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Effects of amine modifiers on the separation of tetramethylrhodamine-labeled mono- and oligosaccharides by capillary zone electrophoresis

Hans D. Osthoff, Keiko Sujino, Monica M. Palcic, Norman J. Dovichi*

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

Abstract

In this work, nine tetramethylrhodamine (TMR) labeled isomeric oligosaccharide derivatives of βGal(1→4) βGlcNAc-O-TMR were separated by capillary zone electrophoresis coupled with laser-induced fluorescence detection. Charged species were created in situ by complexation with borate and phenylborate. Micellar separation was achieved by addition of 10 m*M* sodium dodecylsulfate to the running buffer. We have investigated the effects of adding a homologous series of monoamine modifiers on the separation efficiency of these oligosaccharides. The separation was significantly improved in the presence of the organic modifiers methyl- and ethylamines, but worsened in the presence of propyl- and butylamines. Possible mechanisms of the amine additives are discussed.

Keywords: Buffer composition; Carbohydrates; Amines; Alkylamines; Borate; Phenylborate; Soduim dodecylsulfate; Tetramethylrhodamine

1. Introduction

A variety of methods has been used for the analysis of neutral oligosaccharides by capillary electrophoresis and has been the subject of many reviews [1–8]. These methods include the use of strong alkali, precapillary conversion to ions, in situ conversion to ions, micellar electrokinetic chromatography (MEKC), and size-exclusion methods in packed beds [5].

Oligosaccharides are commonly derivatized for UV–Vis or fluorescence detection. Some frequently used precapillary derivatizing reagents are 6-aminoquinoline [9], 7-aminonapthalene-1,3-disulfonic acid [10], 8-aminonapththalene-1,3,6-trisulfonate [11], and 8-aminopyrene-1,3,6-trisulfonates [12]. Exquisite detection limits can be achieved by coupling CE with laser-induced fluorescence detection (CE–LIF) [13]. For tetramethylrhodamine (TMR) labeled oligosaccharides the limits of detection are below 100 molecules, as demonstrated by this group. [14–17]. The use of TMR has several advantages, among them: stability of the derivative to hydrolysis, high fluorescent quantum yields, and an excitation maximum that matches the output of the green helium–neon laser. Other oligosaccharide labeling reagents that have been employed for CE–LIF are 3-(*p*-carboxybenzoyl)quinoline-2-carboxyaldehyde [18–20] and 9-aminopyrene-1,4,6-trisulfonate [21].

The structural complexity and micro-heterogeneity of carbohydrates requires high resolving power of the separation medium. In CE, charge must be introduced to the molecules. In situ conversion to ions is traditionally done by complexation of *cis*-

*Corresponding author. Tel.: 1-780-492-2845; fax: 1-780-492-8231.

E-mail address: norm.dovichi@ualberta.ca (N.J. Dovichi).

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diols with boric acid or earth alkali metal ions [22]. Many procedures employ micellar reagents such as sodium cholate, cetyltrimethylammonium bromide (CTAB) [23], or sodium dodecyl sulfate (SDS) in MEKC. Often, the dye attached for detection is less polar than the carbohydrate analyte, increasing the partitioning coefficient of the analyte molecules into the pseudostationary phase. Organic modifiers, such as butanol and octanol [24] or ion-pairing reagents such as tris(hydroxymethyl)aminomethane (Tris) [25] or tetrabutylammonium phosphate [26] have also been used to modify the electrophoretic mobility of oligosaccacharides in MEKC.

In this report, we studied a set of TMR-labeled oligosaccharide standards, some of which were studied previously [14–17]. Our interest in these compounds arose from work done on metabolic cytometry, in which the uptake, biosynthesis, and biodegradation of these standards were monitored [27,28]. Several sialylated oligosaccharides were recently synthesized [29] and act as standards for the exploration of additional biosynthetic pathways. We now wish to communicate an improved separation method of this complex mixture in which ethylamine was added to the separation buffer. We have also investigated and compared the effects of adding the organic modifiers methyl-, ethyl-, propyl- and butylamines on the separation.

