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Separation and determination of glycosaminoglycan disaccharides by micellar electrokinetic capillary chromatography for studies of pelt glycosaminoglycans

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ABSTRACT

Capillary electrophoresis based on cetyltrimethylammonium bromide micellar electrokinetic capillary chromatography (MECC) was developed for the separation and determination of glycosaminoglycan (GAG) disaccharide units without derivatization. The influence of changes in several separation conditions was studied, and the separation mechanisms are discussed. Tests of repeatability and linearity were performed for qualitative and quantitative evaluation of the method. The described procedure gives a rapid and efficient determination of GAG disaccharides. Samples of chondroitin sulphates and mink skin were treated with proteases, and the extent of protein cleavage was followed by free zone capillary electrophoresis. The result of the chondroitinase ABC treatment following the protease treatment was evaluated by the MECC method.

INTRODUCTION

Proteoglycans, which are highly glycosylated glycoproteins with distinctive features of the carbohydrate parts, are found in animal connective tissues. The carbohydrate part consists of oligosaccharides and polysaccharides or glycosaminoglycans (GAGs) covalently attached to the polypeptide core of the proteoglycan through a trisaccharide unit. Structural studies of intact proteoglycan molecules indicate that different types of GAG chains can be attached to a single polypeptide, and that large numbers of oligosaccharide units may also be attached to the same core protein [1]. The GAGs contain repeating disaccharide subunits generally consisting of a uronic acid linked to a hexosamine and with various numbers of sulphate groups attached to either the uronic acid or the hexosamine [1,2]. Classification of proteoglycans is based on the structure of the peptide core and the GAG side-chains [3]. The hyaluronate and chondroitin sulphate species have fairly simple structures whereas others, such as dermatan sulphate, heparin sulphate and other heparin species, may contain a large number of different disaccharide units, arranged either in block structures or in less well ordered complex sequences [2].

The GAGs are essential for maintaining the structural integrity of many connective tissues. In addition, their ability to bind water and micro-ions and steric exclusion of some macromolecules may also be of importance [2]. Although

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no detailed structure–function relationships have been found, the presence of sulphate GAGs has been related to normal collagen fibrillogenesis with some influence on the diameter and size of the collagen fibres [4,5]. When the unsulphated hyaluronic acid is the only GAG present in the dermis, thinning of the hair occurs [6], and when the amount of sulphate GAGs increases in the skin, *e.g.*, after testosterone treatment, thickening of the hair occurs [7].

Studies of the cyclic hair growth of various species of animals have revealed that in connection with anagen there is an increase in the amount of sulphated GAGs in skin [8], whereas in telogen there is an increase in the amount of non-sulphated GAGs [9]. Further, the ratio of 4- and 6-sulphated isomers of chondroitin varies with age and in various pathological states [10]. The properties and functions of GAGs make these compounds interesting in connection with the properties and quality of fur animal pelts. Exact structural information on GAGs in skin is therefore needed.

Determinations of individual GAG disaccharides have been based on techniques such as paper chromatography, TLC, paper or cellulose acetate membrane electrophoresis, LC and HPLC [1,11–16]. HPLC methods are usually superior to the other techniques. However, the disadvantages of HPLC include large sample volumes, large solvent volumes, usually long-term analyses, expensive columns, long equilibration and regeneration times of columns and an inability to resolve some of the isomers.

Recently, high-performance capillary electrophoresis (HPCE) as free zone or micellar electrokinetic capillary chromatography (MECC) with sodium dodecyl sulphate (SDS) has been introduced to separate monosaccharides, disaccharides and oligosaccharides with or without derivatization [10,17–21]. However, the published methods using free zone or MECC with SDS do not separate all GAG disaccharides well enough. MECC based on ion pairing and hydrophobic interaction with positively charged cetyltrimethylammonium bromide (CTAB) was very likely to improve the separation of the negatively charged GAG disaccharides, as has been shown for other negatively charged hydrophilic compounds in HPCE [22]. Further, it is preferable to avoid derivatization, because it is a further complication of the method and a time-consuming step. Underivatized and unsaturated GAG disaccharides are well suited for direct UV detection with a characteristic absorbance at 232 nm. Generally, HPCE techniques are rapid and inexpensive, have the potential for high resolution of analytes, require only small amounts of sample and inexpensive fused-silica capillaries are used [10,22,23].

This paper describes an efficient HPCE method based on MECC with CTAB, developed for the determination of individual GAG disaccharide isomers. The parameters studied include the evaluation of the effects of temperature, voltage and pH and CTAB, electrolyte and modifier concentration. Tests of linearity and repeatability were performed. Finally, results from enzymatic cleavages of GAGs from various chondroitins and mink skin and peptides from mink skin and the separation of the mixtures of GAG disaccharides obtained are described. The procedure developed gives a rapid and efficient determination of GAG disaccharides.

EXPERIMENTAL

Apparatus

An ABI Model 270 A and 270 A-HT capillary electrophoresis system (Applied Biosystems, Foster City, CA, USA) was used with a 750 mm × 50 μm I.D. Fused-silica capillary (J & W Scientific, Folsom, CA, USA). The detection point was 522 mm from the injection end of the capillary. Data processing was performed on a Shimadzu (Kyoto, Japan) Chromatopac C-R3A instrument.

