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Nanomolar determination of aminated sugars by capillary electrophoresis

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Abstract

A procedure is described for the determination of aminated monosaccharides at nanomolar concentrations by derivatization with the fluorogenic reagent 3-(*p*-carboxybenzoyl)quinoline-2-carboxyaldehyde (CBQCA) to produce a fluorescent derivative that can be identified and detected by capillary electrophoresis with laser-induced fluorescence detection. Labeling conditions that favor the formation of the CBQCA-aminated sugar derivative over secondary fluorescent products were chosen. Samples as dilute as 1.0×10^{-9} *M* 1-glucosamine were analyzed. The results can be extended to other aminated monosaccharides since they showed similar reaction yields on labeling. The capability for the analysis of mixtures of aminated sugars was also demonstrated. Five labeled aminated sugars were separated by capillary electrophoresis using a running buffer containing a mixture of phenyl boronate and borate. The detection scheme was based on a low-scattering sheath flow cuvette as a postcolumn detector and two photomultiplier tubes that have mutually excluded wavelength ranges to prevent the water Raman band from contributing to the background signal. The system had a limit of detection of 75 zeptomol of fluorescently labeled 1-glucosamine.

1. Introduction

The structural complexity of carbohydrates complicates the establishment of a relationship between their structure and their biological function. Determination of the structure of the saccharide moiety in biomolecules such as glycoproteins, proteoglycans and polysaccharides requires a technique that allows the identification of the individual monosaccharides and determines how they are linked to each other. Selective enzymatic cleavage of complex carbohydrates can be used to determine how monosaccharides are interconnected to form carbohydrates. In addition, a separation and identification technique for monosaccharides that is compatible with the enzymatic cleavage is required.

Separation and identification methods for monosaccharides, such as high-performance liquid chromatography, gas chromatography and thin-layer chromatography, usually require large samples [1]. Ideally, determination of carbohydrate structure requires the handling and analysis of samples in very small volumes. For example, if complex carbohydrates or monosacchaarides from a single cell are to be analyzed, a technique that provides high sensitivity and low detection limits such as capillary electrophoresis with laser-induced fluorescence detection (CE–LIF) is required.

CE–LIF is a useful technique for the trace analysis of biological molecules such as DNA,

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proteins, peptides and carbohydrates [2,3]. However, CE–LIF analyses of saccharides are often more difficult than analyses of other biomolecules, since saccharides do not have a net charge (except for acidic saccharides). Thus, direct separation of saccharides by CE is not possible. The use of borate as a complexing agent in the separation buffer facilitates the formation of carbohydrate–borate complexes that are charged; thus, electrophoretic separation is possible [4–12].

Another difficulty in analyzing carbohydrates by CE–LIF is the lack of a native fluorophore in the molecule, making it necessary to attach a fluorescent tag to the molecule. Some procedures for fluorescent labeling of saccharides use direct attachment of an amine-containing fluorescent tag to the reducing hydroxyl of the saccharide. Reagents such as 2-aminopyridine (AP), 5-aminonaphthalene-2-sulfonate (ANS) and 7-amino-1,3-naphthalenedisulfonate (ANTS) can be conjugated to the anomeric carbon of the saccharide through reductive amination using sodium cyanoborohydride [13–16]. ANTS is an attractive reagent since it fluoresces at longer wavelengths than AP and ANS and it provides the labeled saccharide with three negative charges that contribute to labeled-saccharide electrophoretic mobility while AP and ANS provide no and one negative charge to the labeled saccharide, respectively. Other procedures require reduction of the hydroxyl group at the anomeric carbon to form a primary amine, followed by labeling with an amine-reactive probe. Liu et al. [17] added a primary amino group to the anomeric carbon by reacting the saccharide in the presence of sodium cyanoborohydride and excess of ammonium.

