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Capillary Electrophoresis for the Analysis of Chondroitin Sulfate- and Dermatan Sulfate-Derived Disaccharides

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High-voltage capillary zone electrophoresis (CZE) has been used for the first time in the analysis of non-, mono-, di-, and trisulfated disaccharides derived from chondroitin sulfate, dermatan sulfate, and hyaluronic acid. These glycosaminoglycans are first depolymerized using polysaccharide lyases. The resulting unsaturated disaccharide products can be detected by their ultraviolet absorbance at 232 nm. Different retention times were obtained for each unsaturated disaccharide analyzed by CZE. The application of a constant voltage across a 70-cm fused silica capillary using a single, simple buffer system resolved an eight-component mixture within 40 min. Quantitation of disaccharides derived from chondroitin sulfate using chondroitin ABC lyase (EC 4.2.2.4) and mixtures of unsaturated disaccharide standards was possible requiring only picogram quantities of sample. The disaccharides examined had a net charge of from −1 to −4 and were resolved primarily on the basis of net charge and secondarily on the basis of charge distribution. Two unsulfated disaccharides both containing the same unsaturated uronic acid residue were analyzed. One was from chondroitin having an *N*-acetylgalactosyl residue and one from hyaluronate having an *N*-acetylglucosyl residue. Despite the fact that they differed only by the chirality at one center, these disaccharides were resolved by CZE. CZE is a fast and simple method that represents a powerful new tool for analysis and separation of acidic disaccharide components of glycosaminoglycans.

High-voltage capillary electrophoresis is rapidly becoming a valuable technique for the separation and analysis of biopolymer molecules (1,2). Capillary electrophoresis includes capillary zone electrophoresis (CZE)[2] (3), in which molecules are separated in a fused-silica capillary (25–100 μm i.d.) of the desired length (0.5–1.5 m). When the high voltage is applied, all the components within the capillary (including water) are driven by electroosmotic flow towards the cathode. Resolution and separation efficiency depend on the length of the capillary, charge to mass ratio and molecule mobility, applied voltage, and the pH and type of buffer used (4). Detection is typically by ultraviolet absorbance and fluorescence emission (1).

Capillary electrophoresis provides an alternative to analysis by high-performance liquid chromatography and polyacrylamide gel electrophoresis. Capillary electrophoresis has been used to analyze biopolymers including peptides, proteins, nucleotides, and nucleic acids (1,2,5,6). To date there have been few reports of capillary electrophoresis being applied to the analysis of carbohydrates (7,8). One reason is the absence of charge in most carbohydrates thus requiring either their derivitization (9) or their complexation with charged ions such as borate (7,8). Carbohydrates are also often difficult to detect as they typically do not possess a chromophore.

Glycosaminoglycans (GAGs) are highly charged, linear, sulfated polysaccharides (10) that can be broken down with polysaccharide lyases (11) into acidic oligosaccharides that absorb in the ultraviolet (12,13). Strong anion-exchange SAX–HPLC (13–15) and reversed-phase ion-pairing RPIP–HPLC (16), the primary methods for the analysis of GAG-derived unsaturated disaccharides, rely on ultraviolet detection

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[2] Abbreviations used: CZE, capillary zone electrophoresis; GAGs, glycosaminoglycans; SAX, strong anion-exchange; RPIP, reversed-phase ion-pairing; ΔUA, 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid; S, sulfate; GalNAc, 2-deoxy-2-acetamidogalactopyranose; GlcNAc, 2-deoxy-2-acetamidoglucopyranose; FAB, fast atom bombardment.

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(12,13,17). Suppressed conductivity, another recently reported detection method, has been used with RPIP–HPLC (18). Derivitization, by radiolabeling (19) and fluorescent tagging, is also useful for detecting these carbohydrates (7,9,20).

