection of Δ-disaccharides in fmol levels has also been reported with HPLC by Toyoda et al. [41] and with HPCE by Honda et al. [42], however, these levels of sensitivity were obtained following precolumn derivatization with UV-absorbing substances.

3.2. Applications to the analysis of GAGs and PGs

The analysis of pure HA resulted in one split peak only, corresponding to the anomeric forms of the Δdi-OS_{HA}. Digests of GAGs from different sources, followed by HPCE analysis, showed the presence of various amounts of non-, mono- and oversulphated disaccharides (Table 1). These results were in close agreement with the previously reported composition of these tissues [19, 30–31]. The commonest location of the sulphate was C-4 and C-6 of the galactosamine. Native sulphation in C-2 of the uronic acid was detected in both mono- and disulphated Δ-disaccharides. However, the monosulphated type, Δdi-mono2S, was found only as a small fraction in CS from shark fin cartilage (Fig. 5). The trisulphated Δ-disaccharide and three types of mono- and disulphated Δ-disaccharides were completely separated with the HPCE method

Table 1
Composition of GalAGs from various sources, determined by HPCE[a]

	CSA	CSC	CSB	CSE
Δdi-nonS_{CS}	8.9 (9.0)[b]	1.1 (1.3)	2.2 (2.0)	2.1 (2.1)
Δdi-mono4S	64.3 (63.8)	12.5 (12.1)	72.5 (71.3)	19.4 (20.1)
Δdi-mono6S	18.9 (18.4)	64.0 (63.1)	1.2 (1.0)	9.1 (8.2)
Δdi-(2,6)diS	4.5 (5.0)	12.1 (13.4)	4.2 (3.5)	ND[c]
Δdi-(2,4)diS	ND	2.1 (1.8)	24.5 (24.1)	ND
Δdi-(4,6)diS	ND	ND	ND	69.2 (69.7)
Δdi-(2,4,6)triS	ND	ND	ND	ND

[a] Results are the average of three experiments and are expressed as per cent of the total disaccharides recovered by HPCE after digestion with both chondroitinases ABC and AC. Variations in the amounts of disaccharides measured were less than 5% in all cases.
[b] Values in parentheses were obtained from Ref. 19.
[c] Not detected.

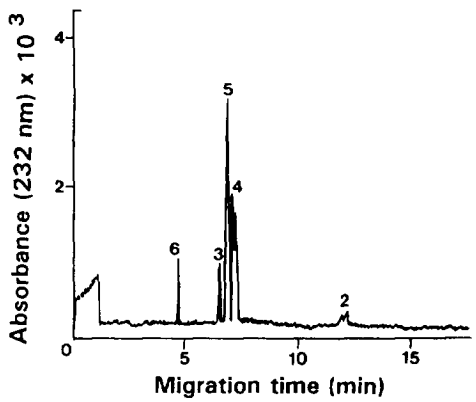


Fig. 5. HPCE analysis of CS isolated from shark fin cartilage after digestion with chondroitinase ABC.

described, even with the larger injection volumes used for analysing samples in the pg range. The identity of the migration times of these di- and trisulphated disaccharides was estimated by chromatography of standard Δ-disaccharide preparations and by observing their chromatographic behaviour after further enzymic treatment. Therefore, digestion of Δdi-di(2,4)S and Δdi-di(2,6)S with chondro-4- and 6-sulphatase, respectively, followed by HPLC analysis of the digests for monosulphated Δ-disaccharides, showed that they both migrated to the position of Δdi-mono2S. The presence of Δdi-di(4,6)S was also confirmed by the detection of Δdi-nonS_{CS} as the only Δ-disaccharide obtained after digestion with both chondro-4- and 6-sulphatases.

As shown in Table 2, the sulphation patterns obtained for the tissue extracted PGs were in close agreement with those obtained with purified GAGs. This indicates that the proposed HPCE method can also be easily and accurately used for the sulphation analysis of PG molecules present in tissue extracts. On the other hand, analysis of GAGs secreted by the two human malignant mesothelioma cell lines showed that this method is also applicable to biological preparations containing minute amounts of GAGs (approximately 1 ng of each of HA and GalAGs per 10^3 mesothelioma cells and less than 20 pg per the same number of benign mesothelial cells) which otherwise can be detected only by radiochemical techniques [43,44]. The sulphation pattern of these GAGs can also be established with the present technique. When the total amount of GalAGs is to be determined, the digest is preferably completed chondro-4- and -6-sulphatases, to collect and concentrate most of the disaccharides in the nonsulphated peak.