2. Experimental

2.1. Materials

A set of 250 m*M* stock solutions including sodium tetraborate (J.T. Baker, Philpsburg, NJ, USA), disodium phosphate (Na_2HPO_4, Fisher, Fair Lawn, NJ, USA), SDS (BDH Biochemicals, Toronto, Canada) and phenylboronic acid (Sigma, St. Louis, MO, USA) was prepared using deionized water and filtered using 0.22 μm pore size filters (BioRad, Mississauga, Canada). The phenyl boronic acid solution was prepared with an equimolar concentration of NaOH. The amines added were either pure (propyl- and butylamines) or aqueous solutions (methyl- and ethylamines) supplied by Aldrich (Milwaukee, WI, USA). The pH of the buffers was adjusted using 0.10 *M* NaOH or 0.50 *M* HCl solutions.

2.2. Sample preparations

The TMR-labeled oligosaccharides used in this study were prepared as previously described [14–17,29] and are listed in Table 1. Sample solutions were 2.5 n*M* in running buffer. Components in the electropherograms were identified by spiking mixtures.

2.3. Capillary electrophoresis

Capillary electrophoresis was performed with a locally constructed instrument [30–32]. Separations were performed in a 31cm×10 μm I.D.×150 μm O.D. fused-silica capillary (Polymicro, Phoenix, AZ, USA) at ambient temperature. The separation buffers typically contained 10 m*M* disodium phosphate, 10 m*M* SDS, 10 m*M* borate and 10 m*M* phenylborate. Detection was performed with a locally constructed ultrasensitive laser-induced fluorescence detector,

Table 1
Tetramethylrhodamine (TMR) labeled oligosaccharide standards

Number	Structure	Trivial name
1	$HO(CH_2)_8CONH(CH_2)_2NHCO$-TMR	Linker arm
2	βGlcNAc-O-TMR	GlcNAc
3	βGal(1→4)βGlcNAc-O-TMR	LacNAc-TMR
4	αFuc(1→2)βGal(1→4)βGlcNAc-O-TMR	H-type II-TMR
5	βGal(1→4)[αFuc(1→3)]βGlcNAc-O-TMR	$Lewis^X$-TMR
6	αFuc(1→2)βGal(1→4)[αFuc(1→3)]βGlcNAc-O-TMR	$Lewis^Y$-TMR
7	αNana(2→6)βGal(1→4)βGlcNAc-O-TMR	2,6-sialylated LacNAc-TMR
8	αNana(2→3)βGal(1→4)βGlcNAc-O-TMR	2,3-sialylated LacNAc-TMR
9	αNana(2→3)βGal(1→4)[αFuc(1→3)]βGlcNAc-O-TMR	Sialylated $Lewis^X$-TMR

based on a sheath-flow cuvette. A 5-mW helium–neon laser beam (Melles Griot, Carlsbad, CA, USA) at 543.5 nm was focussed onto the cuvette. Fluorescence was collected at right angles with a 60×, 0.7 NA microscope objective, filtered with a 590DF35 band-pass filter from Omega Optical (Brattleboro, VT, USA), imaged onto an iris to block stray light, and detected with a R1477 photomultiplier tube operated at 1000 V (Hamamatsu, Middlesex, NJ, USA). Samples were injected hydrodynamically (10 cm height difference, 90 s unless stated otherwise). The separation field was 400 V/cm, set by a CZE 1000R high-voltage power supply (Spellman, Plainview, NY, USA). Data were digitized by a NB-MIO-16XH data acquisition board in a Macintosh computer, controlled by software written in LabView (Version 3.0.1, National Instruments, Austin, TX, USA) and analyzed using the software package Matlab (The Mathworks, Nantick, MA, USA). Data were convoluted with a 0.15 s standard deviation Gaussian filter before presentation.