In general, fluorescent labeling reactions for saccharides require extremely high concentrations of the saccharide and the labeling reagent. Zhao et al. [12], despite attaining the lowest limit of detection for an aminated sugar monomer ever reported (60 molecules), used 1 m*M* 5-carboxytetramethylrhodamine succinimidyl ester to label 20 m*M* aminated sugar. Since the level of some carbohydrates found in biological samples is submicromolar, the labeling scheme should be effective at those levels. In addition, high concentrations of fluorescent labeling reagents can result in overlapping reagent peaks in the electropherograms. Hence techniques that allow labeling of carbohydrates at submicromolar levels and do not result in electropherograms containing reagent peaks are required to handle many biological samples.

Novotny and co-workers introduced the use of 3-(*p*-carboxybenzoyl)quinoline-2-carboxyaldehyde (CBQCA) as a fluorogenic reagent to label monosaccharides and also polysaccharides that have been previously aminated [17–19]. This approach offers the advantage that the reagent does not fluoresce unless it reacts with an amine group. Thus, electropherograms are free of reagent peaks that in other cases may overlap with the saccharide derivative peaks. Using CBQCA for labeling aminated sugars, excitation with 457-nm radiation from an argon ion laser and on-column detection, Liu et al. [8] reported a limit of detection (LOD) in the sub-attomole range.

Here we report a method for CBQCA labeling of monosaccharides and demonstrate the labeling of 10^{-9} *M* 1-glucosamine. The CBQCA derivative of 1-glucosamine is then detected by CE–LIF. The highly sensitive detection scheme is based on the selection of an alternative excitation wavelength that minimizes interference from water Raman scatter. The CE separation of five aminated monosaccharides based on the formation of charged complexes with borate and phenyl boronate is presented to illustrate the compatibility among the labeling technique, CE separation and LIF detection.

2. Experimental

2.1. Instrument

The CE–LIF instrument has been described elsewhere [20]. Non-derivatized capillaries (180 μm O.D., 50 μm I.D. or 142 μm O.D., 10 μm I.D.) were used for the separation. A CZE 1000R high-voltage power supply (Spellman, Plainview, NY, USA) with a maximum output of

29 kV provided a positive high voltage at the injection end (cathodic mode). Usually, CBQCA derivatives of aminated sugars (maximum excitation at 456 nm) are excited with 442-nm radiation from a He–Cd laser or 457-nm radiation from an argon ion laser and the emission is detected at 552 nm [8,9]. In our experiment, fluorescence was excited with 488-nm radiation from a multiple-wavelength argon ion laser (INNOVA 99-4; Coherent, Palo Alto, CA, USA) set at 30 mW. The detector was arranged in order to eliminate the water Raman band (577–596 nm) associated with excitation with 488-nm radiation. Detection was based on light collection by two R1477 photomultiplier tubes (Hamamatsu, Middlesex, NJ, USA). A dichroic beam splitter, 590DRLP@45 (Omega Optical, Brattleboro, VT, USA), reflected light with wavelengths shorter than 590 nm (reflected channel) and transmitted light with wavelengths longer than 590 nm (transmitted channel). Reflected light was sent through a 535DF35 bandpass filter (transmission range 515–555 nm; Omega Optical) before reaching the reflected channel photomultiplier tube. For the transmitted channel, light was transmitted through a 635DF55 bandpass filter (transmission range 605–685 nm; Omega Optical) before reaching the photomultiplier tube. The PMT outputs were digitized using an NB-MIO-16X-18 input/output board (National Instruments, Austin, TX, USA). The digitized signals were summed to obtain a combined signal that includes fluorescence in the 515–555 and 605–685 nm ranges and excludes light from 552.5 to 607.5 nm. The water Raman band is in the range 577–596 nm, which is effectively blocked by this detector configuration, resulting in a lower background and improved limits of detection.

2.2. Preparation of sugar derivatives

Glucose, galactose, mannose, and fucose were reductively aminated to produce their 1-amino-1-deoxy-D-alditols. 2-Amino-2-deoxy-D-glucitol was synthesized by reduction of N-acetylglucosamine. Their synthesis has been described elsewhere [12]. In this paper, the alditols will be referred to as 1-glucosamine, 1-galactosamine, 1-mannosamine, 1-fucosamine and 2-glucosamine.