GAGs exhibit a variety of biological properties and can be altered in disease states (10). The presence of a certain type of GAG or the identification of one with a particular disaccharide composition might give valuable information about the nature of the biological process in which the GAG is involved. Therefore, reliable, reproducible, and sensitive methods for the detection and quantitation of GAGs are needed. A method that could be automated to accurately analyze many samples might have importance to clinical chemistry.

We describe the use of CZE for the analysis of the acidic disaccharide units of chondroitin sulfate, dermatan sulfate, and hyaluronic acid. This method is simple, fast, automatable, and reproducible, giving high sensitivity.

MATERIALS AND METHODS

Materials

ΔUA(1→3)-β-D-GlcNAc (**1**); ΔUA(1→3)-β-D-GalNAc (**2**); ΔUA(1→3)-β-D-GalNAc4S (**3**); ΔUA(1→3)-β-D-GalNAc6S (**4**); ΔUA2S(1→3)-β-D-GalNAc (**5**); ΔUA2S(1→3)-β-D-GalNAc4S (**6**); ΔUA2S(1→3)-β-D-GalNAc6S (**7**); ΔUA(1→3)-β-D-GalNAc4S6S (**8**); and ΔUA2S(1→3)-β-D-GalNAc4S6S (**9**) were prepared directly from hyaluronic acid, chondroitin sulfate, and dermatan sulfate polymers using hyaluronate lyase or chondroitin ABC lyase and purified by SAX–HPLC and recovered in yields comparable to those reported for the preparation of heparin-derived oligosaccharides (21). Alternatively, these disaccharides are available commercially as unsaturated chondro-disaccharide (C-kit) and unsaturated dermato/hyaluro-disaccharide (D-kit) kits for HPLC from Seikagaku America, Inc. (Rockville, MD). Purity of these standards were confirmed by SAX–HPLC (22) and RPIP–HPLC (18), while their identity was confirmed by ^{1}H NMR spectroscopy at 360 MHz and FAB–MS analysis (22).

Chondroitin ABC lyase (EC 4.2.2.4), was from Seikagaku America, while hyaluronate lyase (EC 4.2.2.1, from *Streptomyces hyaluronlyticus*) was from Sigma Chemical (St. Louis, MO). Sodium borate was from Fisher Scientific (Fair Lawn, NJ) and boric acid from Mallinckrodt (Paris, KY). Chondroitin-4-sulfate (chondroitin sulfate A), chondroitin-6-sulfate (chondroitin sulfate C), and dermatan sulfate were prepared by Mathews and Cifonelli of the University of Chicago under NIAMD contract (NO1-AM-5-2205) from river sturgeon (chondroitin sulfates) and from porcine mucosa (dermatan sulfate). Hyaluronic acid from bovine trachea, pronase, bovine lung acetone powder, and Dowex 1X2 ion-exchange resin were from Sigma.

Methods

Preparation of disaccharide standards for analysis. The disaccharide standards were accurately weighed and a 10 mg/ml stock solution of each was prepared in double-distilled water. Portions of these stock solutions were mixed together to prepare standard mixtures and for coinjection studies. The standard solutions were stored frozen at −70°C.

Depolymerization of GAGs for analysis. GAG (10 μg/μl) was treated with the appropriate lyase (2 mU of enzyme/100 μg of substrate) in 5 mM sodium phosphate buffer at p.H. 7.0 for 36 h at 37°C. Depolymerization was monitored by a change in absorbance at 232 nm. Aliquots (10 μl) were removed from the reaction and added to 0.03 M hydrochloric acid (1 ml) and the absorbance measured; the reaction was complete when a constant absorbance was reached.

CZE analysis. The experiments were performed with Dionex Capillary Electrophoresis System (Sunnyvale, CA) equipped with a variable wavelength ultraviolet detector set at 232 nm. Separation and analysis of materials were carried out using a fused silica (externally coated except where the tube passed through the detector) capillary tube (75 μm i.d., 375 μm o.d., 68 cm long) from Dionex. The capillary tube was washed extensively with 0.1 M phosphoric acid, 0.5 M sodium hydroxide, and deionized water then filled with the operating buffer (10 mM sodium borate and 50 mM boric acid, having a pH of 8.8) and inserted into the machine. The sample was injected by gravity injection (1 nl) and electrophoresis was performed at 10 kV using the above operating buffer.

