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Separation of aminated monosaccharides by capillary zone electrophoresis with laser-induced fluorescence detection

Jian Ying Zhao, Paul Diedrich, Yanni Zhang, Ole Hindsgaul, Norman J. Dovichi*

University of Alberta, Department of Chemistry, Edmonton, Alberta T6G 2G2, Canada

Abstract

The six most abundant hexoses found in mammalian carbohydrates were derivatized to serve as standards for an oligosaccharide sequencing project. Glucose, galactose, mannose, and fucose were reductively aminated to produce 1-amino-1-deoxy alditols. Standards for N-acetylglucosamine and N-acetylgalactosamine were synthesized by reducing glucosamine and galactosamine to 2-amino-2-deoxy alditols. The monosaccharides were labeled with 5-carboxytetramethylrhodamine succinimidyl ester. By carefully adjusting the separation buffer, we have separated the sugar derivatives completely. The detection limit is 100 molecules of labeled sugar with a post-column laser-induced fluorescence detection in a sheath flow cuvette.

1. Introduction

Carbohydrates play essential roles in biology such as cell–cell recognition, hormone receptors, virus binding sites, inflammation processes and various other biological functions [1]. In general, analysis of oligosaccharides has proceeded by methods such as methylation analysis, enzymatic degradation, and mass spectrometry in association with nuclear resonance spectroscopy [2]. However, it is difficult to obtain sufficient material for use with those technologies; these methods are usually impractical for analysis of the minute amounts of oligosaccharides found on cell surfaces. Thus, a different method with great separation power and high sensitivity is required to identify and quantify the monosaccharide fragments for oligosaccharide sequencing.

Capillary electrophoresis offers excellent separation efficiency. Also, the small dimensions of the capillary tubes facilitate the analysis of extremely small amount of samples. Normally, the solution volume required is at the order of a nanoliter. Post-column laser-induced fluorescence detection in a sheath flow cuvette has been developed as a remarkable sensitive technique for capillary electrophoresis at the order of zeptomole (i.e. 10^{-21} mol) or less [3]. Since Honda *et al.* [4] reported the capillary electrophoresis results of monosaccharides as their N-(2-pyridyl)glycamines with UV detection in 1989, there is now a growing research interest in carbohydrates using capillary electrophoresis as reviewed recently by Kuhr and Monnig [5].

The first step in sequencing an oligosaccharide is the determination of the monosaccharide composition of the oligosaccharide. High separation efficiency is required because several of the monosaccharides are isomers. The second step of sequencing an oligosaccharide is the specific degradation of the oligomer. An oligosaccharide primary structure can be branched,

* Corresponding author.

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producing a wide variety of linkages between the monomers and a huge number of possible products. So far no method has been devised for oligosaccharide sequencing that is similar to either the Edman degradation of polypeptides or Maxim and Gilbert's chemical degradation of oligonucleotides. New methods for oligosaccharide sequencing may involve labeling of the reducing end of a carbohydrate followed by enzymatic degradation from the non-reducing ends. Alternatively, it may be possible to chemically degrade the sugar chains from their reducing ends.

Model compounds are required to explore these sequencing strategies. The six major hexoses found in mammalian carbohydrates were chosen to serve as standards for oligosaccharide sequencing: glucose, galactose, mannose, fucose, N-acetylglucose and N-acetylgalactose. There are two kinds of detection strategies: one is to detect the sugars directly without any derivatization [7–10], the other is to do derivatization in order to use high sensitivity detection (such as fluorescence detection) [11–13].

In this paper, we describe the derivatization of glucose, galactose, mannose, fucose into their 1-amino-1-deoxy alditols and N-acetylglucosamine and N-acetylgalactosamine as their potentially accessible 2-amino-2-deoxy alditols. The aminated sugars are labeled with the fluorescence dye 5-carboxytetramethylrhodamine succinimidyl ester (TRSE). We will present the separation results of the six labeled sugars. Subzeptomole detection limit of TRSE labeled sugar has been achieved.

