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Short communication

Ultrasensitive capillary electrophoresis of sulfated disaccharides in chondroitin/dermatan sulfates by laser-induced fluorescence after derivatization with 2-aminoacridone

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Abstract

An ultrasensitive capillary electrophoretic method for separating the variously sulfated chondroitin/dermatan sulfate-derived Δ-disaccharides after digestion with chondro/dermatolyases and derivatization with the fluorophore 2-aminoacridone is described. All known mono-, di- and tri-sulfated Δ-disaccharides were completely separated using 15 m*M* orthophosphate buffer (pH 3.0) at 20 kV without any interference of the excess derivatizing reagent. They were detected at the anode (reversed polarity) using either an Ar-ion laser-induced fluorescence (LIF) detector (excitation wavelength 488 nm) or a UV detector. The sensitivity obtained by LIF (0.51 pmol/l) was at least 100 and 10 times higher as compared to those obtained by UV detection at 232 nm of underivatized Δ-disaccharides and at 254 nm of those derivatized with aminoacridone, respectively. The method has been easily applied to the analysis of chondroitin/dermatan sulfates from various tissues at the attogram level, including chondrotin/dermatan sulfates from normal and aneurysmal human abdominal aortas.

Keywords: Disaccharides, sulfated; Chondroitin sulfate; Dermatan sulfate

1. Introduction

Chondroitin sulfate (CS) and dermatan sulfate (DS), usually referred to as galactosaminoglycans (GalAGs), consist of repeating disaccharide units which contain a uronic acid glycosidically linked to galactosamine. They are covalently linked to protein cores forming proteoglycans. GalAGs are highly charged polymers due to the presence of esterified sulfate groups at any available hydroxyl group of the disaccharide unit [1]. Their sulfation plays a key role in determining the physiological characteristics of proteoglycans. Altered sulfation patterns are often encountered in pathological situations [2–4].

GalAGs and their PGs are often found in trace amounts and, consequently, highly sensitive analytical methods are necessary for their analysis and structural characterization (for reviews see Refs. [5]and[6]). Treatment of CS/DS with chondroitinases ABC, AC and/or B is essential to determine disaccharide composition. The obtained Δ-disaccha-

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rides have been previously completely separated utilizing either capillary electrophoresis (CE) or HPLC methods and sensitively detected at 232 nm due to the absorbance of the unsaturated uronic acid in conjunction with the carboxylic group at C-5 [1,7].

In the quest for higher sensitivities, derivatization schemes with fluorescent molecules have been introduced. 2-Aminoacridone (AMAC) is a fluorescent neutral molecule with λ_{exc}=425 nm and λ_{em}=520 nm. It was successfully introduced by Jackson [8] for labeling reducing carbohydrates and separating them by polyacrylamide gel electrophoresis. Kitagawa et al. [9] used AMAC derivatives to analyze glycosaminoglycan-derived disaccharides by HPLC with fluorescence detection and CE with UV detection at 254 nm, since no fluorescence detector was available for CE.

Fluorescence detection is inherently more advantageous than UV spectroscopy because of its high specificity and sensitivity. However, fluorescence detection of carbohydrates in CE has had only marginal gains in sensitivity due to difficulties in focusing a significant amount of light on the optical window of the capillary. The recent utilization of LIF detectors for carbohydrate detection resulted in ultrahigh sensitivities, so that only a few thousand molecules are necessary for detection, because of the potential of the monochromatic laser beam to be focused on a very small area. Moreover, they have the advantage of a rather large linear dynamic range of six or seven orders of magnitude as compared to spectroscopy where only two or three orders can be obtained. Our present study focuses on determining the sensitivities provided by LIF using AMAC derivatives as well as improving separation of all known sulfated Δ-disaccharides present in vertebrates.

2. Experimental

2.1. Chemicals and biological material

$NaBH_3CN$, CS mainly sulfated at C-4 (formerly named CSA) from whale cartilage, DS (previous name CSB) from porcine skin were obtained from Sigma–Aldrich (Steinheim, Germany). CSE was isolated from squid cranial cartilage [10]. GalAGs from normal and human aneurysmal abdominal aortas were isolated according to the protocol proposed by Theocharis et al. [11]. Standard preparations of Δ-di-nonS_{HA}, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose, Δ-di-nonS_{CS}, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose and the variously mono-, di-, and trisulphated Δ-disaccharides were purchased from the Seikagaku Kogyo (Tokyo, Japan). Chondroitinases AC and ABC were also obtained from Seikagaku. 2-Aminoacridone was obtained from Lambda Fluoreszenz Technologie (Graz, Austria). All other chemicals used were of analytical reagent grade.

2.2. Sample treatment

Digestions of the CS and DS preparations with chondroitinases ABC or AC were performed at 37°C for 90 min in 50 m*M* Tris–HCl (pH 7.5), using 0.01 U per 10 μg of uronic acid and 10–100 μl of solution. Mixtures were centrifuged in a Microfuge at 10 000 *g* for 5 min and aliquots were taken for derivatization.