RESULTS AND DISCUSSION

Chondroitin sulfates are a family of glucuronic acid containing galactosaminoglycans (**I**, Fig. 1) that include chondroitin sulfate A, C, D, E, H, and K (23–26), which have disaccharide units with different sulfation patterns. Exhaustive treatment of these chondroitin sulfates with chondroitin AC or ABC lyases affords a mixture of unsaturated disaccharides having structures **2–9** (Fig. 1). The unsaturated uronic acid (ΔUA) residue at the nonreducing end of each disaccharide exhibits an absorbance maxima at 232 nm with ϵ_m 5000–6000 M^{-1} cm^{-1} (12) permitting their detection by ultraviolet spectroscopy. Dermatan sulfate (also called chondroitin sulfate B) is an iduronic acid-containing, sulfated galactosaminoglycan (**II**, Fig. 1) that can similarly be broken down to its constituent disaccharide units (**2–9**, Fig. 1) using chondroitin B or ABC lyase (11). Finally, hyaluronic acid is a glucuronic acid con-

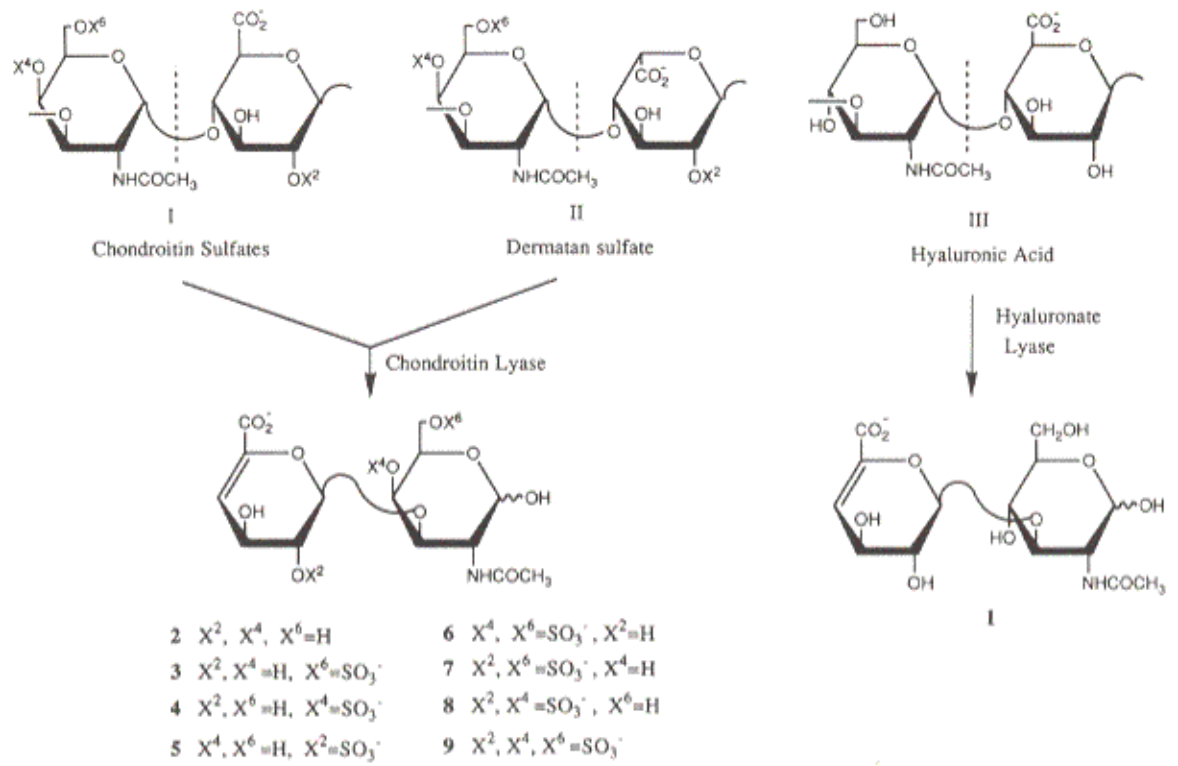

FIG. 1. Structures of the chondroitin sulfates (**I**), dermatan sulfate (**II**), and hyaluronic acid (**III**). The glycosidic linkage that is cleaved by either chondroitin or hyaluronate lyase are shown as are the disaccharide products (**1–9**) of this enzymatic treatment.

taining, nonsulfated glucosaminoglycan (**III,** Fig. 1). Hyaluronic acid can be broken into a single disaccharide **1** on treatment with hyaluronate lyase (11). These alternating 1→3, 1→4 linked GAGs are often found in tissues as extracellular matrix (10). The presence, content, and type of GAG found in a tissue or biological fluid can help identify its source (27) and may be diagnostic of the presence of disease states (10,28).

Analytical approaches have focused on depolymerizing GAGs with polysaccharide lyases and analysis of their disaccharide composition by HPLC (13–16). No single HPLC method resolves mixtures containing disaccharides **2–9.** Without derivitization, the detection limit using either suppressed conductivity or ultraviolet detection is between 10 and 100 pmol (18). Although radiolabeled and fluorescently tagged derivatives should offer more sensitive detection, problems with the chemistry of the derivitization reactions prevent the detection of less than a few picomoles (20).