2.3. CBQCA labeling reaction

Stock solutions ($1.0 \cdot 10^{-2}$ *M*) of the sugar derivatives were prepared in 0.185 *M* sodium hydrogencarbonate (BDH, Toronto, ON, Canada) and stored at 4°C. Dilutions for labeling reactions were made with HPLC-grade water (Fisher Scientific, Nepean, ON, Canada). Dilutions for electrokinetic injections were made with 10 m*M* sodium borate (Fisher Scientific) and 10 m*M* sodium dodecyl sulfate (BDH) buffer (BS buffer). A 10 m*M* stock solution of CBQCA (Molecular Probes, Eugene, OR, USA) was prepared in methanol and stored at −20°C. Potassium cyanide (Molecular Probes) was dissolved in HPLC-grade water to obtain a 0.20 *M* stock solution and stored at 4°C.

The labeling reaction mechanism has been described elsewhere [18]. The reaction starts with the nucleophilic attack of cyanide on CBQCA and then the amine group is incorporated and participates in the formation of a quinoline ring; the resulting derivative has highly fluorescent properties compared with the unreacted CBQCA reagent. The labeling reaction was performed by mixing 5–10 μl of a dilution of the aminated sugar in HPLC-grade water, 10 μl of the CBQCA stock solution and an aliquot of methanol (or phosphate buffer) to make up 23 μl. Then 2 μl of 50 m*M* cyanide were added to the mixture. The 25 μl of reaction mixture contained 4 m*M* CBQCA, 4 m*M* cyanide and 10^{-4}–10^{-9} *M* aminated sugar. After vortex mixing, the mixture was incubated in the dark for 2–10 h. The mixture was vortex mixed every 30 min. A blank was prepared by replacing the aminated sugar aliquot with water. The reaction mixtures could be stored at −20°C for 1 week without noticeable degradation.

The effect of pH on the labeling reaction was determined by using 100 m*M* phosphate buffer instead of methanol to make the reaction volume 25 μl. The reaction mixture contained 4 m*M*

CBQCA and 2 m*M* cyanide and the reaction was carried out for 4 h.

2.4. Separation

Prior to electrokinetic injection, CBQCA derivatives of aminated sugars were diluted to $2.0 \cdot 10^{-7}$ *M* in BS buffer. This concentration is based on a 100% yield for the labeling reaction. For concentrations $\leq 2.0 \cdot 10^{-7}$ *M*, no dilution was performed prior to injection.

Separation buffers were prepared from stock solutions of 0.556 *M* sodium dodecyl sulfate, 0.20 *M* sodium phosphate dibasic acid (Fisher), 0.20 *M* sodium borate and 0.10 *M* phenylboronic acid (Sigma, St. Louis, MO, USA). The pH was adjusted with 1.1 *M* sodium hydroxide (BDH). Although several buffers were investigated for use in the separation, the buffer selected for the separation contained 50 m*M* phenylboronic acid, 20 m*M* phosphate dibasic acid and 20 m*M* borate (pH 9).

3. Results and discussion

3.1. Selection of labeling reaction conditions

The conditions for the labeling reaction were optimized using 1-glucosamine. The conditions required to maximize the reaction yield depended on the concentration of 1-glucosamine. For samples with high concentrations of 1-glucosamine (e.g., 10^{-4} *M*), the labeling reaction had its highest yield when 6 m*M* CBQCA and 8 m*M* cyanide were used. On the other hand, samples with low 1-glucosamine concentrations (e.g., 10^{-9} *M*) could not be detected by CE–LIF when these concentrations of CBQCA and cyanide were used. Samples with low concen-