Materials and reagents

Chondroitin disaccharides (sodium salts) Δ^4-GlcUA→GalNAc (ΔDi-OS; **1**), Δ^4GlcUA→4-O-sulpho-GalNAc (ΔDi-4S; **2**), Δ^4GlcUA→6-O-sulpho-GalNAc (ΔDi-6S; **3**), $\Delta^4$2-O-sulpho-GlcUA→4-sulpho-GalNAc (ΔDi-diS_B; **4**) (Fig. 1), chondroitin sulphate A from bovine trachea (*ca.* 70% and 30% C), chondroitin sulphate B from bovine mucosa (dermatan sulphate, *ca.* 85% and 15% A + C) and chondroitin sulphate

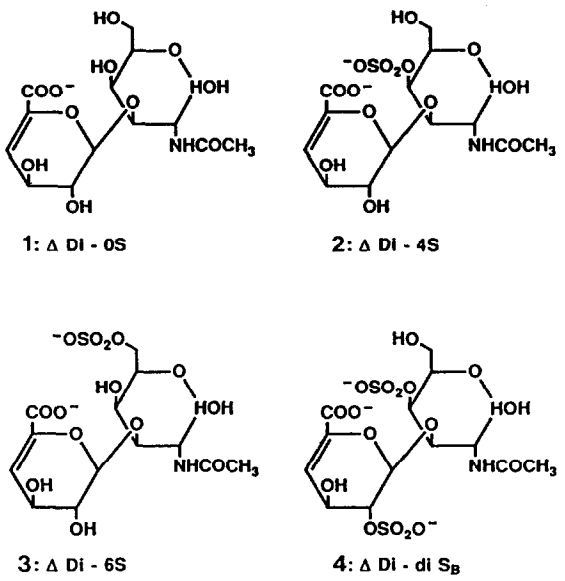

Fig. 1. Structures of the individual GAG disaccharides used in the HPCE analyses.

C from shark cartilage (*ca.* 90% and 10% A), and also chondroitinase ABC (chondroitin ABC lyase; EC 4.2.2.4) and trypsin were obtained from Sigma (St. Louis, MO, USA). Pepsin and pancreatin were purchased from Merck (Darmstadt, Germany). Skin samples were from the back of standard mink pelts, and the samples were obtained from the National Institute of Animal Science, Department of Research in Fur Animals, Foulum, Denmark. The samples were stored at −20°C until used.

Sodium tetraborate and sodium phosphate were obtained from Sigma and CTAB from BDH (Poole, UK). All chemicals were of analytical-reagent grade.

Procedure

Buffer preparations for the HPCE separations were performed according to Michaelsen *et al.* [22]. Samples were introduced from the cathodic end of the capillary by 1-s vacuum injection. Separations were performed at 30–60°C and 10–30 kV. On-column UV detection was at 232 nm unless indicated otherwise. Washing with buffer was done between each analysis for 5 min. After a number of analyses had been carried out, the capillary was washed for 5 min with 1.0 *M* NaOH and for 2 min with water.

Calculations of relative migration times with respect to **2** (*RMT*), normalized peak areas (*NA*), relative normalized peak areas with respect to **2** (*RNA*), resolution (R_s) and the number of theoretical plates (N) were performed according to Michaelsen *et al.* [22].

Enzyme treatments

Chondroitin sulphate A, B and C (1 mg of each) were treated with 0.30 mg of chondroitinase ABC in 100 μl of water plus 900 μl of 50 m*M* Tris–HCl (pH 8.0) buffer. The reaction mixtures were incubated at 37°C for 18 h and the reactions were stopped by transfering the mixtures into 100-μl Dowex 50W-X8 (H^+) columns and collecting the unretained solutes. Samples were then filtered through 0.2-μm filters, which were washed twice with 0.5 ml of water. The filtrates were either analysed directly by HPCE or evaporated to dryness and the residues dissolved in 100 μl water and analysed by HPCE.

Skin samples to be treated with chondroitinase were homogenized with an Ultra Turrax in 50 m*M* Tris–HCl (pH 8.0), enzyme was added and the mixture incubated at 37°C for 18 h. The reaction was stopped and the mixture filtered as described for chondroitin sulphate samples. Pepsin- and pancreatin-treated samples were prepared from skin samples homogenized with an Ultra Turrax in water (*ca.* 50 mg of skin per 2 ml), the pH of the solution was adjusted to 1.5 with HCl, pepsin (5.0 mg) was added and the mixture was incubated at 40°C for 60 min. Tris–HCl (50 m*M*, pH 8.0) was added to adjust the pH to 6.8, followed by pancreatin (5.0 mg) and the mixture (4 ml) was incubated at 40°C for 60 min. A 1-ml volume of this reaction mixture was mixed with 3.0 mg of chondroitinase and incubated at 37°C for 18 h. The reaction was stopped and the mixture filtered as described above for chondroitin sulphate samples. The samples were evaporated and the residues dissolved in one tenth the volume of water.

Fat extraction prior to protease and chondroitinase treatments was performed with either butanol, hexane or diethyl ether, and followed by either pepsin–pancreatin or trypsin treat-

ment. The extraction and homogenization of samples (*ca.* 20 mg) were performed with an Ultra Turrax for 2 min. Butanol (1.0 ml) or hexane (1.0 ml) was added to water (2.0 ml) before the pepsin (2.5 mg) and the pancreatin (2.5 mg) treatments (see above) and to 50 m*M* Tris–HCl (pH 8.0) (2.0 ml) before the trypsin (2.0 mg) treatment. Diethyl ether extractions (2 × 5 ml) of skin samples (*ca.* 20 mg) were performed with a glass spatula, and the samples were dried and homogenized in water or Tris–HCl as above. The aqueous phase was used for protease and chondroitinase treatments (see above). The hexane extraction in Tris–HCl of skin samples resulted in a hexane phase, a gel-like intermediate phase and the Tris–HCl aqueous phase. The gel-like phase and the Tris–HCl phase were treated separately with trypsin.

The protease treatments were followed by HPCE analyses of released peptides. The separation conditions were 20 m*M* citrate buffer (pH 2.5), temperature 30°C, voltage 25 kV, on-column UV detection at 200 nm, and sample introduction from the anodic end of the capillary by 1-s vacuum injection.

