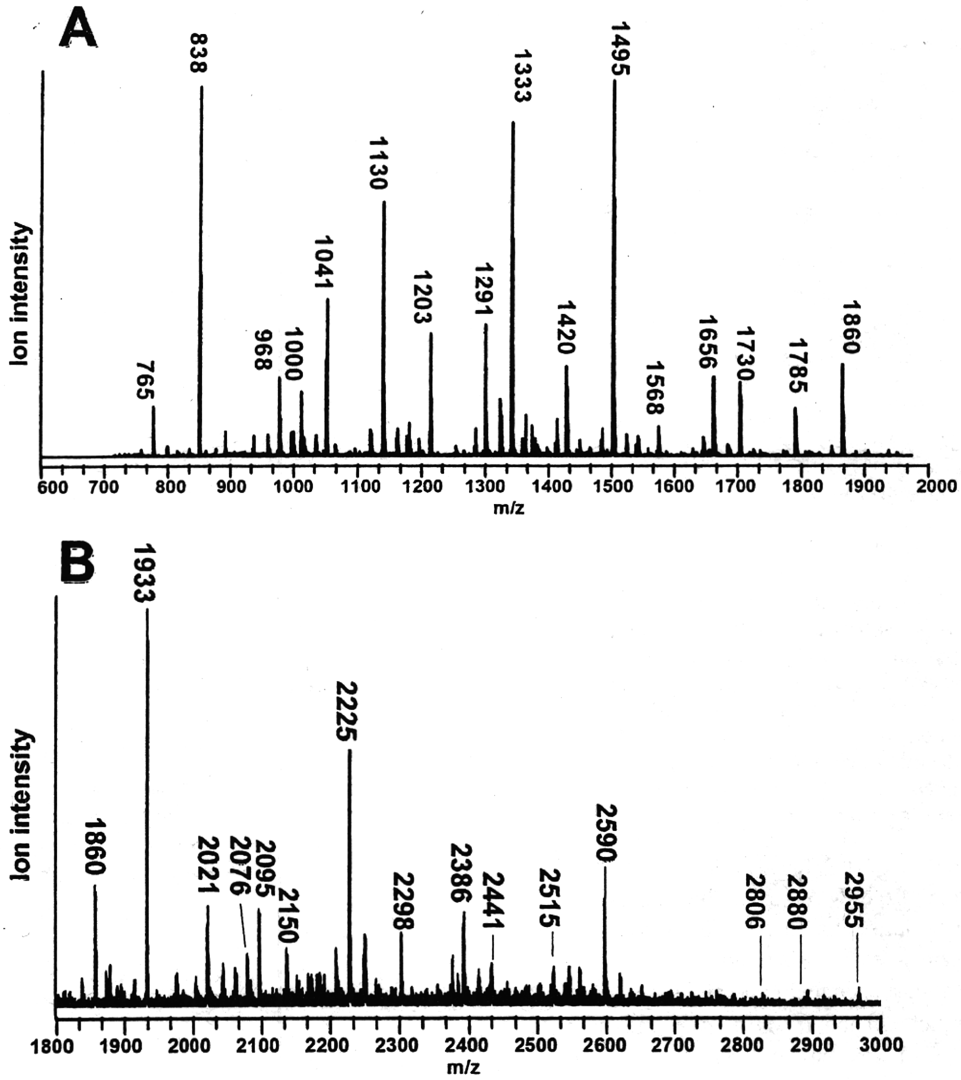

Figure 5. Analysis of O-glycans from MKN45 cells. The results for the low- and high-mass ranges are shown in parts A and B, respectively.

remove the excess phenylhydrazine by washing. Thus, the whole procedure from the releasing of O-glycans to MS measurement was completed within 1.5 h.

On the basis of the studies described above, we applied the present technique to the analysis of O-glycans from bovine submaxillary mucin (BSM). The results are shown in Figure 4.

When using 25 μg of BSM (2.5 μg as the injected amount), we could easily observe the major molecular ions due to O-glycans. Two molecular ions observed at m/z 603 and m/z 619 are due to NeuAcα2-6GalNAc=PHN and NeuGcα2-6GalNAc=PHN, respectively. Two molecular ions observed at m/z 806 and 822 are confirmed as sialyl-core 3 structures, GlcNAcβ1-3(NeuAcα2−6) GalNAc=PHN and GlcNAcβ1-3(NeuGcα2-6) GalNAc=PHN, respectively. Sialic acids of O-glycans in BSM have two forms of *N*-acetyl neuraminic acid and *N*-glycolyl neuraminic acid. Therefore, we could observe two sets of the molecular ions of O-glycans.[11] Even at the 1 μg-level of the sample (0.1 μg as the amount injected to the MALDI plate), we clearly observed the molecular ions of these glycans.

Application to the Analysis of O-Glycans Derived from Cancer Cells. We applied the present method to the analysis of O-glycans expressed on MKN45 cells (a human stomach cancer cell line). O-Glycans of glycopeptide fractions obtained from the whole cells after digestion with Pronase were analyzed by the present method. The results observed for low- and high-molecular ranges are shown in parts A and B of Figure 5, respectively, and the list of the proposed structures based on our previously reported data is summarized in Table 1.[18]

Two molecular ion peaks observed at m/z 838 and m/z 1041 are due to asialo core 2 type structures, Galβ1-3(Galβ1-4GlcNAcβ1-6)-GalNAc=PHN and Galβ 1-3(GlcNAcβ- Galβ1-4GlcNAcβ1-6)-GalNAc=PHN, respectively. Four molecular ion peaks at m/z 1203, 1568, 1933, and 2298 are confirmed as asialo polylactosamine-type O-glycans, Galβ1-3((Galβ-GlcNAcβ)$_n$-Galβ1-4-GlcNAcβ1-6) GalNAc=PHN ($n = 1-4$). The molecular ion peak observed at m/z 765 corresponds to sialyl-T, NeuAcα2-3Galβ1-3GalNAc=PHN. Three molecular ion peaks observed at m/z 968, 1130, and 1333 are assigned as monosialo core 2 type glycans, Galβ1-3(GlcNAcβ1-6)GalNAc=PHN + NeuAc$_1$, Galβ1-3(Galβ1-4GlcNAcβ1-6) GalNAc=PHN + NeuAc$_1$, and Galβ1-3(GlcNAcβ-Galβ1-4GlcNAcβ1-6)GalNAc=PHN + NeuAc$_1$, re-

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(24) Lattova, E.; Kapkova, P.; Krokhin, O.; Perreault, H. *Anal. Chem.* 2006, *78*, 2977–2984.