2. Experimental

2.1. Synthesis standards of six monosaccharides

Derivatization of glucose, galactose, mannose and fucose to produce the amino alditiols required several steps (Fig. 1). The first step is the reaction of a concentrated sugar solution with excess benzylamine [14] until full conversion of reducing sugar to imine product was indicated by thin layer chromatography. Next the reduction

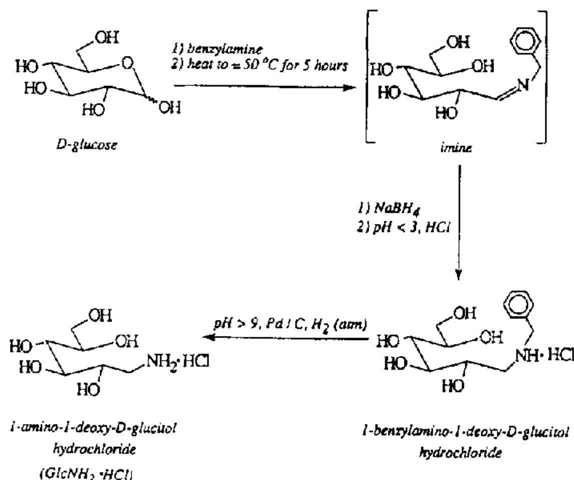

Fig. 1. Example of the reaction scheme used to synthesize 1-amino-1-deoxy alditols.

of the imine by sodium borohydride yielded the 1-benzylamino-1-deoxy derivative [14]. As the benzylamine is in excess, the aqueous solution is quite basic and thus we were able to remove the excess benzylamine by extracting the aqueous solution with dichloromethane. The completion of this process was also monitored by thin-layer chromatography. Dilution of the aqueous layer with methanol followed by acidification and the evaporation served to remove borate esters from the products. Chemicals used were from diverse sources and were of analytical-reagent quality.

The N-benzyl intermediate typically was isolated, recrystallized and characterized with a portion of the recrystallized product being used for the reduction. The debenzylation proceeded by catalytic reduction of the benzylamino intermediate under atmospheric hydrogen using palladium on carbon as the catalyst. This reduction was carried out overnight and yielded the required free amine at the 1 position of the reducing sugar. The 1-amino-1-deoxy alditols were shown to be pure by ^{1}H NMR.

The synthesis of the standards for N-acetylglucosamine and N-acetylgalactosamine is shown in Fig. 2. To reduce the aldehyde in glucosamine or galactosamine, we added the solution of the sugar (glucosamine or galac-

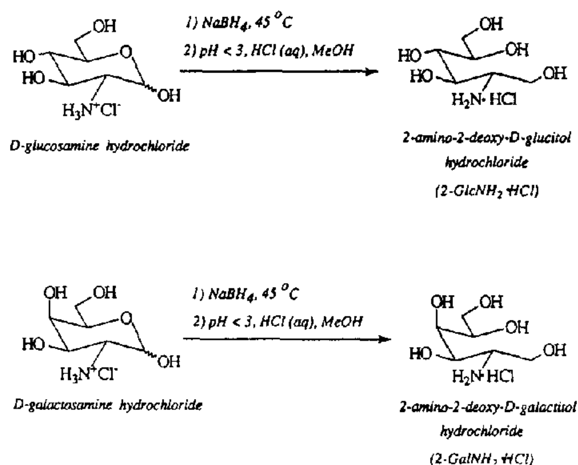

Fig. 2. Reaction schemes used to synthesize 2-amino-2-deoxy alditols.

tosamine hydrochloride) dropwise to a concentrated basic solution of sodium borohydride over a period of approximately 2 h at a temperature of 45°C. After reduction of the amino sugar, the solution was then acidified and concentrated *in vacuo* with methanol three times to remove borate esters. The residue was recrystallized and yielded pure 2-amino-2-deoxy alditols as their hydrochloride salts. Details of the synthesis and characterization of the amines will be reported elsewhere.