RESULTS AND DISCUSSION

The unsaturated uronic acid of the GAG disaccharide unit exhibits an absorbance maximum at 232 nm with a molar absorptivity of 5000–6000 l mol^{-1} cm^{-1} [19]. Various GAGs are found in different animal tissues such as skin, cartilage, bone, arterial walls and intestinal mucosa [2, 24]. The location of and the number of sulphate residues vary both between the GAGs and within the actual group of GAGs [2,24].

A systematic investigation of the influence of changes in the separation conditions on the migration times (*MT*), *RMT*, *NA*, *RNA*, R_s and *N* values were carried out. Chondroitin disaccharide standards **1–4** (Fig. 1) were dissolved in water and analysed under the initially applied separation conditions with an 18 m*M* borate and 30 m*M* phosphate buffer with 50 m*M* CTAB added and adjusted to pH 7.0, a temperature of 30 or 40°C and a voltage set at 20 kV. A complete separation of the disaccharides was obtained (Fig. 2).

The separation principles have been described by Michaelsen *et al.* [22] and Bjergegaard *et al.* [23]. Briefly, the CTAB–MECC separation is based on hydrophobic and ion-pairing interaction of the negatively charged disaccharides, the positively charged CTAB micelles and the CTAB-covered capillary wall. Further, complexation of some of the disaccharides may occur with the borate ions. Compared with published HPCE disaccharide methods, this CTAB system results in a reversal of the electroosmotic flow in the capillary.

Voltage

Increasing the applied voltage from 10 to 30 kV resulted in non-linear reductions in migration times of a factor of 4.5 (Fig. 3). The *MT* values of the compounds decreased with decreasing rate as the applied voltage was increased from 10 to 30 kV. An increase in the field strength is

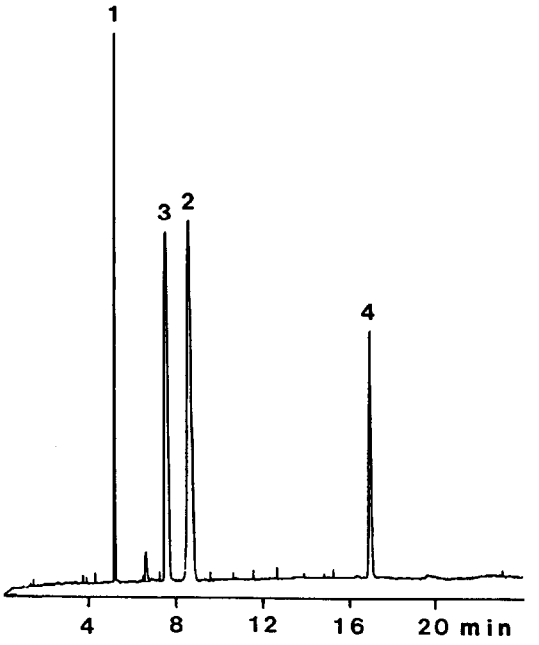

Fig. 2. Electropherogram of the mixture of GAG disaccharides dissolved in water and used in the optimization studies. Peak numbers represent compounds in Fig. 1. Concentrations: **1** = 1.13 m*M*; **2** = 1.60 m*M*; **3** = 3.19 m*M*; **4** = 0.63 m*M*. Conditions: 18 m*M* borate–30 m*M* phosphate–50 m*M* CTAB buffer, adjusted to pH 7.0; temperature, 30°C; voltage, −20 kV; detection wavelength, 232 nm, vacuum injection for 1 s.

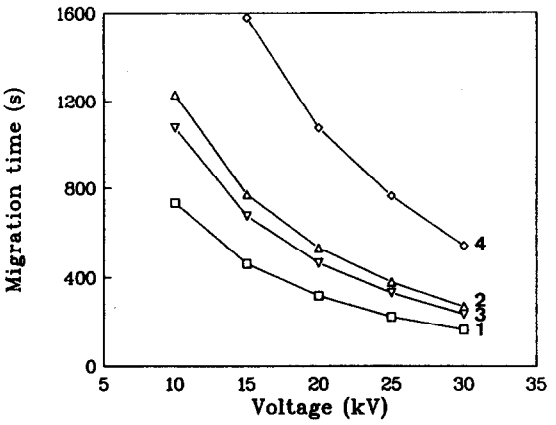

Fig. 3. Influence of applied voltage on migration times of GAG disaccharides. Numbers represent compounds in Fig. 1. Other separation conditions as in Fig. 2.

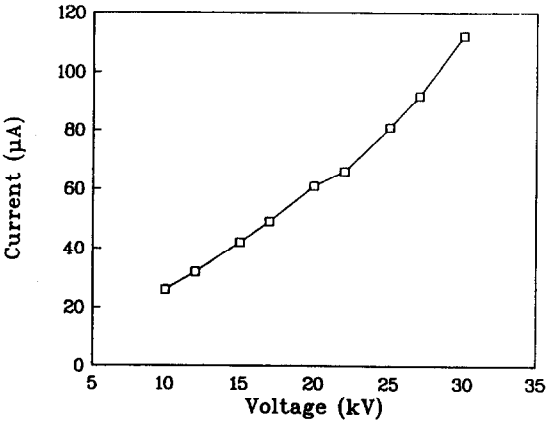

Fig. 4. Current as function of applied voltage. Other separation conditions as in Fig. 2.

expected to give a linear decrease in *MT* due to increasing velocity of the analytes. This was not seen. However, if the temperature increases in the capillary as the voltage is increased, a lower viscosity will result in a further decrease in migration times. Further, increasing temperatures may change the ratio between the micellar phase and the aqueous phase, the shape and size of micelles and the interaction of analytes with CTAB [23,25,26]. Evidence of increasing temperature within the capillary was seen from a plot of voltage *versus* current. Plotting the resulting current against voltages of 10–30 kV showed a linear relationship up to 22 kV, but the current increased relatively more than expected from a linear relationship above 22 kV (Fig. 4). This indicates that the temperature increases in the capillary due to insufficient heat removal at voltages above 22 kV. The concave curves may therefore represent a combination of increasing analyte velocity and temperature effects.