PAGE (29,30) and gradient PAGE (22,28,31) methods permit the high-resolution separation of acidic oligosaccharides on the basis of their molecular size. Unfortunately, gel-based electrophoresis does not separate disaccharides such as **2–9** having nearly the same molecular size (22). In addition, the most sensitive form of detection, silver staining, only weakly detects oligosaccharides having low sulfation levels (<4 sulfate groups/molecule) and is only semiquantitative (28).

CZE analysis of individual disaccharides **1–7** and **9** gave single peaks, consistent with their high level of purity as assessed by SAX–HPLC and RPIP–HPLC. Disaccharide **8** showed a shoulder on CZE analysis indicating the presence of an impurity that was undetected by HPLC and spectroscopic analysis. The nonsulfated disaccharides **1** and **2** eluted first from the capillary followed by the monosulfated **3–5,** the disulfated **6–8,** and the trisulfated **9** disaccharides (Table 1).

A simple mixture containing non-, mono-, di-, and trisulfated disaccharides **1, 3, 6,** and **9** was prepared and analyzed under a variety of experimental conditions to test the affect of voltage, capillary, buffer, and pH on migration time. A doubling of the voltage from 10 to 20 kV reduced the migration time by approximately 50%, but also decreased resolution. The replacement of one capillary tube for a second, identically prepared capillary only slightly affected the migration times. The use of phosphate buffer, in place of borate buffer, at pH 8.8 had a pronounced affect, increasing migration time and decreasing resolution. Under basic conditions borate can complex to vicinal diols in sugars increasing their negative charge and thus increasing their migration time (7,8). Oligosaccharides **1, 2, 3, 4,** and **6** have the

TABLE 1

Migration Time (min) under Various CZE Analysis Conditions (Buffer, Voltage, Capillary)[a,b]

Disaccharide	Sulfation	Net charge	B8.8,7 kV,1	B8.8,10 kV,1	B8.8,20 kV,1	B8.8,28 kV,1	B8.8,20 kV,2	B5,20 kV,1	P8.8,10 kV,1
1	non-	−1	21.9	15.0	7.33	4.50	7.63	10.0	30.0
3	mono-	−2	30.3	20.5	9.38	6.35	9.60	17.7	65.0
6	di-	−3	40.0	27.5	15.1	8.73	16.7	26.7	73.0
9	tri-	−4	63.1	42.0	24.1	13.0	24.9	39.0	78.0

[a] Buffers are: B8.8, 50 mM boric acid and 10 mM sodium borate at pH 8.8; B5, 50 mM boric acid at pH 5; and P8.8, 25 mM phosphoric acid adjusted with 12.5 M sodium hydroxide to pH 8.8. Voltages used were: 7, 10, 20, and 28 kV. Two 68-cm capillary tubes were prepared using identical methods at two different times and were labeled 1 and 2.