Derivatization of GalAG-derived Δ-disaccharides with AMAC was performed as described by Jackson [8] and modified by Kitagawa et al. [9]. Particularly, 1–10 nmol of Δ-disaccharides were evaporated in a microcentrifuge tube at low temperature. A 5 μl volume of a 0.1 *M* AMAC solution in glacial acetic acid–DMSO (3:17, v/v) and 5 μl of 1 *M* $NaCNBH_3$ in water were added to the sample, and the mixture was incubated at 45°C for 2 h. The samples were evaporated to dryness and reconstituted in 10 μl of 50% DMSO.

2.3. Capillary electrophoretic analysis

CE was performed on a Beckman instrument (P/ACE system 5510) fitted with a LIF detector, laser module 488 (3 mW, air-cooled Argon ion with λ_{exc}= 488 nm), a connecting fiber optic cable and interlock cables that run from the laser to the detector. A diode array detector with a window of 800×100 μm set at 254 nm for detection of eluted peaks and at 200–600 nm for recording the spectrum of these peaks was also used. Separation and analysis were carried out

on an uncoated fused-silica capillary tube (75 μm I.D., 55 cm total length, 50 cm effective length to the detector) at 20°C.

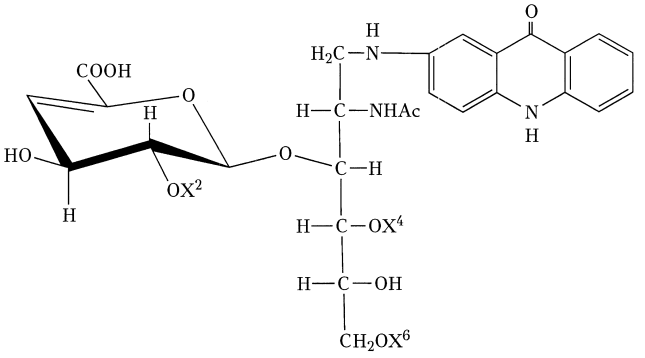
Before each run, the capillary tube was washed with 0.1 *M* NaOH for 1 min, doubly distilled water for 0.5 min and the operating buffer for 4 min. Samples were introduced hydrodynamically at the cathode (reversed polarity) and separated using 15 m*M* orthophosphate buffer (pH 3.0) at 20 kV as it has been earlier suggested for underivatized Δ-disaccharides [7].

3. Results and discussion

3.1. HPCE analysis of AMAC derivatives

The derivatization procedure was simple and rapid. AMAC was attached to the reducing end of GalAG-derived Δ-disaccharides via reductive amination, i.e., the amino group of AMAC reacts with the carbonyl group of the reducing end to form a Schiff base which is further reduced with sodium cyanoborohydride to form a stable secondary amine. The structures of the variously sulfated Δ-disaccharide AMAC derivatives are given in Fig. 1.

The separation of the AMAC GalAG-derived sulfated Δ-disaccharides by CE is given in Fig. 2. All known tri-, di- and monosulfated disaccharides were well resolved within 25 min. A typical electropherogram showing the analysis of the seven variously sulfated standard Δ-disaccharides is depicted in Fig. 2A. Repeated injections gave migration times with a standard deviation of about 1%. Each peak was identified with coinjection experiments. The complete separation of all AMAC derivatives involving also the monosulfated Δ-disaccharide bearing esterified sulfate with the –OH group of C-2 at the uronic acid (Δ-di-mono2S) and the disulfated Δ-di-(2,4)diS or Δ-di-diS_B is reported for the first time. Excess reagent did not interfere with the disaccharide analysis since 2-aminoacridone did not enter the capillary. This is due to the positive charge it acquires at low pH and the reversed polarity mode avoiding, thus, interference in Δ-disulfated disaccharide determination as it has been previously described [9]. In contrast to the CE analysis of underivatized Δ-disaccharides [7], non-sulfated di-



Sulfation	Disaccharide terminology
1. X^2, X^4, X^6 = SO_3H	Δdi-tri(2,4,6)S
2. X^4 = H — X^2, X^6 = SO_3H	Δdi-di(2, 6)S
3. X^6 = H — X^2, X^4 = SO_3H	Δdi-di(2, 4)S
4. X^2 = H — X^4, X^6 = SO_3H	Δdi-di(4, 6)S
5. X^4, X^6 = H — X^2 = SO_3H	Δdi-mono2S
6. X^2, X^6 = H — X^4 = SO_3H	Δdi-mono4S
7. X^2, X^4 = H — X^6 = SO_3H	Δdi-mono6S

Fig. 1. Structure of AMAC derivatives of mono-, di- and trisulfated CS/DS-derived Δ-disaccharides.

