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Capillary zone electrophoresis of oligosaccharides derivatized with N-(4-aminobenzoyl)-L-glutamic acid for ultraviolet absorbance detection¹

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Abstract

A charged and strongly UV-absorbing tag, N-(4-aminobenzoyl)-L-glutamic acid (ABG), was coupled to oligosaccharides by reductive amination under mild conditions. The effectiveness of ABG as a derivatization agent is shown through the separation of isomaltooligosaccharides from a dextran hydrolysate. The minimum detectable quantities in the subpicomole range are demonstrated.

Keywords: Derivatization, electrophoresis; Oligosaccharides; N-(4-Aminobenzoyl)-L-glutamic acid

1. Introduction

Analysis of carbohydrate oligomers plays an important role in a complete characterization of polysaccharides and other glycoconjugates. In spite of the growing need for improved methodologies in the area, there is a lack of sensitive, simple and inexpensive techniques for rapid characterization of oligosaccharide mixtures.

Capillary electrophoresis (CE) with its sensitive detection techniques holds much promise for carbohydrate analysis. The methodological aspects and applications of CE to glycoconjugates have recently been reviewed [1–4]. There are two general ways of analyzing complex carbohydrate samples: (a) without

any sample modification, carbohydrates separated by CE are detected spectroscopically at 195 nm [5], or amperometrically [6,7]; and (b) using a derivatization step for introduction of a chromophore or a fluorophore into the molecules of interest, the derivatized carbohydrates are separated and detected spectroscopically. Such a tagging step can also positively influence a separation in CE through introduction of additional charge to neutral saccharide molecules. Very high detection sensitivity can be achieved through appropriate fluorogenic labeling [8,9] and the use of laser-induced fluorescence. Highly charged tagging reagents, such as aminonaphthalene trisulphonic acid (ANTS) [10,11] and 9-aminopyrene-1,4,6-trisulfonate, have added advantages of a rapid and efficient separation [12].

While fluorescence-tagging procedures provide the utmost in terms of sensitive detection, there is still a need for UV-absorbing tags because they are less expensive, and because CE instruments equipped with UV detectors are readily available. A reagent developed initially for liquid chromatography of

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carbohydrates, 2-aminopyridine, can also be utilized in CE [13,14]. Using this approach, it has been demonstrated that the fluorescent aminopyridine derivatives can also be detected by UV detection (240 nm, excitation wavelength) with good mass sensitivity (low picomoles) [15]. Similarly, the ANTS derivatives can be detected at 214 nm [10]. Derivatization with 2-aminopyridine suffers from some disadvantages. First, the coupling procedure takes place under extreme conditions at which sample decomposition could occur, and the reaction is relatively slow [16]. Second, the formed derivatives are not strongly charged, so that the separation efficiency for carbohydrate oligomers in CE appears relatively low. The strongly absorbing products of reaction between carbohydrates and *p*-aminobenzoic acid were recently investigated as alternative derivatives [17]. Various monosaccharides (aldoses, ketoses, and uronic acids) have been derivatized for CE analysis through the action of ethyl *p*-aminobenzoate [18] or *p*-aminobenzoic acid [19]. Monitoring absorbance at 285 nm and 305 nm for *p*-aminobenzoic acid and ethyl *p*-aminobenzoate derivatives, respectively, appears to facilitate detection with mass sensitivity in the femtomole range. Moreover, the wavelengths close to 300 nm are advantageous in the analysis of complex, "real" samples (e.g., extracts or hydrolyzates) because they are more specific than lower wavelengths. Honda et al. [20] introduced another strongly UV-absorbing chromophore, 3-methyl-1-pyrazolin-5-one (PMP) for the analysis of mono- and oligosaccharides. Still, all these derivatives have some disadvantages for oligosaccharide analysis: they are only weakly charged, resulting in relatively low separation efficiencies. Maltooligosaccharides of up to only 8–9 sugar units could be resolved as borate complexes for both 2-aminopyridine [14] and ethyl *p*-aminobenzoate [19], while the separation of PMP derivatives was slightly better [20].

As an alternative to the above-mentioned reagents, we have investigated the properties of *N*-(4-aminobenzoyl)-*L*-glutamic acid, which possesses more charge in the molecule (than the other reported benzoic derivatives) for the benefit of optimum CE separation and can be detected more specifically and sensitively at wavelengths close to 300 nm (compared to ANTS). This is a useful strategy for the

analysis of complex biological mixtures where underivatized endogenous compounds, absorbing at lower wavelengths, could cause interference. This reagent is shown here to be employed quite easily for derivatization of the aqueous solutions of oligosaccharides. The study was primarily aimed at finding a simple and rapid procedure to analyze complex oligosaccharide mixtures cleaved from various glycoconjugates.