Table 1. O-Glycans Observed in MKN45 Cells

proposed structures	observed molecular mass (m/z)
Asialo Glycans	
Galβ1-3(Galβ1-4GlcNAcβ1-6)GalNAc=PHN	838
Galβ1-3(GlcNAc-Galβ1-4GlcNAcβ1-6)GalNAc=PHN	1041
Galβ1-3(Gal-GlcNAc-Galβ1-4GlcNAcβ1-6)GalNAc=PHN	1203
Galβ1-3((Gal-GlcNAc)$_{2}$-Galβ1-4GlcNAcβ1-6)GalNAc=PHN	1568
Galβ1-3((Gal-GlcNAc)$_{3}$-Galβ1-4GlcNAcβ1-6)GalNAc=PHN	1933
Galβ1-3((Gal-GlcNAc)$_{4}$-Galβ1-4GlcNAcβ1-6)GalNAc=PHN	2298
Monosilao Glycans	
NeuAcα2-3Galβ1-3GalNAc=PHN	765
Galβ1-3(GlcNAcβ1-6)GalNAc=PHN + NeuAc$_{1}$	968
Galβ1-3(Galβ1-4GlcNAcβ1-6)GalNAc=PHN + NeuAc$_{1}$	1130
Galβ1-3(GlcNAc-Galβ1-4GlcNAcβ1-6)GalNAc=PHN + NeuAc$_{1}$	1333
Galβ1-3(Gal-GlcNAc-Galβ1-4GlcNAcβ1-6)GalNAc=PHN + NeuAc$_{1}$	1495
Galβ1-3((Gal-GlcNAc)$_{2}$-Galβ1-4GlcNAcβ1-6)GalNAc=PHN + NeuAc$_{1}$	1860
Galβ1-3((Gal-GlcNAc)$_{3}$-Galβ1-4GlcNAcβ1-6)GalNAc=PHN + NeuAc	2225
Galβ1-3((Gal-GlcNAc)$_{4}$-Galβ1-4GlcNAcβ1-6)GalNAc=PHN + NeuAc	2590
Galβ1-3((Gal-GlcNAc)$_{5}$-Galβ1-4GlcNAcβ1-6)GalNAc=PHN + NeuAc	2955
Disialo Glycans	
NeuAc-Galβ1-3(NeuAc-Galβ1-4GlcNAcβ1-6)GalNAc=PHN	1420
NeuAc-Galβ1-3(NeuAc-Gal-GlcNAc-Galβ1-4GlcNAcβ1-6)GalNAc=PHN	1785
NeuAc-Galβ1-3(NeuAc-(Gal-GlcNAc)$_{2}$-Galβ1-4GlcNAcβ1-6)GalNAc=PHN	2150
NeuAc-Galβ1-3(NeuAc-(Gal-GlcNAc)$_{3}$-Galβ1-4GlcNAcβ1-6)GalNAc=PHN	2515
NeuAc-Galβ1-3(NeuAc-(Gal-GlcNAc)$_{4}$-Galβ1-4GlcNAcβ1-6)GalNAc=PHN	2880
Trisialo Glycans	
NeuAc-Gal-GlcNAc-(NeuAc-Gal-GlcNAc)Galβ1-3(NeuAcα2-6)GalNAc=PHN	2076
NeuAc-Gal-GlcNAc-(NeuAc-Gal-GlcNAc)Galβ1-3(NeuAcα2-6)GalNAc=PHN + Gal-GlcNAc	2441
NeuAc-Gal-GlcNAc-(NeuAc-Gal-GlcNAc)Galβ1-3(NeuAcα2-6)GalNAc=PHN + (Gal-GlcNAc)$_{2}$	2806
Degradation Product	
(Gal-GlcNAc)$_{2}$-Gal=PHN	1000
NeuAc-(Gal-GlcNAc)$_{2}$-Gal=PHN	1291
NeuAc-(Gal-GlcNAc)$_{3}$-Gal=PHN	1656
(Gal-GlcNAc)$_{4}$-Gal=PHN	1730
NeuAc-(Gal-GlcNAc)$_{4}$-Gal=PHN	2021
(Gal-GlcNAc)$_{5}$-Gal=PHN	2095
NeuAc-(Gal-GlcNAc)$_{5}$-Gal=PHN	2386

spectively. Five molecular ion peaks at m/z 1495, 1860, 2225, 2590, and 2955 are due to monosialo polylactosamine-type O-glycans, Galβ1-3((Galβ-GlcNAcβ)$_{n}$-Galβ1-4GlcNAcβ1-6)GalNAc=PHN + NeuAc$_{1}$ ($n = 1-5$). Disialo polylactosamine-type O-glycans were also observed in MKN45 cells. Four molecular ion peaks at m/z 1785, 2150, 2515, and 2880 are confirmed as disialo core 2 type glycans modified with 1−7 units of lactosamine residues. Three molecular ion peaks containing three sialic acid residues were observed at m/z 2076, 2441, and 2806 are due to NeuAc$_{3}$Hex$_{3}$HexNAc$_{3}$=PNH, NeuAc$_{3}$Hex$_{4}$HexNAc$_{4}$=PNH, and NeuAc$_{3}$Hex$_{5}$HexNAc$_{5}$=PNH, respectively. As reported previously, these trisialo glycans are core 1 type glycans having the structures as indicated below.[18]

NeuAcα(Galβ1-4GlcNAcβ)nNeuAcα2-6GalNAc
NeuAcα(Galβ1-4GlcNAcβ)mGalβ1-3

These structures were observed exclusively in MKN45 cells according to our recent research results, and we suggested that these O-glycans might be a specific marker of gastric adenocarcinoma.[18] Seven molecular ion peaks observed at m/z 1000, 1291, 1656, 1730, 2021, 2095, and 2386 are due to the degradation products from these trisialo core 1 type polylactosamine glycans, NeuAc-(Galβ-GlcNAcβ)$_{n}$-Gal=PHN ($n = 1-5$), and the presence of these products means that these glycans occupy the 1-3 linked arm of the glycans because of easy degradation (i.e., peeling). Therefore, these glycans are important keys for structural confirmation of the parent glycans.[18]

Analysis of O-Glycans Derived from Serum Glycoproteins Using AGC-MS System. Another application to the analysis of O-glycans in a serum sample is shown in Figure 6. After brief deionization of the diluted solution using an ultramembrane filter, the solution was easily analyzed by the present system. As indicated in Figure 6A, several molecular ions were observed. The molecular ion peaks observed at m/z 765 with sodium adduct ion (m/z 787) are confirmed as sialyl-T.