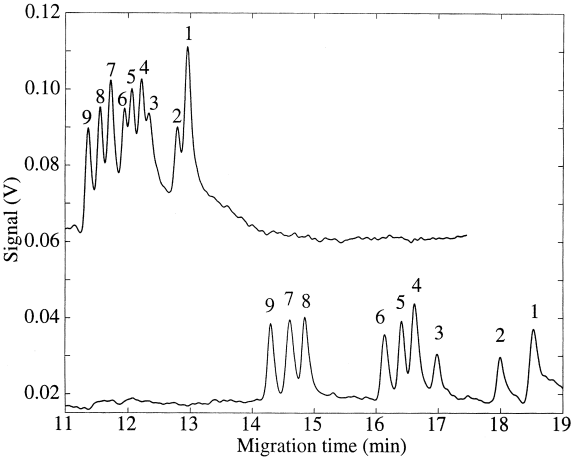

Fig. 1. Separation of 2.5 n*M* oligosaccharides in 10 m*M* borate, 10 m*M* phenylborate, 10 m*M* SDS, 10 m*M* Na_2HPO_4, pH 12.0 buffer. Separation was in a 31 cm×10 μm I.D.×150 μm O.D. bare silica capillary at 400 V/cm. Current was 1.7 μA. Detection was by LIF, excitation at 543 nm and emission at 580 nm. Top trace: No ethylamine added. Bottom trace: 360 m*M* ethylamine added. Numbers in the figure correspond to compounds listed in Table 1.

3. Results

3.1. Separation in the absence of ethylamine at pH 12.0

In the absence of amine modifier at pH 12.0, all peaks elute in a 2-min window (Fig. 1, top trace). Similar separation behavior was observed at lower pH (not shown). Larger, less hydrophobic (Nos. 7–9) molecules elute first. The variation in peak height is due to variation in analyte concentration.

3.2. Separation in the presence of ethylamine at pH 12.0

A sample electropherogram at pH 12.0 with an ethylamine buffer is shown in the bottom trace of Fig. 1. The elution order of two of the sialylated compounds is reversed. In general, the elution times increased when ethylamine was added. Migration times in the presence of ethylamine depend on capillary history, increasing slightly over time (data not shown).

3.3. Separation in the presence of ethylamine at pH 10.2 (reversed polarity)

A typical trace generated at pH 10.2 is shown in Fig. 2. Similar electropherograms with shorter migration times were obtained at pH 9.0 and pH 9.8 (data not shown). Migration times were generally more reproducible at pH 10 than at pH 12. The elution order reversed compared with the pH 12 data — more hydrophobic compounds elute first. Most carbohydrate-bearing compounds show satellite peaks at slightly faster migration times.

3.4. Comparison of methyl-, ethyl-, propyl- and butylamines at pH 12.1 (normal polarity)

The electropherograms generated at pH 12.1 are shown in Fig. 3. Addition of methylamine yields sharper but poorer resolved peaks than the addition of ethylamine. The buffer containing propylamine yields broader, faster migrating peaks, with some loss of separation. The resolution degrades significantly when butylamine is added.