3.3. Determination of GlcA or IdoA clusters in DS

Clustered disaccharide structures in DS, i.e., sequences with a single type of uronic acid only, either GlcA or IdoA, can be studied by separate digestions with chondroitinase AC and B, comparing the obtained values with those obtained after digestion with chondroitinase ABC. A high rate of recovery can help in recognising such

Table 2
HPCE analysis of GalACs in human malignant mesothelioma and in tissue extracted PGs

	Δdi-nonS_{HA}	Δdi-nonS_{CS}	Δdi-mono4S	Δdi-mono6S	Δdi-diS	Δdi-triS
Fibroblast GalAGs[a]	684	425				
Epithelial GalAGs[a]	8187	5460				
Squid skin PG extract[b]	ND	3567	1200	276	1087	257
Chondroitin[b]	ND	310	ND	ND	ND	ND
CSI + CSII[b]	ND	145	198	82	25	ND
Oversulphated CS[b]	ND	152	912	202	894	185

[a] Expressed as pg per 10^3 cells and day in culture. These separations were performed only after sulphatase digestion.
[b] Expressed as ng present in mg tissue dry weight.

GlcA or IdoA clusters, whereas low a recovery indicates that the DS is rich in alternating GlcA- and IdoA-containing disaccharides (cf., Fig. 6A, B and C). The oligosaccharides resulting from such a partial digestion of chondroitinase B contain a terminal Δ-uronic acid derived from IdoA, although the remaining uronic acids in these fragments are GlcA. Corresponding fragments obtained with chondroitinase AC have a GlcA-derived Δ-uronic residue, the remaining ones are IdoA. Three such extra peaks were obtained when the DS was digested with chondroitinase B (Fig. 6C), indicating that most of the GlcA-containing disaccharides are scattered or occur in short sequences. This is also in agreement with our previous results [45], showing that the longer UV-absorbing sequences of this digest consists of one Δ-tetrasaccharide, one Δ-hexasaccharide and one Δ-decasaccharide fragment, the latter containing the linkage region. The exact identity of these oligosaccharide peaks, however, remains to be determined. The present HPCE method, in combination with the various chondroitinases, thus facilitates an understanding of the DS disaccharide sequences. Such an analytical use of chondroitinases AC and B, however, necessitates a careful interpretation of the data obtained. The exact nature of the oligosaccharide fragments remains to be elucidated.

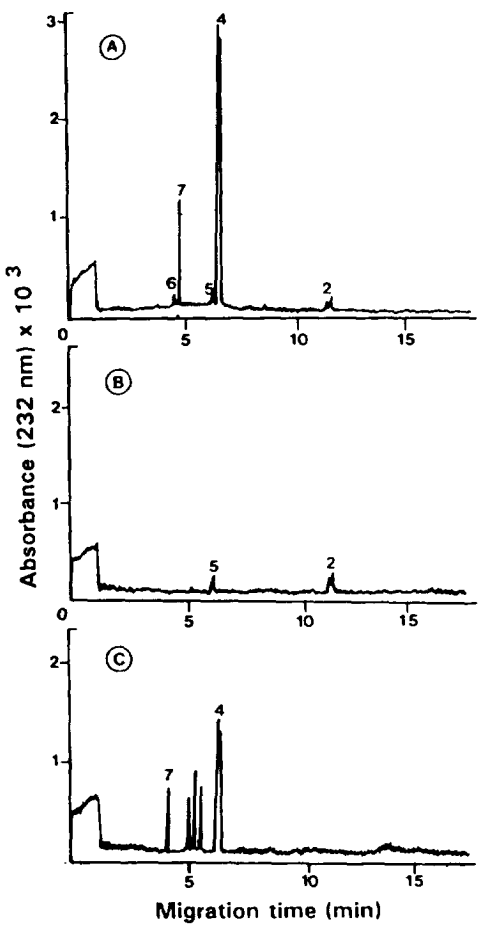


Fig. 6. (A, B and C) Analyses of CSB from porcine skin digested with chondroitinases ABC, AC and B, respectively.

Analysis of various DS digests showed the absence of IdoA-derived Δdi-mono6S, but this was found noted mainly with GlcA, indicating a non-random sulphation during synthesis. The disulphated disaccharides, Δdi-di(2,4)S, Δdi-di(2,6)S and Δdi-di(4,6)S, were obtained from GlcA- and IdoA-containing disaccharides (Table 3). These results indicated that all six disulphated disaccharides tested (GlcA- or IdoA-containing di(2,6)S, di(2,4)S and di(4,6)S can be determined in the same HPCE run.

In conclusion, the presently used HPCE method allows the separation of all the different types of Δ-disaccharides known to be present in chondroitinase-susceptible vertebrate GAGs. The

Table 3
Composition of disulphated disaccharides of various GalAGs, determined by HPCE[a]

	Disulphated Δ-disaccharides		
	(2,6)S	(2,4)S	(4,6)S
CSA	55.7[b]	ND	ND
CSC	57.4[b]	6.0[b]	ND
CS shark fin cartilage	100.0[b]	ND	ND
CSB porcine skin	6.0[c]	93.8[c]	ND
CS squid cartilage	ND	ND	100.0[b]
CS squid skin	ND	ND	10.0[b]

[a] The results are expressed as per cent of the total disulphated Δ-disaccharides recovered by HPLC after digestions with chondroitinase AC and B.
[b] Derived from GlcA-containing disaccharides.
[c] Derived from IdoA-containing disaccharides.

analysis can be performed on the attomole level. In combination with the separate use of chondroitinase AC and B, this analysis can also provide information about the types of uronic acid present.

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