The *RMT*, *NA* and *RNA* values remained nearly constant with increasing voltage, whereas the R_s values decreased from 10 to 20 kV and were constant at higher voltages. The *N* values for **2** and **3** were low and nearly constant, whereas those for **1** had a maximum at 25 kV and the lowest value at 30 kV and **4** had a minimum at 25 kV and a maximum at 15 kV. The expected increase in R_s and *N* with increasing voltage according to the HPCE theory was not observed, which is probably due to the mentioned temperature effects [22,23]. From the results a voltage of 20 kV was chosen.

Temperature

The migration times decreased by a factor of 1.4–1.8 when the temperature was increased from 25 to 60°C. The decrease was linear except for **4**, which indicates a possible change in the interaction of CTAB and **4** and a general decrease in the viscosity of the solvent. The *RMT* values were also constant except for **4**, for which they increased by *ca.* 8%, indicating an increased interaction with CTAB. If borate complexation occurs, only **1–3** are likely to form complexes with borate ions owing to their vicinal hydroxyl groups. Changes in borate complexation therefore cannot explain the changes in the *MT* and *RMT* values for **4**. The *NA* values increased for all disaccharides (Fig. 5) and the *RNA* values were constant. The increasing *NA* values are therefore most likely to be caused by increasing injection volumes as the viscosity of the buffer decreases with increasing temperature. However, the increased injection volume may not alone explain the changes in the *NA* values. Changes in the actual response factors of disaccharides with increased temperature due to changes in borate complexation may be involved, as proposed for other compounds [22].

The R_s values were nearly constant except for a higher value at 25°C for **2–4**. The *N* values

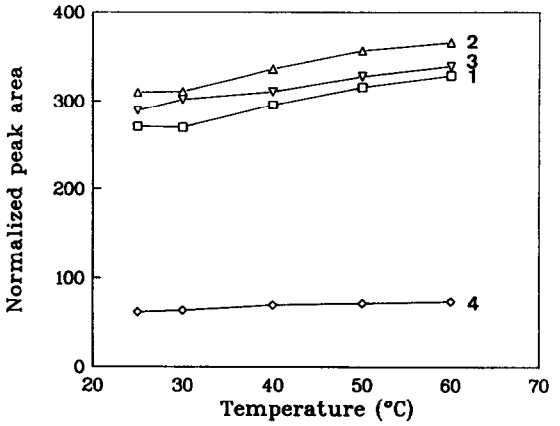

Fig. 5. Influence of temperature on normalized peak areas of GAG disaccharides. Numbers represent compounds in Fig. 1. Other separation conditions as in Fig. 2.

were highest for **1** and **4** at 30°C, whereas for **2** and **3** the *N* values were highest at high temperatures. A temperature of 30 or 40°C was chosen.

Electrolyte concentration

Increasing the electrolyte concentration [borate–phosphate (3:5)] from 16 to 48 m*M* gave interesting results for *MT* and *RMT* values (Fig. 6). The *MT* values increased by a factor of 1.3 for **1** and 1.45 for **4**, and decreased by a factor of 1.3 for **2** and 1.4 for **3**. The *RMT* values increased for **1** and **4** and were constant for **3**. No single mechanism can explain these results.

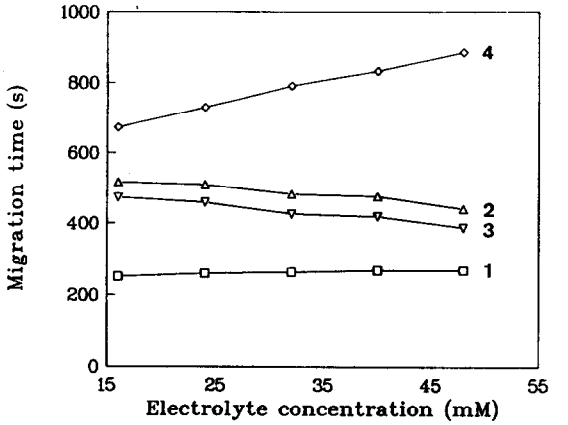

Fig. 6. Influence of electrolyte concentration [borate–phosphate (3:5)] on migration times of GAG disaccharides. Numbers represent compounds in Fig. 1. Other separation conditions as in Fig. 2, except temperature (40°C).

The electroosmotic flow decreases with increasing electrolyte concentration [23,27]. This could explain the increasing *MT* values of **1** and **4**. The higher electrolyte concentration may also lead to temperature increases, resulting in lower *MT* values. However, this was only seen for **2** and **3**. Further, a change in the critical micelle concentration (CMC) of CTAB or changes in the hydrophobic and ion-pairing interaction of GAG disaccharides with CTAB may also be involved [23]. Finally, the effect of the borate concentration on the borate–disaccharide complex concentration and equilibrium may explain some of the observed changes [28]. The observed *MT* values of the compounds are probably a result of a combination of these effects, especially the effects of electroosmotic flow and CTAB interaction.

The *NA* and *RNA* values were constant. The R_s values were constant for **2** and **3**, decreased by a factor of 1.5 for **1** and **3** and increased by a factor of 3.8 for **2** and **4**. This was a result of the large changes in *MT* values. The *N* values varied with the electrolyte concentration, and were generally highest at high electrolyte concentrations. From these results, an electrolyte concentration of 48 m*M* was chosen.