2.2. Labeling reactions

5-carboxytetramethylrhodamine succinimidyl ester (Molecular Probes) was dissolved in DMF to make a $1.28 \cdot 10^{-3}$ *M* stock solution that was stored at −20°C. The $2 \cdot 10^{-2}$ *M* stock aminated sugar solutions were prepared in 0.185 *M* Na_2CO_3 buffer (pH 8.3) and stored at 4°C. To label the sugar, 30 μl sugar stock solution was mixed with 5 μl TRSE solution in a disposable vial. The mixture was vortex mixed and centrifuged a few times during a 1-h reaction period. The reactions were carried out in the dark at room temperature. The mixture was stored at 4°C. A blank solution was prepared by mixing 30 μl 0.185 *M* Na_2CO_3 buffer with 5 μl TRSE stock.

2.3. Standard for detection limit

β-GlcNAc-O-$(CH_2)_8$-CO-NH-CH_2CH_2-NH_2 (GlcNAc = N-acetylglucosamine) is a synthetic sugar with a free amine group. It was labeled with TRSE. The labeled derivative was purified and then dissolved in water. The solution was diluted to $4.8 \cdot 10^{-11}$ *M* by a buffer containing 10 m*M* Na_2HPO_4, 10 m*M* borate and 10 m*M* SDS (pH 9.3) for capillary electrophoresis with laser-induced fluorescence detection.

2.4. Instrument setup

The capillary electrophoresis with fluorescence detection system has been described before [4]. An inexpensive helium-neon laser was used to provide a 0.75 mW beam at λ = 543.5 nm (Melles Griot, Ontario, Canada). The beam was focused with a 10× microscope objective about 200 μm below the exit of the capillary which was fixed inside a locally constructed sheath flow cuvette. Post-column fluorescence was collected at right angles with a 50×, 0.60 numerical aperture microscope objective (Melles Griot) and imaged onto an iris adjusted to block scattered laser light. The emission channel of the instrument had an interference filter with a 580 nm center wavelength and a 40 nm bandpass (Omega Optical, Vermont, USA). A Hamamatsu R1477 photomultiplier tube (CA, USA) was operated at 1200 V at room temperature. The photomultiplier tube output was conditioned with a 0.1-s resistor–capacitor low-pass filter and recorded by a Macintosh IIsi computer.

2.5. Electrophoresis conditions

A 74.4 cm × 10 μm I.D. × 150 μm O.D. uncoated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was used for separations. Sample injections were performed electrokinetically at 2500 V for 5 s. Electrophoresis was driven at 29 kV provided by a high-voltage power supply (Spellman CZE 1000R).

2.6. Separation buffers

Stock buffer solutions were prepared in water and filtered with a 0.22-μm pore-size membrane. These stock solutions included 0.2 *M* Na_2HPO_4, 0.1 *M* phenyl boronic acid, 0.2 *M* borate and 0.556 *M* sodium dodecyl sulfate (SDS). Separation buffers were prepared from mixtures of these stock solutions. For example, most separations were performed with a 10 m*M* Na_2HPO_4, 10 m*M* borate, 18 m*M* phenyl boronic acid and 10 m*M* SDS (pH 9.3).

3. Results and discussion

3.1. The dye

5-Carboxytetramethylrhodamine succinimidyl ester has a maximum absorbance at $\lambda = 552$ nm and maximum emission at $\lambda \approx 570$ nm in a pH 9.3 buffer (BS) consisting of 10 m*M* SDS, 10 m*M* Na_2HPO_4 and 10 m*M* borate. The experimental molar absorptivity is $8.0 \cdot 10^4$ M^{-1} cm^{-1} in the BS buffer. Figs. 3 and 4 present the electropherograms of the dye diluted in the BS buffer. The result from the electropherogram generated immediately after dilution gives three peaks. The very last peak corresponds to the succinimidyl ester of the dye, while the other two peaks are generated by the hydrolysis products of the dye. Fig. 4 was generated after 22 h in solution; the increase in the relative height of the hydrolysis peaks indicates the poor stability of the succinimidyl ester in the basic buffer.

3.2. The labeling reactions

Fig. 5 shows the electropherogram of TRSE-labeled mannose-NH_2. The separation buffer is 20 m*M* Na_2HPO_4, 20 m*M* phenylboronic acid, 10 m*M* borate and 10 m*M* SDS. In addition to the hydrolysis products, there is only one peak corresponding to the labeled derivative. There are two competitive reactions taking place when we mix the TRSE with an aminated sugar in a basic buffer: the labeling reaction between TRSE and the aminated sugar, and the hydrolysis of TRSE. The other five sugars also gave single labeling results and the same hydrolysis products of the dye.