The molecular ion peak observed at m/z 1056 corresponds to disialyl-T. The molecular ion peaks observed at m/z 562 with sodium adduct ion m/z 584 are due to a peeling product (NeuAc α2-3Gal=PNH). After the releasing reaction is performed in the flow of an aqueous LiOH solution, the reaction mixture is passed through a cartridge packed with cation exchange resin. Therefore, the effluent from the device does not contain cation(s) as examined by the absence of Li adduct ion in the MS spectra. Then, the effluent from the device is mixed with diluted phenylhydrazine solution and spotted onto the MALDI plate. Accordingly, the phenylhydrazone on the MALDI plate theoretically only shows protonated ions. In this case, however, diluted phenylhydrazine solution probably contains a trace amount of sodium ion, and the MS gives sodium adduct ions as well as protonated ions.

To confirm these peaks, we also analyzed the O-glycans obtained from the split effluent (collected by the fraction collector)

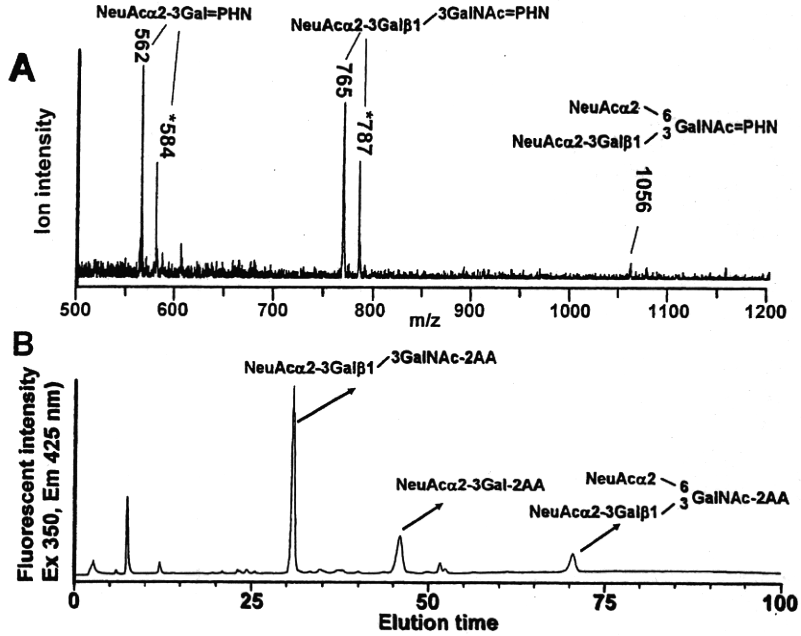

Figure 6. Analysis of O-glycans in a pooled serum sample. (A) MS analysis of the O-glycans by the present method. (B) Analysis of the O-glycans in a pooled serum sample by HPLC. The nine tenth portions of the O-glycans released by the autoglycan releasing system-MS system were labeled with 2-AA and analyzed by HPLC. Analytical conditions for HPLC: column, Asahi Shodex NH2P-50 4E (4.6 mm × 250 mm); eluent, solvent A, 2% CH_3COOH in acetonitrile; solvent B, 5% CH_3COOH/3% triethylamine in water, gradient condition, linear gradient (30−95% solvent B) from 2 to 82 min, maintained for 20 min.

as 2-AA derivatives using HPLC (Figure 6B). Each of the peaks was collected and compared with the standard samples by MALDI-TOF MS as reported previously.[18] The most abundant peak observed at 31 min is due to sialyl-T which shows the molecular ion at m/z 765. The peak observed at 71 min is derived from disialyl-T which shows the molecular ion at m/z 1085. We also observed the peak due to the peeling product (NeuAcα2-3Gal-2AA) at 45 min. These results were in good agreement with the direct MS analysis as shown in Figure 6A, although the relative abundances of these glycans are different between HPLC and MS methods. The HPLC/CE-fluorescent detection method affords accurate amounts of the glycans based on the fluorescent intensity of the 2-aminobenzoic acid residue at the reducing terminal. A combination of both methods will be a powerful tool for accurate diagnosis of the disease or disease states.

CONCLUSIONS

We have been developing an automatic device for releasing O-glycans from the mucin-type glycoproteins.[17−19] In the present paper, we connected the device to a spotter machine for MALDI MS measurement and attempted direct measurement of O-glycans. Because we could not observe the molecular ions of free glycans by direct measurement due to their low sensitivity and the presence of contaminated materials during releasing reactions, we labeled the released O-glycans with phenylhydrazine by *in situ* derivatization to achieve highly sensitive detection of O-glycans by MS measurement. The derivatization reaction proceeded under mild conditions even in the presence of 2,5-dihydroxybenzoic acid (matrix material). Accordingly, we performed the analysis from the glycan releasing reaction to MS measurement within 1.5 h.

The system allows MS analysis of O-glycans of bovine submaxillary mucin even when using 1 μg of the protein sample (actual sample amount 100 ng). We applied the present technique to the analysis of the O-glycans expressed on MKN45 cells derived from human stomach adenocarcinoma and found that trisialo-O-glycans were present abundantly as reported previously.[18] In addition, O-glycans in a pooled serum sample were also successfully analyzed. It is well-known that quantitative analysis is often difficult in MS measurement. Fluorescence detection by HPLC/CE gives robust and reproducible results in quantitative analysis. The data in Figure 6 indicate that the proposed method using the hyphenated glycan-releasing and MS analysis shows similar glycan profiles with those obtained by HPLC, but the relative abundances of the glycans are somewhat different from those observed by HPLC/CE analysis. However, extremely high-throughput characteristics of the present method will be quite important in routine analysis of glycans for clinical use. We believe that the present technique is the primary attempt to use MS measurement for routine clinical diagnostic works.