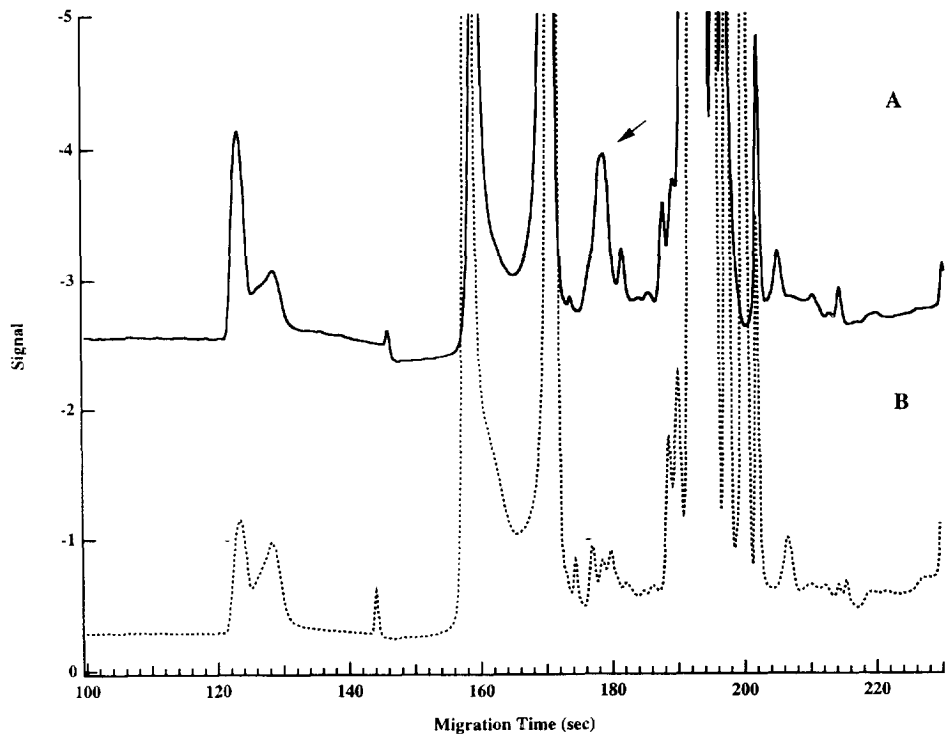

Fig. 1. Electropherogram of a fluorescently labeled sample containing (A) 10^{-9} *M* 1-glucosamine and (B) a reaction blank. Reaction conditions: (A) 10^{-9} *M* 1-glucosamine–4 m*M* CBQCA–4 m*M* KCN for 10 h at room temperature; (B) 4 m*M* CBQCA–4 m*M* KCN for 10 h at room temperature. Separation conditions: 41.5 cm × 50 μm I.D. capillary; running buffer, 10 m*M* borate–10 m*M* SDS; 400 V/cm. Injection conditions: 2500 V for 5 s.

trations of 1-glucosamine had their highest yield when 4 m*M* CBQCA and 4 m*M* cyanide were used in the labeling reaction.

Fig. 1A shows an electropherogram of a sample containing $1.0 \cdot 310^{-9}$ *M* 1-glucosamine that was labeled with CBQCA and detected by CE–LIF. The blank peaks attributed to secondary reactions between CBQCA and cyanide (Fig. 1b) are much larger than the peak of the CBQCA derivative of 1-glucosamine. It has been observed that cyanide can induce condensation of two aromatic aldehydes to form an α-hydroxy ketone; many condensation products are possible [21]. Formation of cyanohydrins is another possible explanation for the secondary peaks that appear in the electropherograms [22]. However, the peak of the CBQCA derivative elutes in a region, 170–190 s, where the electropherogram baseline is relatively flat. If the concentrations of CBQCA or cyanide used in the labeling reaction are higher than 4 m*M* each, the baseline is irregular in the elution region of the CBQCA derivative, making its detection impossible at this low concentration. A method to eliminate the fluorescent secondary products for the labeling reaction of insulin B with FQ (a fluorogenic reagent that has similar chemistry to CBQCA) could be a good alternative to obtain cleaner electropherograms [23].