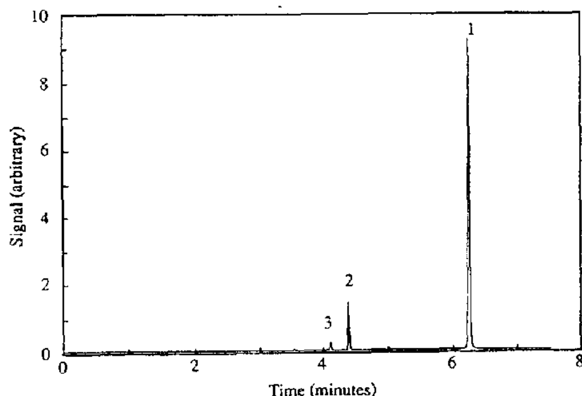

Fig. 3. Capillary zone electropherogram of TRSE (immediately after dilution from its stock solution by electrophoresis buffer). Conditions: electrophoresis buffer = 10 m*M* borate with 10 m*M* SDS (pH 9.3); capillary 59.8 cm × 10 μm I.D.; electrophoresis voltage = 29 kV. Peaks: 1 = TRSE; 2 = one product of TRSE hydrolysis; 3 = another product of TRSE hydrolysis. Note the short retention time with this buffer system.

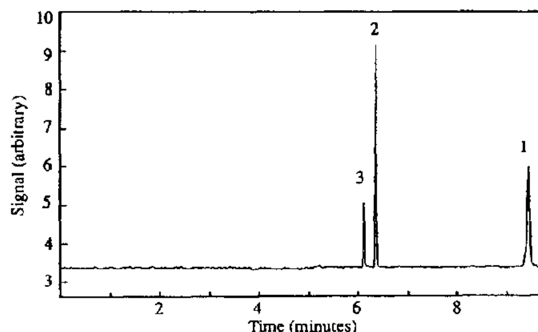

Fig. 4. Capillary zone electropherogram of TRSE (22 h at room temperature after dilution from its stock solution by electrophoresis buffer). Conditions: electrophoresis buffer = 10 m*M* borate with 10 m*M* SDS (pH 9.3); capillary = 73.8 cm × 50 μm I.D.; electrophoresis voltage = 29 kV. Peaks: 1 = TRSE; 2 = one product of TRSE hydrolysis; 3 = another product of TRSE hydrolysis. The retention time is longer than in Fig. 3 because the capillary was longer.

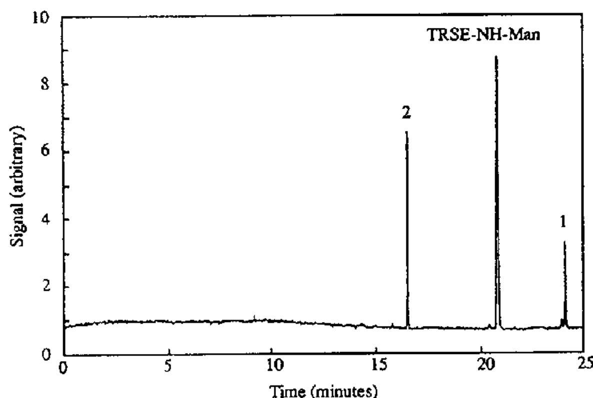

Fig. 5. Capillary zone electropherogram of aminated mannose labeled with TRSE. Conditions: electrophoresis buffer = 20 mM phenyl boronic acid, 20 mM Na_2HPO_4, 10 mM borate with 10 mM SDS (pH 9.3); capillary = 74.4 cm × 10 μm I.D.; electrophoresis voltage = 29 kV. Peaks: 1 = TRSE; 2 = one product of TRSE hydrolysis. Note that the addition of phenyl boronic acid decreases the mobility of the sugar compared with Fig. 4.