2. Experimental

2.1. Materials

N-(4-Aminobenzoyl)-*L*-glutamic acid was purchased from Fluka (Buchs, Switzerland). Dextran (from *Leuconostoc mesenteroides*, M_w 18 300) and maltooligosaccharide standards were all from Sigma (St. Louis, MO, USA). All other common chemicals, also purchased from Sigma, were of the highest available purity.

2.2. High-performance capillary electrophoresis (HPCE)

A commercial apparatus (Model 3850, ISCO, Lincoln, NE, USA) for capillary electrophoresis was equipped with a UV absorbance detector and with an accessory allowing semi-automatic capillary operation and a programmed electrokinetic injection. An uncoated capillary (Polymicro Technologies, Phoenix, AZ, USA), used in our experiments, was of 75 μm I.D. and 360 μm O.D. A capillary, coated with a copolymer of hydroxypropylcellulose and hydroxyethyl methacrylate, was prepared according to a recently developed procedure [21].

2.3. Derivatization procedure

The reagent solution was prepared freshly before derivatization by adding 20 mg sodium cyanoborohydride to 1-ml volume of 4% (w/v) *N*-(4-aminobenzoyl)-*L*-glutamic acid (ABG) in 50% (v/v) acetic acid. One part of the reagent solution was mixed with three parts of the aqueous sample solution containing up to 2 mg/ml of a carbohydrate sample. This mixture was heated in a screw-capped glass vial

for 2–3 h, at 90°C, in a water bath. Subsequently, the vial was cooled and, depending on the original carbohydrate concentration, diluted appropriately prior to the electrophoretic analysis.

2.4. Dextran hydrolysis

A 10-mg amount of dextran (M_w 18 300) was dissolved in 1 ml 0.2 M hydrochloric acid and kept for 2 h in a boiling-water bath. An aliquot obtained from the partially hydrolyzed dextran solution was derivatized as described above (Section 2.3).

2.5. UV-Vis spectral measurements

Maltopentaose (2 mg/ml) was derivatized through the above procedure (Section 2.3). A 100- μ l volume of the resulting mixture was applied onto the column of Bio-Gel P-2 (0.6 \times 17 cm) and eluted with water at the flow-rate of 1.0 ml/h. Fractions (0.5 ml) were collected, while absorbance at 273 nm was monitored. Two major fractions were separated. The fractions representing the maxima of the two separated peaks (the first fraction located within 2 and 3 ml volumes, and the second fraction between 6 and 7 ml) were appropriately diluted and their spectra recorded. The first eluted peak corresponded to a derivatized oligosaccharide, while the second fraction belonged to the remaining reagent. For comparison and estimation of the molar absorptivity, the spectrum of pure ABG in water was also measured. The spectra were recorded on a Shimadzu Model UV-160 (Kyoto, Japan) spectrophotometer.

3. Results and discussion

3.1. Derivatization reaction

The derivatization utilizes typical conditions of reductive amination described already [17–19] in detail for *p*-aminobenzoic acid and its derivatives. In the first step, the imine derivative is formed through condensation of the aldehydic group of the carbohydrate with the primary amino group of ABG. Subsequently, the derivative is reduced by sodium cyanoborohydride to a more stable secondary amine.

The progression of the reaction was evaluated

through a comparison of the derivatized maltopentaose peak areas. Dependence of the relative reaction yield upon the concentration of ABG (monitored after 1 and 2 h) is shown in Fig. 1 (curves a and b). The measurements indicated that derivatization of maltopentaose was finished in approximately 2 h. Concentration of acetic acid was set at 12% (v/v), as, during preliminary testing, we learnt that the acetic acid concentrations above 10% guaranteed the maximum efficiency of acid catalysis. Additionally, 5 mg/ml of sodium cyanoborohydride in the final mixture was found to ensure the reductive step and was, therefore, kept constant in all remaining work. For complex mixtures with varying concentrations of different carbohydrates, we recommend that the derivatization reaction be carried out for approximately 3 h at 90°C.

The derivatized maltopentaose was separated from the remaining reagent by gel filtration on a column of Bio-Gel P-2. The corresponding spectra are shown in Fig. 2. The spectrum of the ABG reagent after derivatization reaction was exactly the same as that of pure ABG, with the absorption maximum at 273 nm. The molar absorptivity of pure, commercial ABG was measured and calculated to be 17 000 at 273 nm. This value is slightly lower than that measured for *p*-aminobenzoic acid (18 200) at 289 nm [22]. However, one can observe a significant shift in the absorbance maximum after derivatization; derivatized maltopentaose has a broad maximum around 291 nm. This phenomenon is actually favorable for enhancing the specificity of detection and one can assume that a similar shift in the absorption maximum also exists for other derivatized carbohydrates.