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Determination of sulfate ester content in sulfated oligo- and poly-saccharides by capillary electrophoresis with indirect UV detection

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ABSTRACT: Carbohydrates having sulfate groups such as glycosaminoglycans and chemically synthesized sucrose sulfate show interesting and important biological activities. We adapted CE with indirect UV detection technique to the determination of sulfate ester in sulfated carbohydrates, which were previously hydrolyzed with HCl. The liberated sulfate ion was analyzed using a background electrolyte consisting of triethanolamine-buffered chromate with hexamethonium bromide. Sulfate contents of glucose 3-sulfate and sucrose octasulfate used as a model were in good agreement with theoretical values (accuracy, 95.9–96.7 and 97.4–101.9%, respectively), and relative standard deviation values run-to-run were 0.977 and 1.90%, respectively. We applied the method to the determination of the sulfate contents of some glycosaminoglycan samples and showed that the contents were in good agreement with those calculated from sulfur content. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: sulfate; glycosaminoglycans; capillary electrophoresis indirect UV detection

Introduction

Sulfation of hydroxyl and amino groups ($-O-SO_3H$ and $-NH-SO_3H$) is one of the common modifications of carbohydrates and is often observed in various glycoconjugates such as proteoglycans or mucin-like glycoproteins. Sulfated carbohydrates are widely distributed in animals as the major constituents of proteoglycans and are biologically active molecules involved in various biological events (Hooper *et al.*, 1996; Honke and Taniguchi, 2002; Wu, 2006). Functions of sulfated carbohydrates strongly depend on the presence and spatial positioning in the molecules. Degree of sulfation on carbohydrates is also closely related to biological activities such as blood coagulation, signal transduction and cell–cell interaction (Lindahl *et al.*, 1983; Villanueva, 1984; Hemmerich and Rosen, 1994; Small *et al.*, 1996; Tsuboi *et al.*, 1996). Chemical sulfation of carbohydrates often affords compounds showing novel biological activities such as anti-HIV activities (Katsuraya *et al.*, 1994, 1999; Yoshida *et al.*, 1995; Hattori *et al.*, 1998). Sulfated carbohydrates also have potential as pharmaceuticals (Werz and Seeberger, 2005). Sucrose octasulfate, 'Sucralfate' and the chemically synthesized octasulfated pentasaccharide 'Arixtra' are used as antiulcer and anticoagulant drugs, respectively (Candelli *et al.*, 2000; Giangrande, 2002).

In view of these interesting features of sulfated carbohydrates, assessment of sulfate content is important not only for the understanding of their biological significance but also the development and manufacturing of novel bioactive sulfated carbohydrates. Several methods have been developed for the determination of sulfate content of carbohydrates. Classical methods are based on the colorimetric determination of the inorganic sulfate ion liberated from sulfated carbohydrates by acid hydrolysis, such as chelating barium ions with rhodizonate (Terho and Hartiala, 1971; Roy and Turner, 1982). Srinivasan *et al.* achieved the determination of microgram quantity of sulfate ion based on the formation of stable complex of sulfate ester with *n*-butylamine and achieved determination of microgram quantity of sulfate ion (Srinivasan *et al.*, 1970). Unfortunately, these methods are not suitable for the determination of a small amount of sulfate ester in complex carbohydrate, because they are time-consuming and not sensitive enough, require a significant amount of material, and are prone to interference from the other ions. Compared with these conventional methods, ion chromatography (IC) demonstrates increased specificity and sensitivity

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Abbreviations used: BGE, background electrolyte; CSA, chondroitin sulfate A; DMF, *N,N*-dimethylformamide; DS, dermatan sulfate; GAG, glycosaminoglycan; HA, hyaluronic acid; HMB, hexamethonium bromide; HP, heparin; HS, heparan sulfate; TBA, tributylamine; TEA, triethanolamine.

as well as the inherent ability for the determination of various inorganic ions, and has been applied to the analysis of the ions in the samples from biological, environmental and industrial origins (Lopez-Ruiz, 2000). The sulfate contents in glycoproteins or GAGs were successfully determined by IC (Cole and Evrovski, 1997; Toida *et al.*, 1999). Toida *et al.* liberated the sulfate ion from chemically O-sulfated GAGs by acid hydrolysis, and determined it by a combination of IC and conductivity detection (Tadano-Aritomi *et al.*, 2001).

Capillary electrophoresis (CE) is a powerful tool for separation of inorganic ions with high resolving power. Its performance is comparable with that of IC, and has become one of the standard tools for the analysis of inorganic ions in environmental, biomedical, clinical and industrial samples (Fritz, 2000; Timerbaev, 2002, 2004; Johns *et al.*, 2003; Pacakova *et al.*, 2003; Paull and King, 2003). CE allows rapid analysis with high resolution and exhibits good capabilities in quantitative analysis, making it well suited for routine analysis of sulfate content of carbohydrates. Although the detection in CE is usually performed by direct UV detection, most inorganic ions lack a chromophore and cannot be detected using common direct UV detection. Therefore, indirect UV detection technique is usually used for determination of inorganic ions. Indirect UV detection adds an UV-absorbing co-ion (called the probe) to the background electrolyte (BGE) and this probe is displaced by migration, causing a negative signal. Indirect UV detection is an effective alternative detection technique for inorganic ions. The attractive performance of the CE method has been employed for the assay of sulfotransferase activity (Saillard *et al.*, 1999). Thus, CE is considered a useful alternative to the well-established IC method for routine analysis of sulfate content of carbohydrates.