3.3. Plate counts

Electrophoresis peaks for the labeled sugars are always broader than the peaks for the dye or the hydrolysis products. Table 1 lists the plate counts for the dyes and the sugars. The dye molecules generate plate counts that are about five times larger than the plate counts produced for the sugar. This difference in plate count is surprising and may reflect slow kinetics of complexation with the sugars, leading to additional band-broadening. Further work is required to clarify the origin of this enhanced peak width.

Table 1
Plate counts for labeled sugars and labeling reagent

TRSE	$1.5 \cdot 10^6$
TRSE (hydrolysis product)	$1.4 \cdot 10^6$
$GlcNH_2$	$3.1 \cdot 10^5$
2-$GlcNH_2$	$4.0 \cdot 10^5$
2-$GalNH_2$	$2.4 \cdot 10^5$
$ManNH_2$	$2.6 \cdot 10^5$
$GalNH_2$	$3.1 \cdot 10^5$
$FucNH_2$	$2.2 \cdot 10^5$

The separation was performed in 20 mM phenyl boronic acid, 20 mM Na_2HPO_4, 10 mM borate, 10 mM SDS (pH 9.3). The 74.4 cm long capillary was operated at 29 kV. Gal = Galactose; Glc = glucose; Fuc = fucose; Man = mannose.

3.4. Detection limit

Fig. 6 shows the electropherogram of the synthetic sugar labeled with TRSE. A 9-pl volume of the $4.8 \cdot 10^{-11}$ M solution was injected (800 V for 5 s), which corresponds to $4.3 \cdot 10^{-22}$

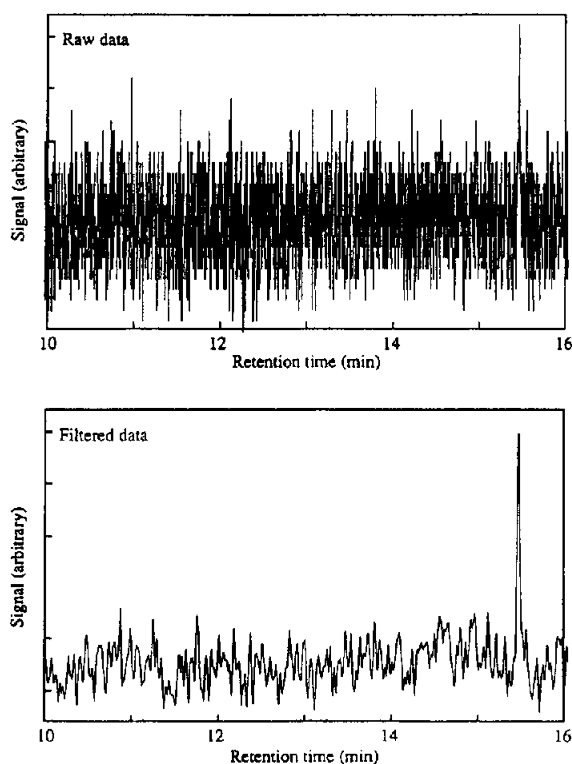

Fig. 6. Capillary zone electropherogram of the synthetic sugar labeled with TRSE. Conditions: electrophoresis buffer = 10 mM Na_2HPO_4, 10 mM borate with 10 mM SDS (pH 9.3); capillary = 74.4 cm × 10 μm I.D.; electrophoresis voltage = 29 kV. The top panel shows the raw data, sampled at 10 Hz and passed through a 0.1-s time-constant low pass filter. The bottom panel presents the data after convolution with a 0.6-s wide Gaussian function. Detection limits (3 σ) are roughly 60 molecules injected onto the capillary.

mol or 260 analyte molecules. The bottom panel of the figure shows the smoothed version of the data, where the data have been convoluted with a 0.6-s wide Gaussian-shaped filter. According to Knoll's method [13], the calculated detection limit is 60 molecules for the smoothed data.