We also checked the efficiency of derivatization for dilute maltopentaose solutions. The derivatization conditions were identical to those described above, except that the time of derivatization was increased to 4 h. The samples were run in 0.2 M sodium borate buffer (pH 10.0) at 6 kV, with detection at 291 nm. The peak areas were calculated and their linear dependence on maltopentaose concentration in un-derivatized sample solutions was observed for the range of 10–500 μ g/ml (12–600 μ M). Knowing the value of molar absorptivity for ABG, we could roughly estimate the amount of derivatized maltopentaose present in the zone detected at the lowest

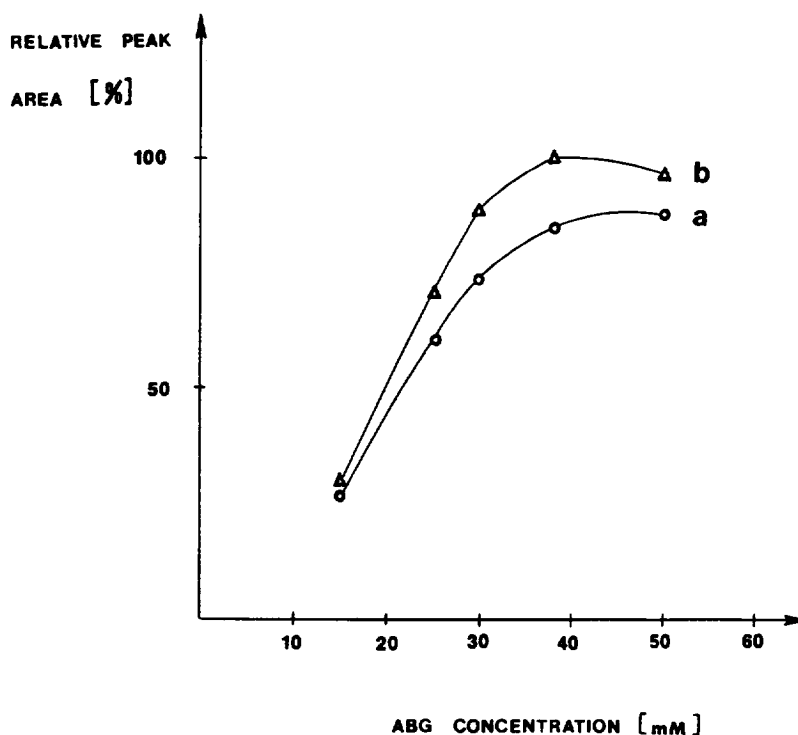


Fig. 1. Dependence of the reaction yield (expressed arbitrarily as relative peak areas of maltopentaose derivative) on the concentration of ABG in the derivatization mixture. Reaction time: (a) 1 h; (b) 2 h. The concentrations of additional components: 2 mg/ml maltopentaose; 5 mg/ml sodium cyanoborohydride; 12% (v/v) acetic acid. Electrophoretic conditions: voltage, 10 kV; buffer: 0.1 M sodium borate, pH 8.5; electrokinetic injection, 3 s (5 kV); uncoated capillary, 75 μ m I.D. and 360 μ m O.D.; effective length, 50 cm; total length, 70 cm. Prior to analysis, the samples were diluted 25-fold.

concentration of maltopentaose in an underivatized sample to be ca. 170 fmoles. The optimization and evaluation of the derivatization procedure for a model compound, maltopentaose, was done orientationally, as we experienced slight differences in the derivatization of various oligosaccharides.

3.2. Electrophoretic separations using uncoated capillaries

Optimization of the separation conditions was achieved through testing the migration behavior of derivatized maltopentaose and maltohexaose. Their resolution, R_s , was calculated as follows: $R_s = 2(t_p - t_H)/(w_p + w_H)$, where t_p and t_H are the migration times for the derivatized maltopentaose and maltohexaose, and w_p and w_H are the widths of their peaks, respectively. As seen in Fig. 3, the best resolution at a constant borate concentration (100

mM) was achieved at pH 10.0. Fig. 4 shows a typical separation of maltopentaose and maltohexaose at this pH. Subsequently, the best resolution at pH 10.0 was found at 200 mM borate concentration. One should mention that at higher borate concentrations (>0.2 M), the electroosmotic flow is significantly reduced, so that the migration times of the derivatized carbohydrates become excessive and the peaks are diffusionally broadened. The electrokinetic injection is also less effective at higher borate concentrations due to a lowered electroosmotic flow (especially for smaller oligosaccharides with higher electrophoretic mobilities). This problem could be partly overcome through the use of hydrodynamic injection.