CTAB concentration

Increasing the CTAB concentration from 10 to 60 m*M* resulted in large changes in some of the parameters. Disaccharides **2** and **3** moved from positions in front of the peak normally seen between **1** and **2** at 10 m*M* to positions after this peak at 30 m*M* and higher concentrations of CTAB (Fig. 7). The *MT* values increased for **2–4** and were constant for **1** with increasing CTAB concentration (Fig. 8). The *RMT* values decreased for **1** and **4** and were constant for **3**. Increasing *MT* values are expected due to the increase in the ratio of the micellar phase to that of the aqueous phase and possible changes in the electroosmotic flow [22]. The constant value for **1** compared with the other diaccharides may be explained by a stronger ion-pairing effect with CTAB compared with hydrophobic interaction for the more charged disaccharides **2–4**. The fact that the more negatively charged disacharides

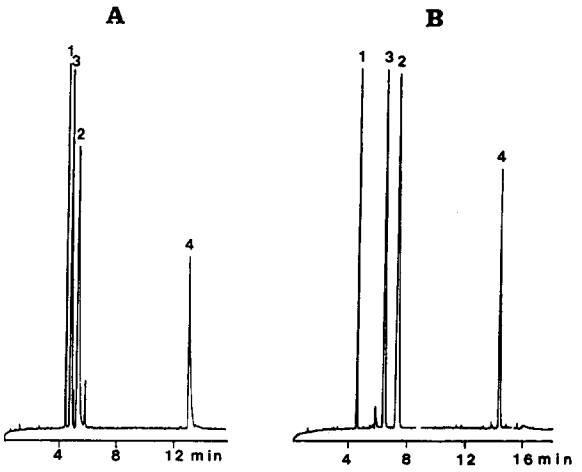

Fig. 7. Electropherograms of the GAG disaccharides at (A) 10 m*M* and (B) 40 m*M* CTAB. Peak numbers represent compounds in Fig. 1. Other separation conditions as in Fig. 2, except temperature (40°C).

migrate at a lower velocity than **1** in spite of positive polarity at the detection end also shows the great importance of ion pairing. This observation is also in agreement with results obtained for other negatively charged analytes in the CTAB system [22].

The *NA* values decreased and the *RNA* values were constant for all analytes with increasing CTAB concentration. A lower injection volume due to the higher viscosity of the buffer can probably explain these results. The R_s values increased with increasing CTAB concentration and the *N* values were highest at 50 m*M* CTAB. A CTAB concentration of 50 m*M* was chosen.

pH

Changing the pH from 6.0 to 8.0 slightly increased the migration times for all the disaccharides. The *RMT* values were nearly constant. The *NA* and *RNA* values were constant except at the low pH of 6.0 (Fig. 9). The changes observed are probably caused by changes in the actual response factors of disaccharides. The possible effects of complexation with borate ions at high pH values [28] are illustrated in Fig. 10. At pH 7.0 four disaccharide peaks are seen, at pH 7.5 peaks **1**, **2** and **3** are each followed by a minor peak and at pH 8.0 only four disaccharide peaks are seen again. Disaccharide **4** is less likely to yield complexes with borate owing to the lack of vicinal hydroxyl groups [28], whereas disaccharides **1**, **2** and **3** can form complexes with borate in the tetrahydroxyborate form at high pH values. Hoffstetter-Kuhn *et al.* [28] mentioned pH values above 8.0. Although equilibria between disaccharides and borate ions are expected to be dynamic [28], changing the pH from 7.0 to 8.0 might result in the two forms of the disaccharides **1**, **2** and **3** seen.

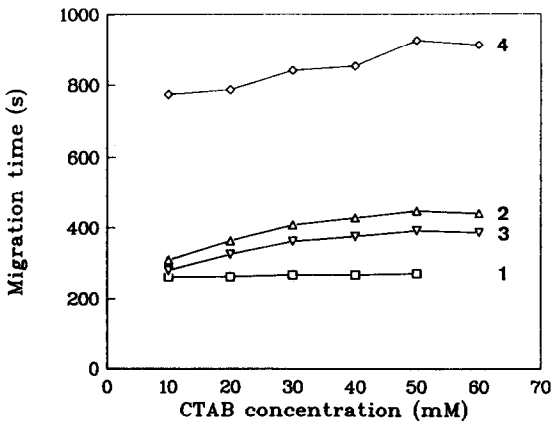

Fig. 8. Influence of CTAB concentration on migration times of GAG disaccharides. Numbers represent compounds in Fig. 1. Other separation conditions as in Fig. 2, except temperature (40°C).

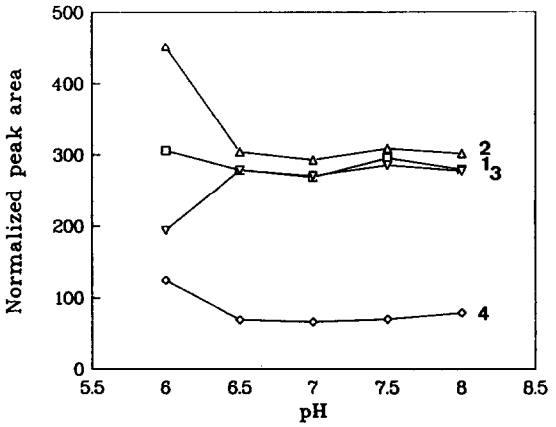

Fig. 9. Influence of pH on normalized peak areas of GAG disaccharides. Numbers represent compounds in Fig. 1. Other separation conditions as in Fig. 2, except temperature (40°C).