In the present study, we developed a method using capillary electrophoresis with indirect UV detection to the determination of sulfate content of sulfated oligo-/polysaccharides, and applied the method for the determination of sulfate content in some sulfated GAGs and the monitoring of chemically sulfation reaction of polysaccharides. The present method will provide a robust method for the analysis of sulfated carbohydrates using routinely available laboratory instrumentation.

Experimental

Materials

Hexamethonium bromide, glucose 3-sulfate (sodium salt, 98% purity by HPLC), sucrose octasulfate (sodium salt) and heparin from bovine intestinal mucosa were purchased from Sigma (St Louis, MO, USA). Hyaluronic acid (from *Streptococcus zooepidemicus*) was purchased from Nacalai tesque (Uji, Kyoto, Japan). Chondroitin sulfate A (from whale cartilage), dermatan sulfate (from pig skin) and heparan sulfate (from bovine kidney) were purchased from Seikagaku Biobusiness (Chiyoda-ku, Tokyo, Japan). Tributylamine (TBA), *N,N*-dimethylformamide (DMF) and pyridine–sulfur trioxide complex were obtained from Wako Pure Chemicals (Dosho-machi, Osaka, Japan). All other chemicals and reagents were of the highest grade or HPLC grade. Running buffers and aqueous solutions were prepared with water purified with a Milli-Q purification system (Millipore, Bedford, MS, USA).

Sulfation of Chondroitin Sulfate A

Chemical sulfation of chondroitin sulfate A was performed according to the method reported by Maruyama *et al.* (1998). The sodium salt (10 mg) of chondroitin sulfate A (from whale cartilage) was dissolved in 1 mL of 5% TBA–HCl water (pH 2.8), and then the solution was lyophilized to dryness to give the tributylammonium salt. The salt was dissolved in 1 mL of DMF, and pyridine–sulfur trioxide complex (10, 50, 100 and 250 mg) was added. After incubating the mixture for 1 h at 40°C, the reaction was terminated by addition of water (1 mL). The reaction product was precipitated with cold ethanol (6 mL) saturated with anhydrous sodium acetate, collected by centrifugation at 4°C, then dissolved in water followed by dialysis against water to remove salts and lyophilized. We obtained $PSCS_{10}$ (11.8 mg), $PSCS_{50}$ (14.1 mg), $PSCS_{100}$ (17.2 mg) and $PSCS_{250}$ (18.6 mg), respectively, by changing the amount of pyridine–sulfur trioxide complex.

Sample and Standard Solutions

Standard solutions of sulfate ion were prepared by dissolving an accurately weighed amount of sodium sulfate (300 mg) in water (10 mL; 210 mM). A series of standard solutions of sulfate ion for calibration curve were prepared by appropriate dilution of the standard solution with water. Sample solution of sulfated oligo-/polysaccharides was also prepared by dissolving an accurately weighed amount (1.00 mg) in of water (1 mL).

CE Analysis of Sulfate Ion with Indirect UV Detection

CE was performed with a Beckman P/ACE MDQ system equipped with a UV detector (Beckman Coulter, Fullerton, CA, USA). A fused silica capillary (50 μm i.d., 56 cm effective length, 66 cm total length, from Agilent Technologies) was used throughout the work. The background electrolyte was composed of 10 mM CrO_3–2 mM hexamethonium bromide in 10% MeOH–water (pH 8.0) adjusted with triethanolamine. The background electrolyte was passed through a cellulose acetate membrane filter (0.2 μm). Prior to the first run, the capillary was rinsed with 0.1 M NaOH for 10 min, followed by washing with water for 10 min, and then filled with the background electrolyte. The capillary was conditioned by pre-electrophoresis (−20 kV) for 10 min. After washing the capillary with water and filling with the background electrolyte, samples were automatically injected using pressure injection mode at 1.0 psi for 10 s. Electrophoresis was performed at −20 kV using reverse polarity. Detection was carried out with monitoring the UV absorption at 254 nm. The negative peaks due to the presence of anions in the background of CrO_3 were automatically converted into positive peaks by Beckman 32 Karat software version 4.0 (Beckman Coulter).

Hydrolysis of Sulfated Carbohydrates

A standard solution (20 μL) of glucose 3-sulfate was mixed with 20 μL of 1 M HCl and the mixture was kept at 100°C in a polypropylene tube for specified times. After cooling the mixture to room temperature, the solution (40 μL) was diluted with 1000 μL of water. An aqueous solutions (1000 μL) of $NaNO_3$ was added to the mixture as internal standard and an aliquot (20 μL) was used for the analysis of sulfate ion.

Standard Procedure for the Determination of Sulfate Content in Sulfated Oligo- and Polysaccharides

A solution (20 μL) of sodium salt of sulfated oligo- or polysaccharides was mixed with 20 μL of 1 M HCl, and the mixture was kept at 100°C for 2 h. After cooling the mixture to room temperature, water and the internal standard solutions were added to the mixture as described above and an aliquot was used for the determination of sulfate ion. The content of sulfate ion was calculated from the calibration curve obtained from standard solutions of Na_2SO_4. The percentages of sulfate content were calculated using the following equation:

$$SO_3\,(w/w) = (\text{wt of } SO_4^{2-} \times (\text{wt of } SO_3/\text{wt of } SO_4)/\\ \text{wt of sample}) \times 100$$

where wt (weight) of $SO_3 = 80$, wt of $SO_4^{2-} = 96$, wt of SO_4^{2-} (μg) in hydrolysates is the amount calculated using the calibration curve, and wt of the sample is the amount of the sample in micrograms.

Results and Discussion

Principle

The method is based on the acid hydrolysis of sulfonic acid ester (-O-SO_3H and –NH-SO_3H) followed by the determination of the released sulfate ion (SO_4^{2-}) with CE with indirect UV detection technique. Sulfated oligo-/polysaccharides are hydrolyzed with HCl to produce SO_4^{2-}. In CE, indirect detection is conveniently available for the detection of compounds which do not have chromophores/fluorophores. When non-UV absorbing sulfate ion passes through the UV detector, the zone of SO_4^{2-} causes a negative signal in the background electrolyte containing a UV-absorbing compound as probe. The output polarity of the detection is reversed so that a positive peak is obtained. The content of sulfate ester in parent compounds is calculated using the calibration curve obtained from a standard solution of Na_2SO_4.