3.5. Separation buffer effects

The six standards have similar structure. Five are isomeric. After they are labeled with relatively large TRSE fluorophore, the structural differences between them are minor. Fortunately, the complexation between sugar hydroxyl groups and borate can be used to enhance the mobility differences between the labeled monosaccharides. We first tried to use borate buffer, but we could not separate the six sugars. Also, we were unable to separate the isomers with buffers containing borate, phosphate and SDS (BPS buffer). However, addition of phenyl boronic acid to the BPS buffer improved resolution markedly. The separation, shown in Fig. 7, revealed that borate and phenyl boronic acid have opposite effects on the electrophoretic mobilities of two pairs of labeled monosac-

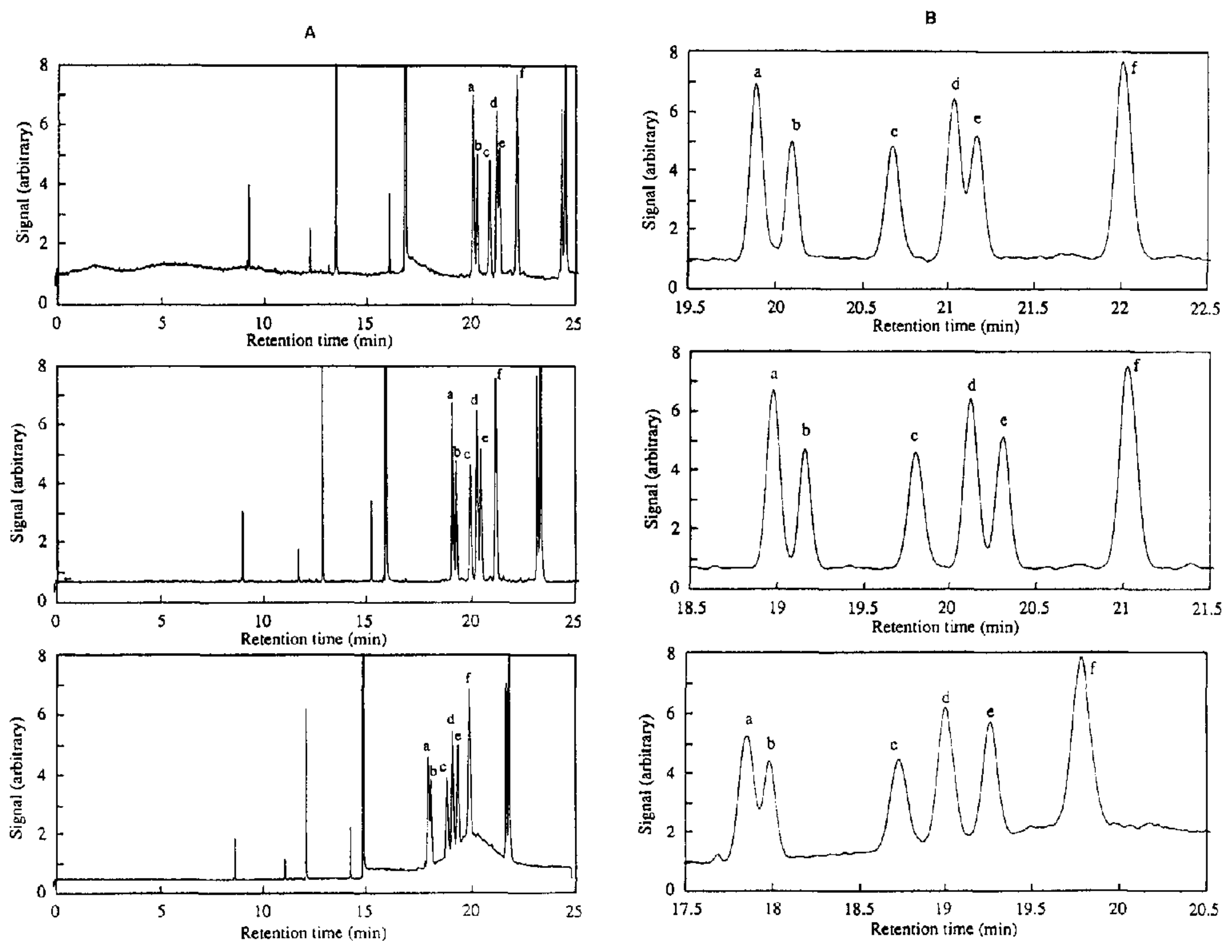

Fig. 7. Separation electropherograms of six aminated monosaccharides labeled with TRSE. The electrophoresis buffer for the top panels was 20 m*M* phenyl boronic acid, 20 m*M* Na_2HPO_4, 10 m*M* borate with 10 m*M* SDS (pH 9.3). The middle panels used 18 m*M* phenyl boronic acid in the buffer, while the bottom panels used 15 m*M* phenyl boronic acid. The capillary was 74.4 cm × 10 μm I.D. and the electrophoresis voltage was 29 kV. Peaks: a = $GlcNH_2$; b = 2-$GlcNH_2$; c = 2-$GalNH_2$; d = $ManNH_2$; e = $GalNH_2$; f = $FucNH_2$. (A) Entire electropherogram; (B) close-up of the labeled monosaccharide peaks.

charides GlcNH-TRSE and 2-GlcNH-TRSE or ManNH-TRSE and GalNH-TRSe. A higher concentration of borate gives better separation between GlcNH-TRSE and 2-GalNH-TRSE, but more overlap between ManNH-TSE and GalNH-TRSE. The optimum separation buffer composition is 18 m*M* phenyl boronic acid, 20 m*M* Na_2HPO_4, 10 m*M* borate with 10 m*M* SDS (pH 9.3). This buffer can completely separate the six TRSE-labeled monosaccharides within 22 min, with no interference from hydrolysis products of the dye molecules.

4. Conclusions