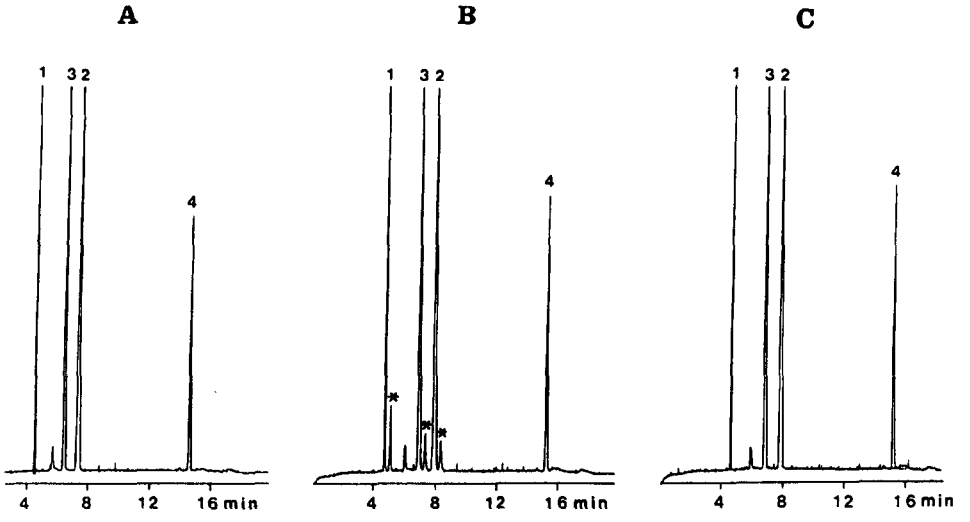

Fig. 10. Electropherograms of the GAG diaccharides at (A) pH 7.0, (B) pH 7.5 and (C) pH 8.0. Peak numbers represent compounds in Fig. 1. The minor peaks following each of the major disaccharides **1**, **2** and **3** are marked with asterisks. Other separation conditions as in Fig. 2, except temperature (40°C).

Modifier

Increasing the concentration of 1-propanol in the buffer from 1 to 10% resulted in increased *MT* values for **1** and **4**, whereas the *MT* values for **2** and **3** remained constant. Increasing amounts of organic modifiers except acetonitrile cause reductions in the electroosmotic flow [22] and less interaction of analytes with detergent. The former will increase and the latter will decrease the migration times. Both effects are probably responsible for the observed results, as the migration times of **1** and **4** increased and those of **2** and **3** remained constant. The influence on migration times therefore varies with different types of compounds, which further supports the mechanism of hydrophobic interaction with detergent. The *NA* values decreased and the *RNA* values were constant with increasing modifier concentration. The changes in *NA* values are probably a result of changes in the relative response factors of the compounds [22,23] in combination with changes in the viscosity of the buffer.

Repeatability

The repeatabilities of the *MT*, *RMT*, *NA* and *RNA* values were determined from fifteen analyses of the four GAG disaccharides. The buffer was changed between each five analyses at the injection end, and the separation conditions were as in Fig. 2. The repeatabilities of the *MT* values were very good, with relative standard deviations below 0.44% (Table I). However, transforming *MT* values to *RMT* values slightly improved the repeatabilities. The repeatabilities of the *NA* values were good with relative standard deviations below 2.05% (Table I). Trans-

TABLE I

RELATIVE STANDARD DEVIATIONS OF MIGRATION TIMES (*MT*), RELATIVE MIGRATION TIMES (*RMT*), NORMALIZED PEAK AREAS (*NA*) AND RELATIVE NORMALIZED PEAK AREAS (*RNA*) FOR GAG DISACCAHRIDES

Compound numbers as in Fig. 1 and separation conditions as in Fig. 2.

Parameter	Relative standard deviation (%) ($n = 15$)			
	1	**2**	**3**	**4**
MT	0.33	0.24	0.23	0.44
RMT[a]	0.29	0.07	–	0.36
NA	1.48	1.85	1.96	2.05
RNA[a]	0.93	1.19	–	1.26

[a] Relative to **2**.

forming *NA* values to relative values (*RNA*) also improved these repeatabilities. The *RMT* and *RNA* values correspond to the use of internal standards in analyses, which is generally recommended [22]. The results show that the repeatabilities are good and at an acceptable level for qualitative and quantitative analyses.

Linearity

The linearity of the method was determined from five repetitions at five concentrations of each of the four GAG disaccharides. The concentration ranges were 0.6–3.0 m*M* for **1**, 0.4–2.1 m*M* for **2**, 1.5–7.6 m*M* for **3** and 0.5–2.7 m*M* for **4**. The buffer was changed between each five analyses at the injection end, and the separation conditions were as in Fig. 2. The correlation between increasing concentration and the corresponding peak areas was calculated form linear regression analyses by the least-squares method for peak areas. The correlation coefficients obtained were 0.9975 for **1**, 0.9987 for **2**, 0.9982 for **3** and 0.9976 for **4**. These results show that this CTAB method gives a linear increase in peak area with increasing concentration of GAG disaccharides in the samples.

Mesityl oxide was included in the analyses in order to establish whether the small peak between **1** and **3** was a peak of neutral compounds following the electroosmotic flow. The peak of mesityl oxide, however, appeared between **3** and **2**. This indicates that **1** and **3** migrate faster than and **2** and **4** more slowly than the electroosmotic flow.

In conclusion, the separation conditions have large effects on the separation parameters, and no single effect can explain the changes observed. However, complete separations can be obtained and determinations can be performed from *NA* or *RNA* values. The use of *RNA* values corresponds to the use of internal standards in analyses, which is always recommended in order to obtain more accurate and reproducible results.

This CTAB method gives shorter times of analysis than the method described by Al-Hakim and Linhardt [19], as the separation of the four compounds is complete in less than 18 min, compared with 30 min under the optimum separation conditions in the method described by Al-Hakim and Linhardt [19]. Further, the resolution of **2** and **3** is higher in this method than that described by Al-Hakim and Linhardt [19]. Carney and Osborne [10] obtained very high peak efficiencies with a phosphate–borate–SDS buffer, but the resolution of **2** and **3** was higher in the present study and the peaks were baseline separated. However, when Carney and Osborne [10] used an orthophosphoric acid buffer, they also obtained a high resolution of **2** and **3**. Finally, Honda *et al.* [20] obtained high peak efficiencies and relatively good separations of phenylmethylpyrazolone-derivatized GAG disaccharides in a borate buffer. However, the derivatization step is time consuming and the increased sensitivity obtained by derivatization is offset by the increase in analysis time.