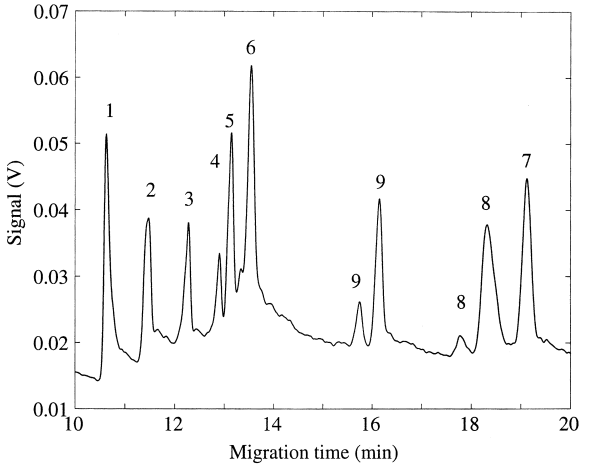

Fig. 2. Separation in reversed polarity mode of 2.5 n*M* oligosaccharides in 10 m*M* borate, 10 m*M* phenylborate, 10 m*M* SDS, 10 m*M* Na_2HPO_4, 360 m*M* $EtNH_3Cl$, pH 10.2 buffer adjusted with 0.10 *M* NaOH. Separation was at 400 V/cm. Current was 10.3 μA. Injection was by siphoning, 10 cm height difference, for 360 s.

4. Discussion

4.1. Dynamic coating of capillary wall

Ethylammonium cation has a pK_a of 10.8. At pH 12.0, roughly 10% of the compound is in the acid form. The capillary walls are dynamically coated with the residual ethylammonium ion. Ammonium cations adsorbing to the capillary wall are known to affect the electroosmotic flow (EOF) [33]. The dynamic nature of the coating process and the dissolution of the capillary wall explain the observed drifts in the migration time. We measured the migration time of a neutral marker by injecting a short plug of 15% methanol in running buffer. Migration of the methanol into the fluorescence detector causes a refractive index perturbation that scatters the laser beam, which generates a peak in the fluorescence signal. The observed migration times were 168 s and 184 s in the absence and presence of ethylamine, respectively. This increase in migration time is too small to account for the improved resolution observed in Fig. 1. However, the capillary walls become more hydrophobic in the presence of the amine.

At pH 10.2, the majority of the additive is present as the weak acid, and sufficient ethylammonium is adsorbed to the capillary wall to reduce the EOF to near zero. In Fig. 2, the net movement of the analytes is in same direction as the movement of the SDS micelles. The order of elution is therefore reversed compared to higher pH.

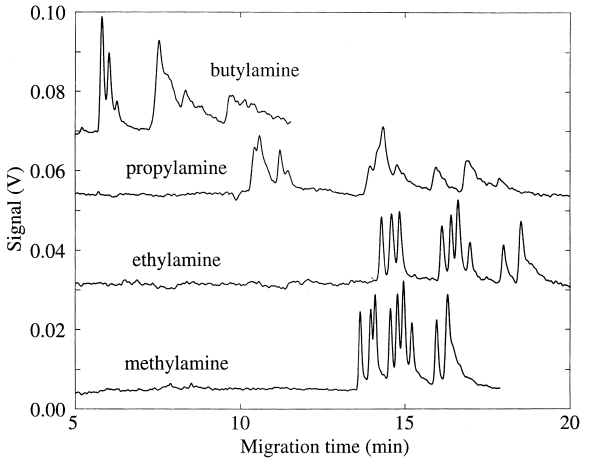

Fig. 3. Effect of adding equal concentrations of a series of monoamines to the running buffer at pH 12.1. Conditions are otherwise the same as in Fig. 1.

4.2. Increased partitioning coefficient into pseudostationary phase

As stated above, the change in EOF alone does not account for the change in resolution of the analytes. The improved resolution in the presence of ethylamine (Fig. 1) can be explained by changes in the partitioning coefficient into the SDS micelles. We discuss several hypotheses:

4.2.1. Amine-analyte ion pair mechanism

It is plausible that improved resolution is due to an ion-pairing mechanism, as was postulated for the buffer additives Tris [25] and tetrabutylammonium ion [26]. When the pH of the running buffer was adjusted with NH_4OH (aq) instead of NaOH (aq) in the absence of ethylamine (data not shown), compounds 7 and 8 co-migrate, indicating that some ion pairing by ethylamine to the sialic acids occurs. However, the breakdown of resolution in the presence of propyl- and butylamines indicates that ion-

pairing alone does not provide a sufficient explanation for the improved resolution with ethylamine.