The pH of the labeling reaction has been known to affect the yield of the CBQCA labeling reactions of different amine-containing molecules. Amino acids and small peptides have their highest reaction yield when the labeling reaction is carried out at pH 8.5–9.5; reaction yields for larger peptides are pH insensitive [18] and sugar reaction yields are highest at pH 7.0 [9]. The yield of the 5-h labeling reaction of 10^{-4} *M* 1-glucosamine with CBQCA varied with pH. For pH 7.1, 8.0 and 9.0 the relative yield was 0.5 ± 0.1, 1.0 ± 0.1 and 0.6 ± 0.1, respectively. If no phosphate buffer was used to regulate the pH in the sample, the reaction yield was about 40 times lower. Despite the low reaction yield when the reaction mixture lacks phosphate buffer, samples with low concentrations of 1-glucosamine (e.g., 10^{-9} *M*) were labeled without addition of such buffer. Since samples containing low concentrations of 1-glucosamine cannot be diluted prior to injection, the presence of phosphate buffer results in an unstable baseline around the elution time of the CBQCA derivative of 1-glucosamine in addition to tailing of the peak. To compensate for the slow kinetics when no phosphate buffer is used, the reaction time was extended to 10 h.

3.2. Sample concentration range for labeling of 1-glucosamine

Samples containing $1.0 \cdot 10^{-4}$–$1.0 \cdot 10^{-9}$ *M* 1-glucosamine were labeled using 4 m*M* CBQCA and 4 m*M* cyanide; these reagent concentrations allowed labeling of 1-glucosamine over the whole concentration range. The labeling reaction was carried out at room temperature for 10 h without using phosphate buffer in the reaction mixture. Fig. 2 shows a plot of signal intensity corrected for dilution versus concentration of 1-glucosamine in the sample prior to derivatization. The non-linearity at the low end of the concentration range is attributed to the presence of methanol in the sample. As the 1-glucosamine concentration was decreased, less dilution was required prior to injection, resulting in higher concentrations of methanol in the injected dilution. The presence of methanol resulted in an increased local electric field during injection and a resultant increase in the amount of sample injected.

3.3. Limit of detection for CBQCA-labeled 1-glucosamine

The LOD (three times the standard deviation of the background) for the fluorescently labeled sugar is calculated from the injection volume [24] and the total concentration of fluorescently labeled aminated sugar. The LOD for fluorescently labeled 1-glucosamine is 75 zeptomol and $4.5 \cdot 10^{-11}$ *M* 1-glucosamine (based on a labeling reaction of 10^{-4} *M* and diluted to $5 \cdot 10^{-7}$ *M* prior to injection). This estimate is conservative, since it assumes 100% labeling of the aminated sugar. This LOD is more than one order of magnitude lower than those reported by Novotny's group for the labeling of monosac-