Chondroitin sulphates

Chondroitin sulphate A, B and C were treated with chondroitinase ABC under conditions described by Yamagata and co-workers [29,30]. The analyses performed with the sample filtrates (see Experimental) gave well separated peaks, as seen in Fig. 11. The identities of the peaks are based on spiking with reference standards (**1–4**) and on data in refs. 10, 16, and 19. The compounds found in the three chondroitin sulphates are also in agreement with information of purity according to the manufacturer.

In chondroitin sulphate B, α-L-iduronic acid dominates. However, it is not possible to distinguish between β-O-glucuronic acid, β-O-galaturonic acid and α-L-iduronic acid originally present in GAGs from disaccharides formed after chondroitinase cleavage, because of the identical structures of these compounds in disaccharides after enzymatic cleavage.

The redissolved samples, which were concentrated by a factor of ten compared with the sample filtrates, had too high concentrations of solutes to give proper HPCE separations. A 5–10-fold dilution of the redissolved samples resulted in well separated peaks. However, after the evaporation, dissolution of the residue and dilution, at least two new peaks appeared between **1** and **3** (Fig. 11). Further, the normalized peak areas of the redissolved disaccharides were

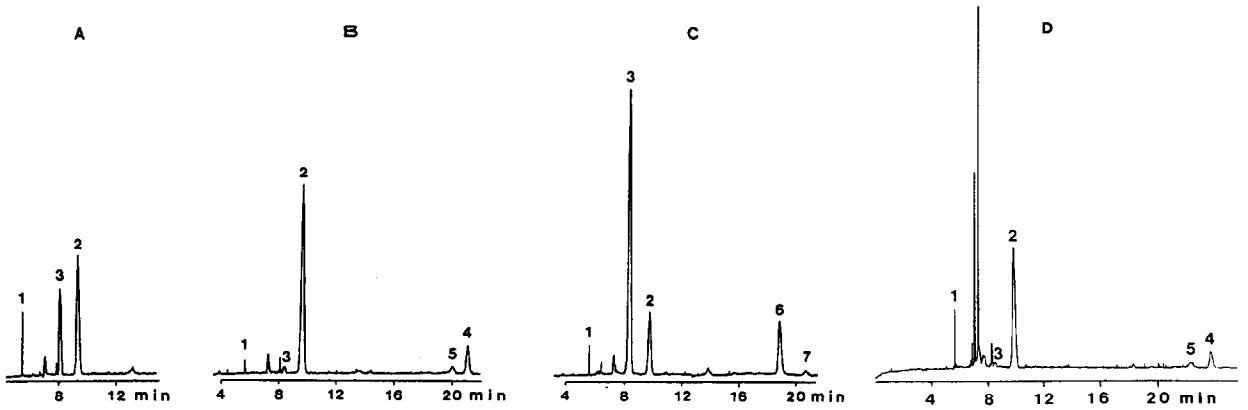

Fig. 11. Electropherograms of disaccharides obtained after chondroitinase ABC treatment of (A) chondroitin sulphate A (0.5 mg/ml), (B) chondroitin sulphate B (0.6 mg/ml) and (C) chondroitin sulphate C (0.9 mg/ml); (D) as (B) but sample evaporated and residue dissolved in an equal volume. Peak numbers **1–4** represent compounds in Fig. 1, **5** = ΔDi-diS; **6** = Δ^4-2-O-sulpho-GlcUA→6-O-sulpho-GalNAc (ΔDi-diS_D); **7** = Δ_4-GlcUA→4,6-bis-O-sulpho-GalNAc (ΔDi-diS_E). Separation conditions as in Fig. 2.

lower than expected. Instability of the GAG disaccharides during evaporation of the solvent is the probable reason for the additional peaks and the lower *NA* values.

Skin samples

Five experiments were performed with skin samples from mink. In the first two experiments only chondroitinase treatment was applied, whereas in the other experiments protease treatments with either pepsin and pancreatin or trypsin were applied prior to chondroitinase treatment. The first chondroitinase treatment (0.3 mg) of skin samples (12.9 mg) showed too small amounts of GAGs to be positively identified. A phosphate buffer was tested for the protease treatments. However, very poor HPCE separations were obtained after the chondroitinase treatment, possibly owing to the high ionic strength in the sample. HPCE analyses of samples in 50 m*M* Tris–HCl buffer (pH 8.0) gave good separations, hence this buffer was chosen for the pancreatin, trypsin and chondroitinase treatments of skin samples.

Increasing the chondroitinase and sample amounts to 3.2 and 53.6 mg, respectively, and treating the samples with pepsin and pancreatin prior to chondroitinase treatment improved the peak sizes (Fig. 12A). Peaks appearing at the same positions as those for the disaccharides in Fig. 2 are marked with the corresponding numbers. However, owing to instability of GAGs during the applied evaporation and redissolution of samples (see above) and the possible presence of other anions after the Dowex cation exchanger, other peaks were also seen. Comparing the electrophrograms of samples analysed at 232, 260, 280 and 320 nm with those for chondroitinase-treated chondroitin sulphate B analysed at the same wavelengths did, however, indicate that **3** and **2** were peaks 1 and 2, respectively, after the large peak at 6.1 min. Further work on peak identification is needed to obtain a precise peak identification. Several peaks were seen in the skin sample at 260 and 280 nm, but none at 320 nm. The analyses of chondroitin sulphate B showed a very small peak of **2** at 260 nm and no peak at 280 nm. The observed peaks at 260 and 280 nm from the skin sample are therefore not GAG disaccharides. The peaks could represent nucleic acids and nucleotides or peptides with aromatic amino acids and negatively charged at pH 7.0.