[b] Analysis was performed five times on a mixture of 1, 3, 6, and 9 (1 ng of each) using a third capillary (identical to 1 and 2) with B8.8, at 20 kV. The average retention times and standard deviations were: 1, 6.87, 0.05; 3, 9.37, 0.06; 6, 13.3, 0.1; and 9, 18.5, 0.3. The average standard deviation was 12% of the absolute peak area but only 4% of the relative peak area (where each peak is expressed as the percentage of total peak area in each electropherogram). These data indicate that a major source of error was variation of injection volume or changes in sample concentration by evaporation during analysis (the autosampler typically contained only 10 μl of each sample). The use of an internal standard should improve quantitation.

2,3-hydroxyl groups of their ΔUA residue available for complexation. Contrary to our expectations, monosulfated disaccharides **3** and **4**, capable of complexation, migrate faster than **5**, while disulfated disaccharide **6** migrates faster than **7** and **8**. Also contrary to our understanding of borate complexation, lowering the pH of the borate buffer to **5** increased disaccharide migration times.

Experimental conditions became very important when the goal was to separate disaccharides having identical net charge. (i.e., **1** from **2**, **3** from **4** or **5**, and **6** from **7** or **8**). The use of boric acid–sodium borate buffer, pH 8.8 and 10 kV, gave the optimal resolution between components having identical net charge (Fig. 2). The tailing on the front of the peak, corresponding to **2**, was reduced at lower voltages while the tailing on the rear of the peak, corresponding to **9**, was reduced at higher voltages. The separation of nonsulfated disaccharides **1** and **2** (Fig. 3) is particularly important as these not only have an identical net charge, but that charge is located in the same position in each molecule. The only difference between these two disaccharides is the chirality at the 4-position in the hexosamine residue resulting in a GlcNAc residue in **1** and a GalNAc residue in **2**.

Chondroitin 4- and 6-sulfate **I** and dermatan sulfate **II** reference standards (32) were depolymerized using chondroitin ABC lyase. The reaction products were directly analyzed by CZE (Fig. 4). Chondroitin ABC lyase-depolymerized chondroitin 4-sulfate gave 0.42, 1.59, and 97.9 mol% of disaccharides **2**, **3**, and **4**, respectively. This result compares well with disaccharide content reported by Mathews and Cifonelli of 2, 6, and 92 mol% (32,33). Chondroitin ABC lyase depolymerized chondroitin 6-sulfate gave 8.1, 73.9, and 18 mol% of disaccharides **2**, **3**, and **4**, respectively. Again this is consistent with a reported analysis of 11, 74, and 15 mol% (32,33). Both chondroitin ABC and B lyases, used to depolymerize dermatan sulfate, gave identical analysis of 0.6, 4.2, 48, and 28 mol% of disaccharides **2**, **3**, **4**, and **6–8**. An additional peak corresponding to 20 mol% was also observed. The reported analysis was 1, 7, 68, and 14 mol% of disaccharides **2**, **3**, **4**, and **6** as well as 10 mol%