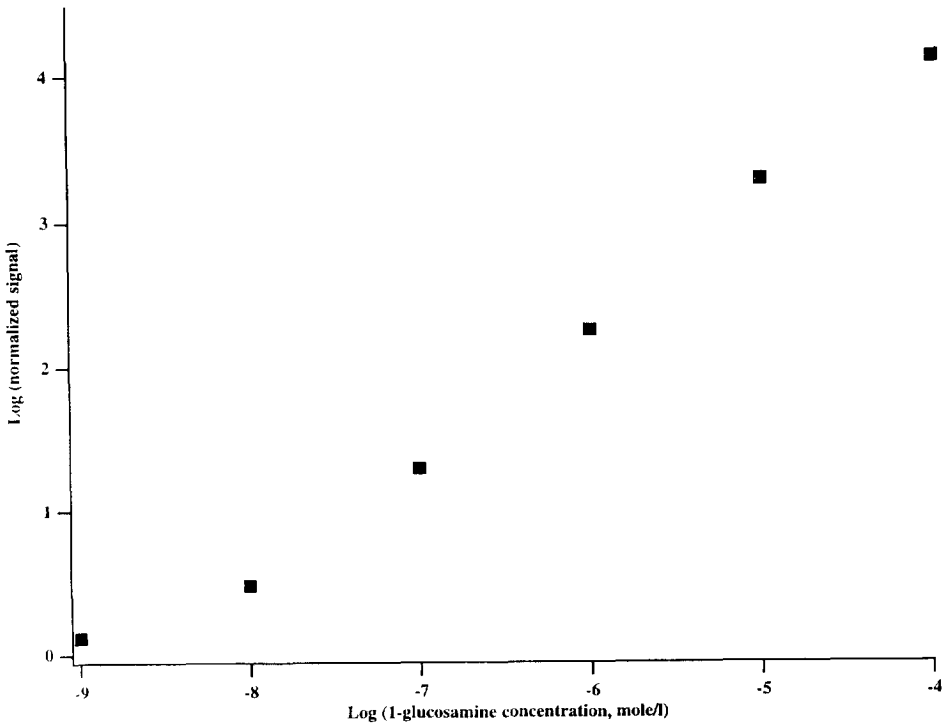

Fig. 2. Concentration range of 1-glucosamine for an effective labeling reaction. Reaction conditions: 10^{-9}–10^{-4} *M* 1-glucosamine–4 m*M* CBQCA–4 m*M* KCN for 5 h at room temperature. Separation conditions as in Fig. 1. Injection conditions: prior to injection (2500 V for 5 s) samples were diluted to $1.0 \cdot 10^{-8}$ *M* (labeled and unlabeled) 1-glucosamine, except for labeling reaction samples containing 10^{-9}–10^{-8} *M* 1-glucosamine.

charides with CBQCA (2.3 attomol for 1-mannosamine, 1.3 attomol for 1-glucosamine, 0.5 attomol for 1-galactosamine [8] and 240 attomol for 1-galactosamine) [9]. The improvement in the LOD reported here is attributed to the use of a sheath flow cuvette with low light scattering as a postcolumn detector, which results in low background and elimination of the main water Raman band from the background through improved spectral filtering. Novotny's group used the 457-nm radiation from an argon ion laser for excitation since it has better overlap with the absorption profile of the derivative; however, the wavelength of the water Raman band coincides with the maximum fluorescence, increasing the background signal.

3.4. Reaction yield for different aminated sugars

As expected, the yield of the CBQCA labeling reaction for the five aminated sugars studied here is not the same. Fig. 3 shows the electropherograms of the CBQCA derivative of the five different aminated sugars labeled, injected and separated under identical conditions. The relative yields were calculated from the relative area under the peak for each aminated sugar. The highest yield was observed for 1-fucosamine (100%) and the lowest for 1-mannosamine (26%). The yields for 1-galactosamine, 2-glucosamine, and 1-glucosamine were 91%, 84% and 66%, respectively. These results suggest that limits of detection for the other aminated sugars will be of the same order of magnitude as for 1-glucosamine.

3.5. Separation of CBQCA derivatives of five aminated sugars

The five aminated sugars investigated, 1-glucosamine, 1-mannosamine, 1-fucosamine, 1-galactosamine and 2-glucosamine, form CBQCA