Selection of the Background Electrolyte

Selection of the electrolyte (e.g. co-ion and electroosmotic modifier) is important for the sensitive and quantitative determination of sulfate ion. In the present study, chromate ion was selected as the UV absorbing co-ion (probe ion) because of its ionic mobility being close to that of SO_4^{2-}, which ensures high peak symmetry (Johns *et al.*, 2003; Pacakova *et al.*, 2003). We had to pay attention on the presence of high excess amount of chloride ion (Cl^-) in the sample solutions due to the HCl employed for the hydrolysis of sulfate-containing carbohydrates. Inorganic anions by CE are usually analyzed under negative polarity using electroosmotic modifiers such as cationic surfactant, polymer and amines, which improve resolutions of the ions (Haddad *et al.*, 1999; Harakuwe *et al.*, 1999; Kaniansky *et al.*, 1999). Muzikar *et al.* (2003) reported the determination of trace amount of inorganic anions (e.g. SO_4^{2-} or NO_3^-) in the presence of large excess of Cl^- using an electrolyte consisting of triethanolamine (TEA)-buffered chromate with hexamethonium bromide (HMB) as electroosmotic modifier. In the present study, SO_4^{2-} (0.21 mM as Na_2SO_4) was successfully analyzed in the presence of 50 mM HCl using this condition, and Cl^- and SO_4^{2-} were completely resolved and observed at 4.50 and 4.85 min, respectively (Fig. 1a).

We employed nitrate ion (NO_3^-) as internal standard (50 µg/mL, 0.59 mM; Fig. 1b). Ions of SO_4^{2-} and NO_3^- with a huge amount of Cl^- were completely resolved within 5 min. Based on these results, 10 mM CrO_3–2 mM HMB in 10% MeOH–water (pH 8.0 adjusted with TEA) was selected as the background electrolyte throughout the present study.

Linearity and Limit of Detection

The calibration curve for absolute peak area of sulfate ion showed good linearity between 5.0 and 625 µg/mL ($y = 65.4x + 0.58$, $R = 0.9996$; Fig. 2a). In the case of correction of the injection amount by internal standard, the calibration curve exhibited excellent linearity ($y = 0.015x + 0.22$, $R = 0.9999$; Fig. 2b). Both lower limit of detection (LOD) and lower limit of quantification (LOQ) were evaluated on the basis of the standard deviation (σ) and slope (S) from the calibration curve of SO_4^{2-}. In the present conditions, LOD ($= 3\sigma/S$) and LOQ ($= 10\sigma/S$) were 0.934 and 3.113 µg/mL, respectively.

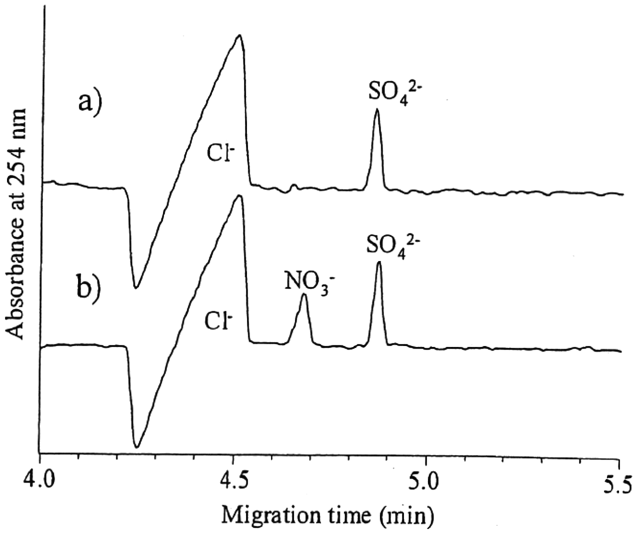

Figure 1. Separation of inorganic anions by CE with indirect UV detection: (a) 0.21 mM sulfate and 50 mM chloride; (b) 0.21 mM sulfate, 50 mM chloride, and 0.59 mM nitrate. Background electrolyte: 10 mM CrO_3/2 mM hexamethonium bromide in 10% MeOH–water at pH 8.0 adjusted with triethanolamine. Capillary: a fused silica capillary (i.d., 50 µm; effective length 56 cm). Applied voltage, –20 kV; temperature, 25°C; sample injection, hydrodynamic injection (1.0 psi, 10 s); detection, indirect UV absorption at 254 nm.

Reproducibility

Run-to-run reproducibility of migration times of SO_4^{2-} and NO_3^- was evaluated using a mixture of 30 µg/mL Na_2SO_4 and 50 µg/mL $NaNO_3$. Migration times of SO_4^{2-} and NO_3^- were 4.96 ± 0.08 and 4.68 ± 0.07, respectively. The relative standard deviation (RSD) was less than 1.4 and 1.6%, respectively ($n = 5$).

Precision

We obtained RSD values in absolute determination of SO_4^{2-} using standard solutions of Na_2SO_4 at 5, 30 and 312.5 µg/mL. The RSD (%) of SO_4^{2-} peak area were 4.58, 2.24 and 2.83%, respectively ($n = 5$; Table 1). In contrast, when using the internal standard (NO_3^-), the RSD (%) of SO_4^{2-} was 1.84, 0.69 and 1.69%, respectively ($n = 5$; Table 1).

Optimization of Conditions for Liberation of SO_4^{2-} by Acid Hydrolysis

Conditions for liberation of SO_4^{2-} from sulfated carbohydrates by acid hydrolysis with HCl were optimized using glucose 3-sulfate as model. After hydrolysis of glucose 3-sulfate with 1 M HCl at 100°C for specified intervals, a portion of the reaction mixture was diluted with water. An aqueous solution of the internal standard ($NaNO_3$) was added, and the released SO_4^{2-} was determined according to the conditions described above.

The content of SO_4^{2-} in the reaction mixture was dependent on hydrolysis time and the hydrolysis was completed within 2 h as shown in Fig. 3(a, b). The excess chloride ion and free glucose produced by hydrolysis in the mixture did not show interference in the determination of SO_4^{2-}. The amount of sulfate ion in glucose 3-sulfate was estimated as 28.1 w/w% (0.96 mol/mol), and showed good agreement with the theoretical values (28.4%). Recoveries were 95.9–96.7% ($n = 5$).