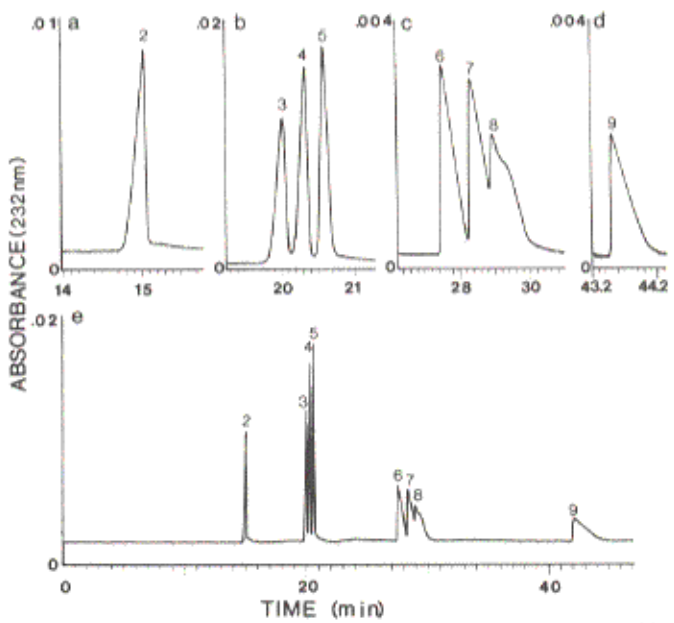

FIG. 2. Electropherograms showing the analysis of 1 nl mixture containing 1 ng/nl each of disaccharides **2–9**. (a–d) are expanded portions of electropherogram (e) that show individual components **2**, **3–5**, **6–8**, and **9**, respectively. (e) is the complete electropherogram showing resolution of this eight-component mixture. The identity of individual peaks was confirmed by coinjection with standards (not shown).

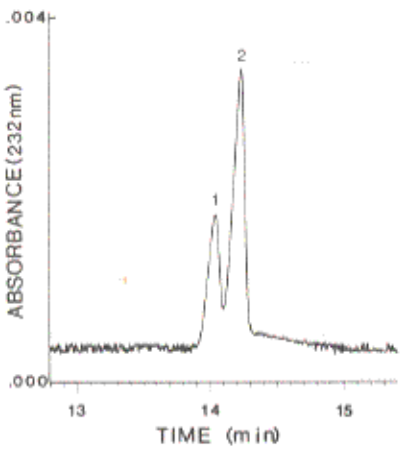

FIG. 3. Electropherogram of 1 nl of a mixture containing 1 ng/nl each of disaccharide **1** and **2.** Detection was by ultraviolet absorbance at 232 nm.

chondroitin ABC lyase-resistant hexosamine derivatives (32,33). Gradient PAGE analysis of the same chondroitin ABC lyase-depolymerized dermatan sulfate (22) showed a band corresponding to disaccharide products as well as a band corresponding to tetrasaccharide products. These data, taken together with the elution position on CZE, suggest that the unidentified product (at 20 mol%) is a disulfated tetrasaccharide having a net charge of −4.

In addition to its utility in identifying the various disaccharides of chondroitin sulfate, dermatan sulfate, and hyaluronic acid, CZE can be used to quantitate the amount of each component present in a mixture. Using disaccharide standards, 10 μl of a mixture containing 10 μg each of disaccharides **1, 3, 6,** and **9** was prepared and 1 nl was analyzed. Peaks corresponding to each component were clearly visible and no other peaks were detected. A series of dilutions were prepared corresponding to 200 and 40 pg/nl, and 800 and 160 fg/nl. Peaks corresponding to **1, 3, 6,** and **9** were observed in all but the last dilution. The peak area of each component is plotted as a function of the amount analyzed in Fig. 5. Trisulfated disaccharide **9,** giving a short broad peak, is detected with greater sensitivity than disaccharides **1, 3,** and **6,** having reduced sulfation and giving tall narrow peaks. A plot of peak area as a function of amount in femtomoles would further accentuate these sensitivity differences. The detection sensitivity was 400 fg corresponding to from 500 amol to 1 fmol. In complex mixtures, the conditions giving the best overall separation may result in some components being insufficiently separated to obtain good quantitation (i.e., **7** and **8,** Fig. 2). In such cases, it may be necessary to adjust conditions to optimize the separation of these individual components.