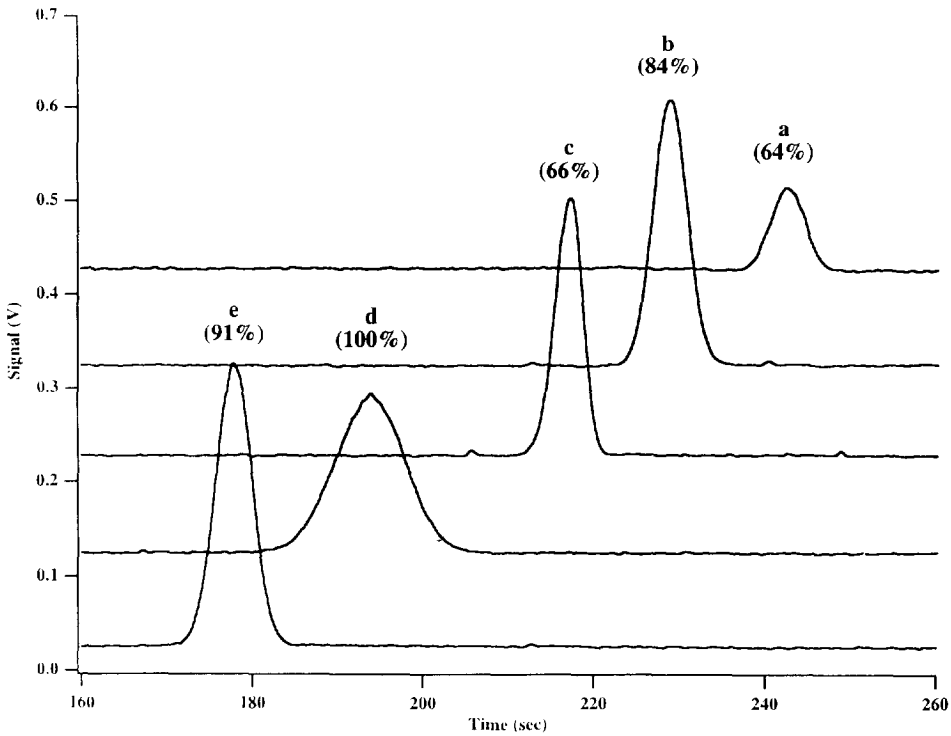

Fig. 3. Comparison of the electropherograms for five aminated monosaccharides labeled under identical conditions. (a) 1-Mannosamine; (b) 2-glucosamine; (c) 1-glucosamine; (d) 1-fucosamine; (e) 1-galactosamine. The time axis does not represent the migration time since peak positions have been offset. Relative area under the peak is indicated in parentheses. Reaction conditions: monosaccharides (10^{-4} *M*) were labeled individually; 4 m*M* CBQCA–2 m*M* KCN (pH 7.1) for 2 h at 50°C. Separation conditions as in Fig. 1 except for 49.6-cm long capillary. Injection conditions: samples were diluted to $1.0 \cdot 10^{-6}$ *M* (labeled and unlabeled) aminated monosaccharide prior to injection (1000 V for 5 s).

derivatives that are expected to have similar electrophoretic mobilities since they have similar molecular masses and the same net charge. They cannot be resolved in a conventional electrophoretic separation based on mass-to-charge ratio. However, if borate or phenylboronate is added to the running buffer, the derivatives can be separated since borate and phenylboronate complex with the CBQCA derivatives. Borate complexation is due to the interaction of hydroxyl groups in the sugar moiety with tetrahydroxyborate ions $B[OH]_4^-$, one of the borate species present at pH 8–12 [4,5]. The charge of the complex is proportional to its formation constant. For example, favorable configurations (with high formation constants) such as the *cis*-oriented pair of hydroxyl groups at C-2 and C-4 and the *cis*-1,2-diol configuration of the monosaccharide will have a greater net charge. Differences in mass-to-charge ratio for the various complexes allow for their electrophoretic separation.

The use of borate in the running buffer for the separation of monosaccharides or their derivatives has been used by several workers [5,8,9,11]. However, based on their electropherograms, complete separation of the five aminated sugars used here was not achieved. Evidence that the use of phenylboronate in the running buffer allows better separations of monosaccharides has also been published by Zhao et al. [12] for derivatives of aminated sugars labeled with 5-carboxytetramethylrhodamine succinimydyl ester. They reported the separation of the derivatives of 1-glucosamine, 2-glucosamine, 1-mannosamine, 1-galactosamine, 2-galactosamine, and 1-fucosamine.