4.2.2. Amine–micelle partitioning competes with analyte–micelle partitioning

The amines, being present at relatively high concentration, likely partition into the micelle, causing the micelles to swell. Charge reduction of the micelles by the amines was not a significant factor, since the migration times of the analytes increased in the presence of the small amines. However, competitive partitioning between the analyte and the amine is consistent with the observation that the smaller, less polar analyte (compounds 1 and 2), which partitioned into the micelles more efficiently, are better resolved than the larger, more polar molecules. In the presence of more hydrophobic propyl- and butylamines the partitioning then would become so inefficient for the analytes that the resolution breaks down.

4.2.3. Amine–Borate complexation changes hydrophobicity of borate–analyte complex

Boric acids and phenylboronic acid require the presence of hydroxide ion in order to efficiently complex the *cis*-diols occurring in oligosaccharides [5]. In other words, the complexing species is the borate or phenylboronate anion (Fig. 4, structure I). It is known [34] that amines form complexes with borates as depicted in Fig. 4, structure III. This complex may also form an ester with a *cis*-diol, giving rise to structures IV and V in Fig. 4. The presence of the hydrophobic R group increases the overall hydrophobicity of the borate–analyte complex. Therefore, the partitioning coefficient increases. We speculate that the neutral species (structure IV) is the dominant species at pH 10. This molecule is expected to partition preferentially into the micelles, where it would be protected from exchange with polar hydroxy ion.

4.3. Satellite peaks

Addition of ethylamine to the separation buffer caused the formation of small leading peaks for the largest oligosaccharides. The origin of these satellite peaks is not clear, but may reflect the presence of structural isomers of the labeling dye molecule.

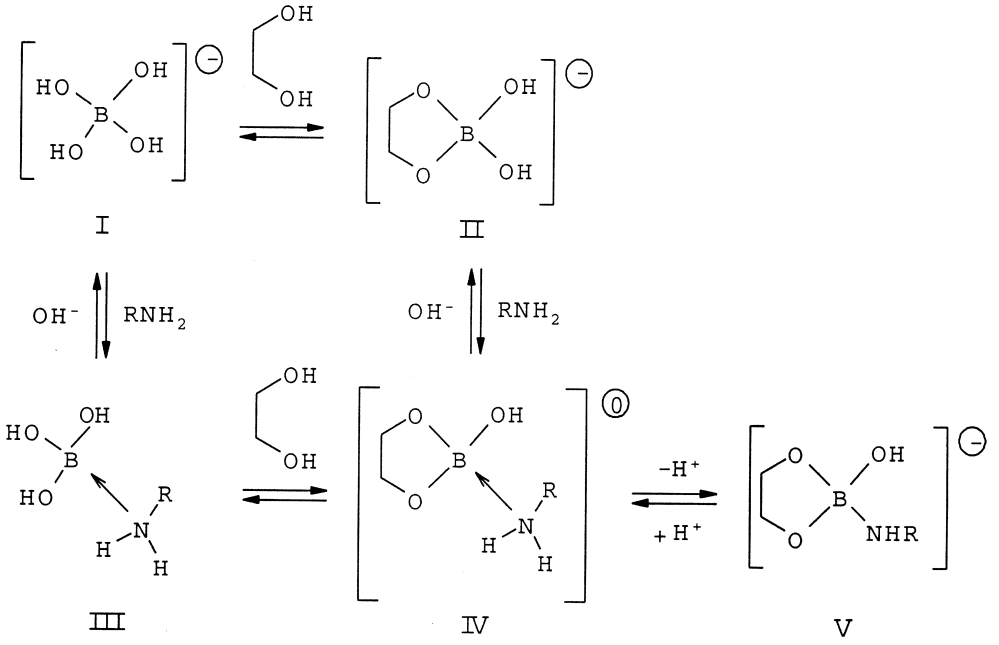

Fig. 4. Postulated mechanism for the complexation of borate with *cis*-diols in the presence of an amine.

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