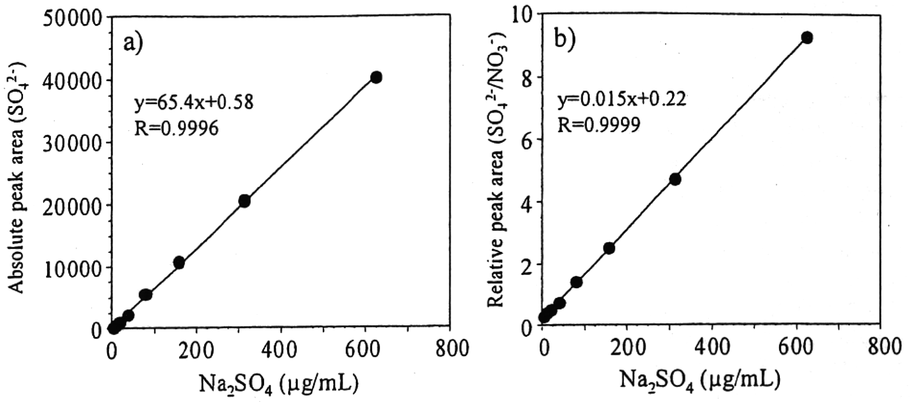

Figure 2. Calibration curve for determination of sulfate ions. (a) Concentration of Na_2SO_4 vs absolute peak area of SO_4^{2-}. (b) Concentration of Na_2SO_4 vs relative peak area (SO_4^{2-}/NO_3^-).

Table 1. Precision results of determination of sulfate ion at three different concentrations

Run	Peak area								
	5 μg/mL Na_2SO_4			30 μg/mL Na_2SO_4			312.5 μg/mL Na_2SO_4		
	SO_4^{2-}	NO_3^-	SO_4^{2-}/NO_3^-	SO_4^{2-}	NO_3^-	SO_4^{2}/NO_3^-	SO_4^{2-}	NO_3^-	SO_4^{2-}/NO_3^-
1	462	361	1.280	2755	2043	1.349	28041	21505	1.304
2	492	378	1.302	2647	1957	1.353	29945	22869	1.309
3	506	402	1.259	2798	2092	1.337	28762	22214	1.295
4	476	362	1.315	2695	1989	1.355	27945	22290	1.254
5	518	409	1.267	2679	2008	1.334	29013	22452	1.292
Average	491	382	1.284	2715	2018	1.346	28741	22266	1.291
SD	22.5	22.3	0.024	60.8	51.9	0.009	813.6	495.1	0.022
RSD%	4.58	5.83	1.838	2.24	2.57	0.687	2.83	2.22	1.694

We evaluated linearity, repeatability, precision and lower limit of detection using sucrose octasulfate. Sucrose octasulfate is a cytoprotective drug widely used to prevent or treat several gastrointestinal diseases such as gastro-esophageal reflux, gastritis, peptic ulcer, stress ulcer and dyspepsia (Lam and Ching, 1994; Candelli *et al.*, 2000). The sulfate content found in the hydrolysate of sucrose octasulfate showed a good linear relationship with sucrose octasulfate (0.03–1 mg/mL). The value for the relative standard deviation ($n = 5$) of determination of sucrose octasulfate was 1.90% at 250 μg/mL. The limit of detection was 7.8 μg/mL as a solution of sucrose octasulfate sodium salt. When a solution (250 μg/mL) of sucrose octasulfate sodium salt was used, the sulfate content of one batch was 53.1% (accuracy 97.4–101.9%, $n = 5$), which is very close to the theoretical value (54.2%).

Determination of Sulfate Content in Various GAG Samples

Glycosaminoglycans (GAGs) are a family of highly complex and polydisperse linear polysaccharides that display a variety of important biological roles (Jackson *et al.*, 1991; Scott, 1992; Bourin and Lindahl, 1993; Sugahara and Kitagawa, 2000). GAGs are categorized into some main structural groups: hyaluronic acid (HA), chondroitin sulfate A (CSA), dermatan sulfate (DS), heparin (HP) and heparan sulfate (HS) (Zaia, 2009). The structural complexity is compounded by their sequence heterogeneity, primarily caused by variation of the degree and position of sulfate groups. We applied the present method to the determination of sulfate content in some GAG samples. The results are shown in Fig. 4 and Table 2.

Among five GAG samples used in the study, CSA, DS, HP and HS showed sulfate contents of 14.2, 15.0, 25.1 and 11.5%, respectively. HP is mainly composed of trisulfated disaccharide units, α(1-4)-linked L-iduronic acid, which is 2-*O*-sulfated, and D-glucosamine, which is *N*- and 6-*O*-sulfated (Zaia, 2009). Therefore, the sulfate content of HP is higher than those of CSA and DS, which are sulfated at only 4-OH of GalNAc in the disaccharide unit. The sulfate content of HS is lower than those of other sulfated GAGs, because HS from bovine kidney contains unsulfated repeating disaccharide units (GlcAβ1–4GlcNAc) as the major component (~60%), and contains the monosulfated GlcA β1–4GlcNAc (~25%) and the di- or tri-sulfated IdoAα1–4GlcNAc (~15%) (Zaia, 2009). HA, composed of non-sulfated disaccharide units, does not contain sulfate. When hydrolysis step was not included, we did not observe sulfate ions in the electropherograms for all these GAG samples (data not shown). This indicates that inorganic sulfate ion was negligible in the sample. The sulfate contents found in five GAGs were in good agreement with those calculated from sulfur contents provided by the manufacturer (Table 2).