Here, the separation of the CBQCA deriva-

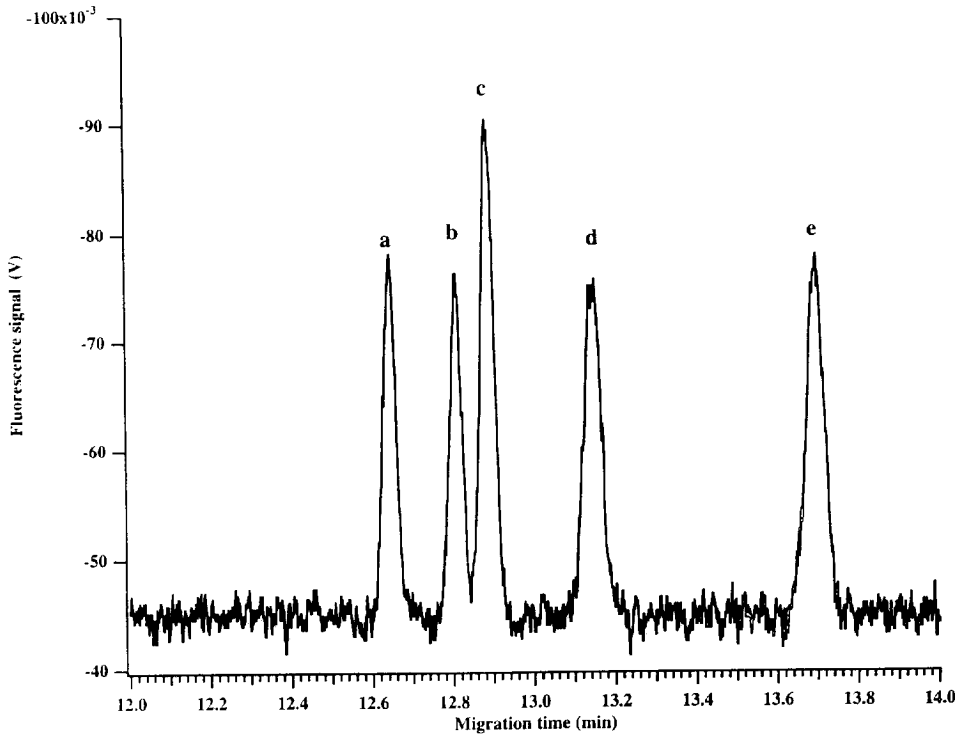

Fig. 4. Separation of five CBQCA derivatives of monosaccharides. (a) 1-Mannosamine; (b) 2-glucosamine; (c) 1-glucosamine; (d) 1-fucosamine; (e) 1-galactosamine. Reaction conditions: monosaccharides ($1 \cdot 10^{-4}$ *M*) were labeled individually, 4 m*M* CBQCA–4 m*M* KCN for 5 h at room temperature. Separation conditions: 72.5 cm × 10 μm I.D. capillary; running buffer, 20 m*M* phosphate–50 m*M* phenylboronate–20 m*M* borate; 400 V/cm. Injection conditions: samples were diluted to $2.0 \cdot 10^{-7}$ *M* (labeled and unlabeled) aminated monosaccharide prior to injection (2.5 kV for 5 s).

tives of 1-glucosamine, 2-glucosamine, 1-mannosamine, 1-galactosamine and 1-fucosamine was possible with the running buffer 50 m*M* phenylboronate–20 m*M* phosphate–20 m*M* borate. Fig. 4 shows the separation of the CBQCA derivatives of the five aminated sugars. In separations using 30 m*M* (or lower) phenylboronate, the CBQCA derivatives for 1-glucosamine and 2-glucosamine were not resolved. Use of higher concentrations of borate (in the absence of phenylboronate) did not allow the complete separation of the five aminated sugars. At present, no information on the interaction of boronate with carbohydrates is available. The complex stability is expected to be different for boronate complexation than it is for borate, since the bulky phenyl group would introduce some steric hindrance. The interaction of the hydroxyl groups in the sugar moiety with trihydroxyboronate, $B(Ph)(OH)_3$ would increase the mass of the monosaccharide but it would not affect the charge of the monosaccharide. Competitive equilibrium between boronate and borate for the monosaccharide would increase the structural differences among the different monosaccharide complexes, resulting in a better separation. Competitive borate and phenylboronate complexation may prove to be useful for the separation of other monosaccharides and oligosaccharides.

4. Conclusions

Samples with concentrations as low as $1.0 \cdot 10^{-9}$ *M* 1-glucosamine or other aminated monosaccharides can be fluorescently tagged using CBQCA as a fluorogenic reagent. This sensitivi-

ty results from the selection of labeling conditions that favor the formation of the CBQCA sugar derivative over the secondary fluorescent products. Further improvement comes from the use of a fluorescent detector with low background signals. The separation of five basic aminated sugars using a mixture of phenylboronate and borate in the separation buffer was also demonstrated. These results illustrate a promising methodology for the separation and identification of very low levels of monosaccharides.

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