5-Carboxytetramethylrhodamine succinimidyl ester is a useful fluorescent label for aminated sugar monomers. The labeling reaction can be done at room temperature within 1 h. The TRSE labeled sugars are very stable at 4°C in basic buffers. Unfortunately, the reaction requires high sugar concentration because of the competition between the labeling reaction and the hydrolysis of the dye. Therefore, in order to analyze real sugar samples, we need to choose a more practical fluorescence dye, which has ether better stability in basic buffer or generates much lower background signals.

The detection limit of the TRSE labeled monosaccharide is 60 molecules in this capillary electrophoresis system with post-column laser-induced fluorescence detection in a sheath flow cuvette. The next step in developing an oligosaccharide sequencing protocol is to develop a strategy for degrading the oligosaccharide chains.

5. Acknowledgements

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References

[1] C.C. Sweeley and H.A. Nunez, *Annu. Rev. Biochem.*, 54 (1986) 765–801.
[2] Y.F. Cheng and N.J. Dovichi, *Science (Washington, D.C.)*, 242 (1988) 562–564.
[3] J.Y. Zhao, D.Y. Chen and N.J. Dovichi, *J. Chromatogr.*, 608 (1992) 117–120.
[4] S. Honda, S. Iwase, A. Makino and S. Fujwara, *Anal. Biochem.*, 176 (1989) 72–77.
[5] W.G. Kuhr and C.A. Monnig, *Anal. Chem.*, 64 (1992) 389R–407R.
[6] D.J. Bornhop, T.G. Nolan and N.J. Dovichi, *J. Chromatogr.*, 384 (1987) 181–187.
[7] T.W. Garner and E.S. Yeung, *J. Chromatogr.*, 515 (1990) 639–644.
[8] S. Hoffsteffer-Kuhn, A. Paulus, E. Gassmann and H.M. Widmer, *Anal. Chem.*, 63 (1991) 1541–1547.
[9] A.E. Vomdran and P.J. Oefner, *Chromatographia*, 33 (1992) 163–168.
[10] J. Liu, O. Shirota, D. Wiesler and M. Novotny, *Proc. Natl. Acad. Sci. U.S.A.*, 88 (1991) 2302–2306.
[11] W. Nashabeh and Z. El Rassi, *J. Chromatogr.*, 600 (1992) 279–287.
[12] R.J. Stack and M.T. Sullivan, *Glycobiology*, 2 (1992) 85–92.
[13] J.E. Knoll, *J. Chromatogr. Sci.*, 23 (1985) 422–425.
[14] K. Bock, I. Christiansen-Brams and M. Meldal, *J. Carbohydr. Chem.*, 11 (1992) 813–817.