Application to the Monitoring of Chemical Sulfation of Chondroitin Sulfate

Chemical modification of polysaccharides such as sulfation affords novel biological activity, and has been well studied (Srinivasan *et al.*, 1970; Suzuki *et al.*, 2001). We synthesized some preparations of sulfated chondroitin sulfate having different degree of sulfation by changing the amount of pyridine–sulfur

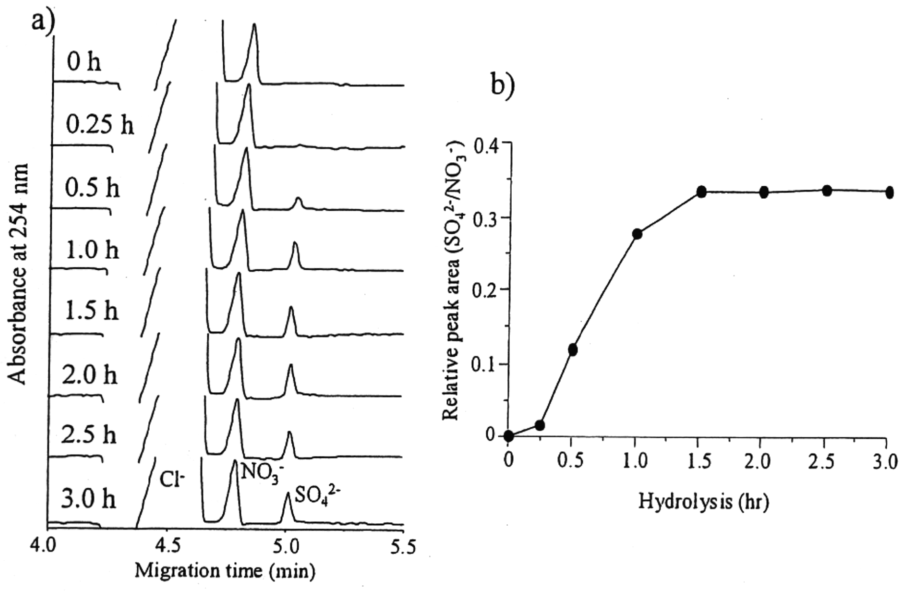

Figure 3. Time course of sulfate liberation during hydrolysis of the glucose 3-sulfate with HCl. (a) Electropherograms of the reaction mixture after hydrolysis. (b) Time course of liberation of sulfate ion from glucose 3-sulfate.

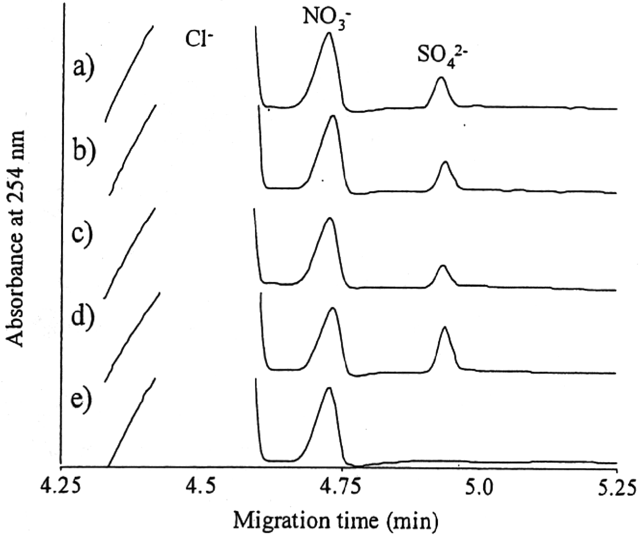

Figure 4. Determination of the sulfate content of some glycosaminoglycans. (a) Chondroitin sulfate A; (b) dermatan sulfate; (c) heparan sulfate; (d) heparin; and (e) hyaluronic acid.

Table 2. Sulfate contents of GAGs

GAGs	Total sulfate (%)[a]	
	Present method	Schoniger method[b]
Hyaluronic acid	n.d.[c]	<0.5
Chondroitin sulfate A	14.2 ± 0.5	15.0
Dermatan sulfate	15.0 ± 0.5	15.5
Heparin	25.1 ± 0.5	25.6
Heparan sulfate	11.5 ± 0.5	11.3

[a] Calculated from the dry weight of GAGs.

[b] Total sulfate contents were calculated from sufur contents provided by manufacturer.

[c] Not detected.

trioxide complex according to the method reported by Maruyama *et al.* (1998). The $PSCS_{10}$, $PSCS_{50}$, $PSCS_{100}$ and $PSCS_{250}$ were obtained from 10 mg CS using 10, 50, 100, and 250 mg pyridine–sulfur complex, respectively. Each preparation was analyzed by the present technique (Table 3).

Sulfate contents calculated from the preparations of $PSCS_{10}$, $PSCS_{50}$, $PSCS_{100}$ and $PSCS_{250}$ were 24.7, 38.5, 45.5 and 48.09%, respectively. The results showed that the amount of pyridine–surfur trioxide complex used in the reaction caused remarkable differences in the sulfate contents, and showed that the present method is useful for monitoring the degree of sulfation during chemical sulfation of oligo-/polysaccharides.

Conclusion

In the present study, we developed a simple, robust and reliable method for the determination of sulfate content in sulfated carbohydrates using CE with indirect UV detection. The background electrolyte consisting of TEA-buffered chromate with HMB is the most appropriate for the analysis of sulfate ion liberated from parent compounds after hydrolysis with HCl.

We applied the present method to the determination of sulfate content in some sulfated GAG samples such as chondroitin sulfate, dermatan sulfate, heparin and heparan sulfate. The sulfate contents found in these GAGs were in good agreement with those obtained by conventional methods. We also applied the method to the determination of sulfate content in chemically sulfated chondroitin sulfate, and revealed the degree of sulfation.

Easy operation of the proposed technique is useful for the determination of sulfate content of sulfated oligo-/polysaccharides. The present method is suitable for routine analysis of sulfate content of carbohydrates.

Table 3. Sulfate contents of chemically sulfated chondroitin sulfate

Sample	Amount of pyridine–sufur trioxide complex	Total sulfate content (%)	Degree of sulfation (%)[b]
CSA[a]	—	14.2	27.5
PSCS$_{10}$	10 mg	24.7	47.6
PSCS$_{50}$	50 mg	38.5	74.6
PSCS$_{100}$	100 mg	45.5	88.2
PSCS$_{250}$	250 mg	48.1	93.2

[a] Chondroitin sulfate A from whale cartilage.

[b] Relative percentage to theoretical value (51.6%) of fully sulfated CSA-30mer.

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