HPCE analyses of released peptides were made in order to evaluate whether the pepsin and pancreatin treatments had been performed for a long enough period. The electropherograms of pepsin (1 h)-and pepsin and pancreatin (1 h + 1 h)-treated skin sample were nearly identical, whereas the pepsin (24 h)-treated

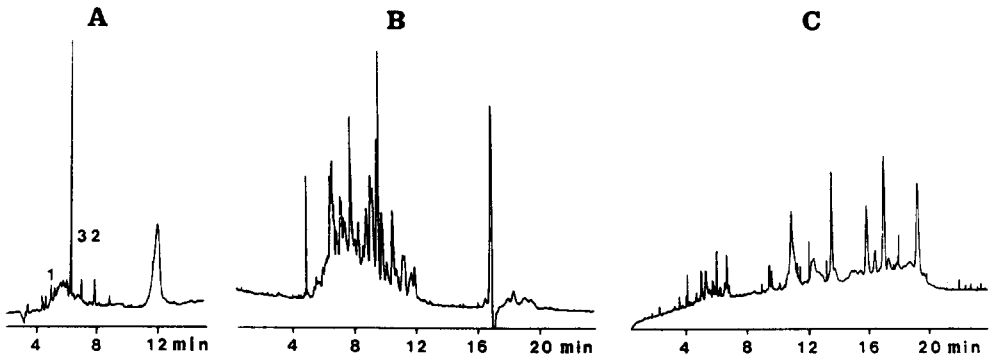

Fig. 12. (A) Electropherogram from GAG analysis of a sample of mink skin (53.6 mg) after pepsin and pancreatin treatment, followed by chondroitinase (3.2 mg) treatment in 50 m*M* Tris–HCl buffer (pH 6.8). Peak numbers represent compounds in Fig. 1. Separation conditions as in Fig. 2, except temperature (40°C). (B) Electropherogram of peptides of a mink skin sample (23.4 mg) after butanol extraction (1 ml) in 50 m*M* Tris–HCl buffer (pH 8.0) (2.0 ml) followed by trypsin treatment (2 mg) at 37°C for 18 h. Separation conditions as under Experimental. (C) Electropherogram from GAG analysis of a butanol-extracted, trypsin-treated skin sample [see (B)] after chondroitinase treatment. Separation conditions as in Fig. 2.

sample showed a large increase in low-molecular-mass peptides. This indicated that the 1-h incubation periods were too short for skin samples, although these time periods are used in protease treatments to cleave proteins in feed samples prior to gravimetric analysis of dietary fibres [23].

Trypsin treatment with and without removal of fat compared with pepsin and pancreatin treatment also with and without removal of fat were tested to improve the GAG analyses. Three different solvents were used for fat extraction: butanol, hexane and diethyl ether. The HPCE analyses of peptides after the trypsin treatment showed large amounts of low-molecular-mass compared with high-molecular-mass peptides (Fig. 12B), except for the diethyl ether-extracted sample. No marked changes were seen between 18 and 23 h of protease treatment, and therefore it can be concluded that the longest time of treatment does not improve the cleavage of proteins in the skin sample.

The protease-treated skin sample after the hexane extraction seemed to yield larger amounts of peptide when the amounts in the aqueous and the gel-like phases were added than the results obtained after the other fat extraction methods. However, the additional gel-like intermediate phase is inconvenient for routine analyses. Further, the amount of peptide obtained after the diethyl ether extraction was very small. Therefore, the butanol treatment seems to be the best choice for fat extraction of skin samples prior to trypsin treatment, and the trypsin treatment improved the amount of peptides released compared with the pepsin–pancreatin treatment.

HPCE analyses by the GAG disaccharide method performed before chondroitinase treatment and Dowex purification showed some peaks in the electropherograms. HPCE analyses of GAG disaccharides after chondroitinase treatment showed new peaks in addition to the peaks seen before the enzyme treatment (Fig. 12C). Repeated Dowex purification (100-μl column) resulted in fewer and smaller peaks in the electropherograms, but large differences in the electropherograms were seen between the various treatments. However, some of the peaks seen are probably a result of the evaporation and redissolution of the samples after the chondroitinase treatment and the repeated column purification, as discussed before. The peaks that disappeared were probably not GAG disaccharides, as the GAGs were not removed by the Dowex purification of the chondroitinase-treated chondroitin sulphates. The effect of the repeated Dowex purification is probably due to overloading of the columns in the first purification.

It can be concluded that the column purification method can probably be used to remove impurities in samples prior to GAG disaccharide analysis. At present the exact identification of GAG disaccharides in the skin samples is not clean-up procedures do not have to be as extensive as in HPLC and the peak efficiency and resolution are superior in HPCE.

CONCLUSIONS

Generally, HPCE methods of analyses have many advantages over HPLC methods. HPCE methods are inexpensive and small amounts of reagents and sample are needed. The sample clean-up procedures do not have to be as extensive as in HPLC and the peak efficiency and resolution are superior in HPCE.

The HPCE method based on CTAB–MECC with reversal of the electroosmotic flow can be applied to GAG disaccharide analyses. Compared with the HPCE methods described previously, this CTAB–MECC method might allow other compounds to be detected simultaneously with the GAG disaccharides or omitted from the analysis, depending on which material is being analysed. The possibility of rapid and easy changes in the separation conditions makes it easy to adjust the conditions for actual samples. Analysing GAG disaccharides in skin samples might need pre-isolation of GAG-containing proteoglycans or derivatization of the GAGs to obtain reasonable detection limits without interfering compounds.

This study demonstrates the possibilities of using HPCE for method development for protease and chondroitinase treatments of skin samples. Crude samples after protease treatments can be analysed directly in order to evaluate the extent of protein cleavage. This results in rapid answers as to whether prolonged treatment will result in further cleavage of proteins. The applied procedure with butanol fat extraction of skin samples and trypsin treatment followed by chondroitinase treatment seems to give the most promising results. However, further work including tests with other proteases and establishment of the identities of peaks appearing after evaporation and redissolution of samples is necessary in order to obtain a complete procedure for the analysis of GAG disaccharides in skin samples.

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