In conclusion, this paper demonstrates that it is possible to separate all the lyase-produced chondroitin sulfate, dermatan sulfate, and hyaluronic acid disaccharides in a single analysis. Preliminary studies have been performed on crude chondroitin sulfate prepared from

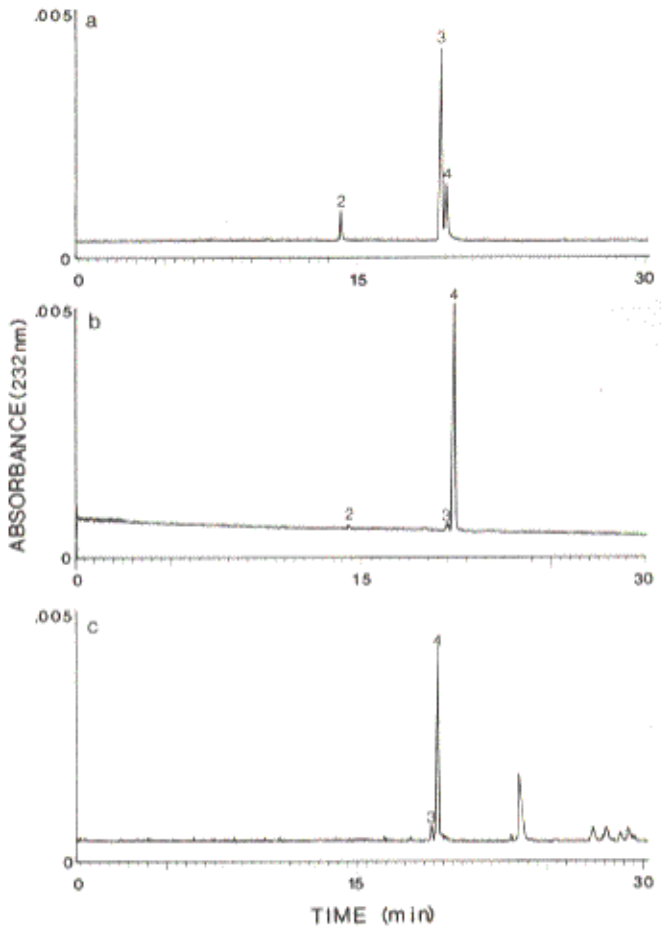

FIG. 4. Electropherograms showing the analysis of 1 nl containing 1 ng of chondroitin ABC lyase depolymerized chondroitin-6-sulfate (a), chondroitin-4-sulfate (b), and dermatan sulfate (c).

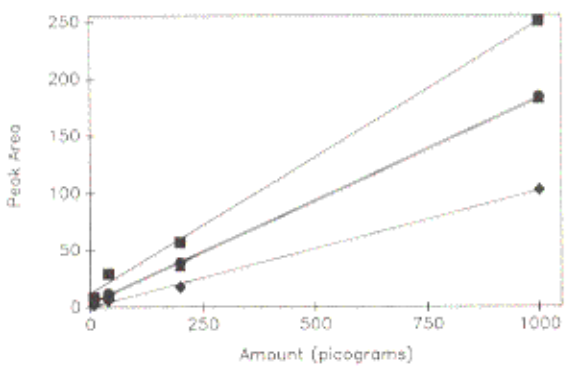

FIG. 5. Standard curves for the analysis of a mixture of disaccharides **1** (♦), **3** (▲), **6** (●), and **9** (■) showing peak area as a function of amount in nanograms. Linear regression gave slope, intercept and r values for **1, 3, 6,** and **9** of: 0.102, 0.434, 0.999; 0.179, 2.98, 0.999; 0.179, 4.40, 0.999; and 0.238, 12.13, 0.999.

bovine lung acetone powder by pronase treatment followed by ion-exchange on a Dowex resin and dialysis. Treatment with chondroitin ABC lyase results in monosulfated disaccharides detected by CZE demonstrating that this method is applicable on relatively crude samples provided they are salt free. This method may also be useful in the analysis of other GAGs by first treating these with the appropriate polysaccharide lyase. Finally, the sensitivity of detection of this method surpasses all other analytical methods previously described.

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