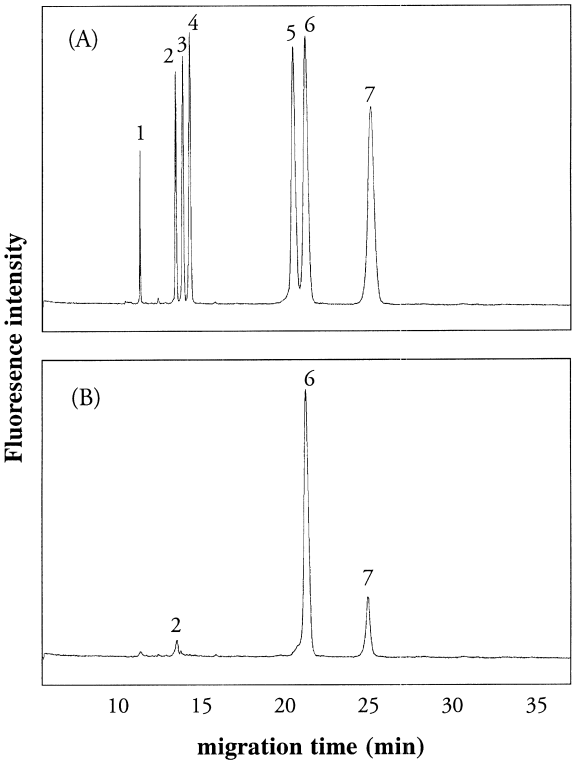


Fig. 2. Typical electropherograms showing the separation of standard sulfated Δ-disaccharides (A) and those present in CSA (B) obtained following digestion with chondroitinases ABC and AC. CE was performed with 15 m*M* orthophosphate buffer (pH 3.0) as operating buffer at 20 kV and 25°C using LIF detection with Ar-ion laser source.

saccharides did not appear on the electropherogram and this may well be attributed to the weak positive charge of their AMAC derivatives. The most striking finding was that the separation was not pH-sensitive in the area of pH 2.5–4.0 suggesting a robust procedure.

Analysis of AMAC derivatives was recorded by a LIF detector (air-cooled Ar-ion laser source). LIF detection led to a concentration detection limit of 0.51 p*M* (0.3 amol injected amount) estimated as the quantity of the Δ-disaccharide producing a signal three times the baseline noise. When compared with UV detection at 254 nm (concentration detection limit 5–8 p*M*), LIF detection improved the detection limit at least by a factor of ten. Derivatization and detection by an Ar-ion LIF detector offered concentration detection limits at least 100-times higher than those obtained by UV detection at 232 nm of underivatized GalAG-derived Δ-disaccharides (50–375 p*M* estimated as signal-to-noise ratio equal to 3).

3.2. Application to the analysis of GalAGs from various sources

This method was applied to the analysis of GalAGs from various sources. A typical electropherogram obtained for CSA is presented in Fig. 2A. The obtained compositions of CS/DS-derived sulfated Δ-disaccharides from various tissues were in complete accordance to those previously reported (Table 1).

It has been described [11] that the major GAG type in normal aortas is CS, overwhelming the DS content by a factor of three. In aneurysmal abdominal aortas a 65% decrease in CS content was noted, whereas that of DS remains almost constant [11]. The present method was applied to the analysis of aortic GalAGs isolated following treatment with chondroitinases ABC and AC and gel-permeation chromatography [11]. As shown in Table 1, structural alterations in disaccharide composition of GalAGs correspond to statistically significant decreases ($P<$ 0.001) of 6-sulfated disaccharide and the considerable alteration of disulfated disaccharides. The modified sulfation profiles in aneurysmal aortas may well be related with the development of the disease [12]. The present method produced results that are in good agreement with those obtained by the application of the HPCE [7] and HPLC methods [1], using detection of underivatized disaccharides at 231 nm.

As it has been previously described [7,13] the sulfation pattern of GalAGs can be accurately described according to their susceptibility to different lyases. In combination with the different susceptibility to chondroitinases ABC, AC or B the glucuronic/iduronic acid containing repeats could also be estimated by the present analytical method.

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Table 1
Disaccharide composition of GalAGs from various sources, determined as 2-AMAC derivatives by HPCE[a]

Disaccharides	CSA (whale cartilage)	CSB (porcine skin)	CSE (squid cranial cartilage)	Normal aortas	Aneurysmal aortas
Δ-di-mono4S	70.58±4.2 (63.8)[b]	74.13±4.4 (71.3)	19.81±1.2 (20.1)	23.6±1.4	37.3±1.6
Δ-di-mono6S	20.75±2.0 (18.4)	1.23±0.2 (1.0)	9.29±0.7 (8.2)	72.0±2.3	59.1±3.1
Δ-di-(2, 6)diS	4.94±0.5 (5.0)	4.29±0.6 (3.5)	ND	2.7±0.3	0.64±0.05
Δ-di-(2, 4)diS	ND[c]	25.05±1.5 (24.1)	ND	1.1±0.1	1.0±0.1
Δ-di-(4, 6)diS	ND	ND	70.68±3.3 (69.7)	0.5±0.05	1.9±0.12
Δ-di-(2, 4, 6)triS	ND	ND	ND	ND	ND

[a] Results are the average±SD of three experiments and are expressed as per cent of the total disaccharides recovered by HPCE after digestion with both chondroitinases ABC and AC.
[b] Values in parentheses were obtained from Ref. [1].
[c] Not detected.

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