The acidic pH (around 3.5) of the derivatization mixture is advantageous for a direct electrokinetic injection, as the carboxylic groups of ABG are only partially ionized (pK_a -values of glutamic acid carboxyls are 2.19 and 4.25, respectively [23]) so that

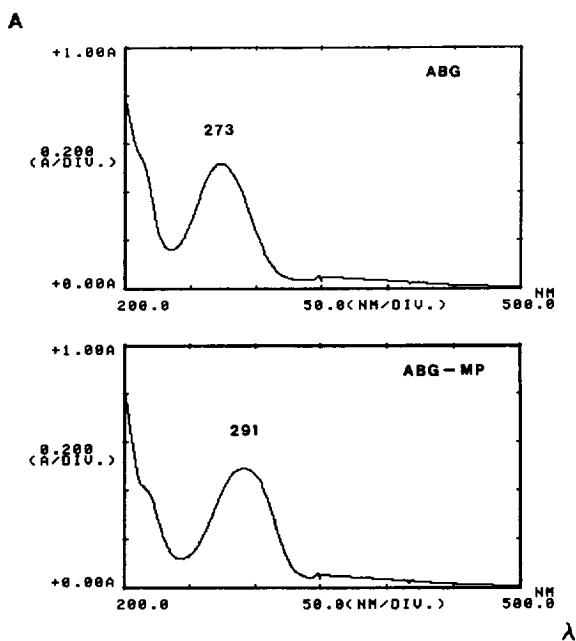


Fig. 2. Absorbance spectra of the derivatization reagent *N*-(4-aminobenzoyl)-*L*-glutamic acid (ABG) and the derivatized maltopentaose (ABG-MP), separated on a Bio-Gel P-2 column after derivatization.

during the time of injection, the derivatized carbohydrates do not migrate significantly in the direction opposite to that of the electroosmotic flow. A high borate concentration is also favorable for efficient complexation of carbohydrates, limiting band dispersion.

The best separations of various oligosaccharide mixtures (5–20 sugar units) were typically achieved at voltages between 60 and 120 V/cm (see Fig. 5). However, at such voltages, the separation of mono- and/or disaccharides from the ABG reagent and the impurities present in commercially available ABG becomes poor. Fortunately, such impurities do not directly disturb the analysis of higher oligosaccharides in a typical glycoconjugate analysis. Purification of the commercially available ABG reagent may be necessary for monosaccharide analyses.

3.3. Separation of derivatized oligosaccharides in a coated capillary

As expected, the separation efficiency for borate-complexed oligosaccharides in a coated capillary

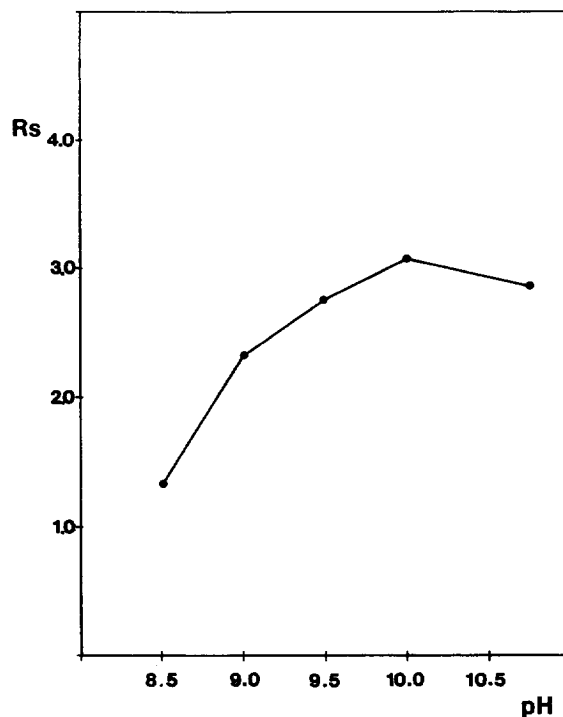


Fig. 3. pH-dependence of the resolution, R_s , between the ABG-derivatized maltohexaose and maltopentaose solutes at a constant borate concentration (100 mM). Electrophoretic conditions: voltage, 10 kV; electrokinetic injection; uncoated capillary, as described in Section 2.2.

without electroosmotic displacement was substantially better than in an uncoated capillary (compare Figs. 5 and 6). Here, we could record oligosaccharides of up to about 40 glucose units without a loss of resolving power. As a capillary coating material, we have employed a hydrolytically stable hydroxypropylcellulose treatment tested recently in our laboratory [21]. The optimum pH range for the separation of ABG-derivatized oligosaccharides from hydrolyzed dextran in such a coated capillary was 8.5–9.0 (Tris–borate buffer). At pH higher than 9.0, the coating generated a small electroosmotic flow which acted against slowly migrating higher oligosaccharides.

While the sample analyzed in a coated and uncoated capillary was the same, the experimental conditions differed: in the uncoated capillary, we used a concentrated (0.2 M) sodium borate buffer of high pH (10.0), while in the coated capillary, a

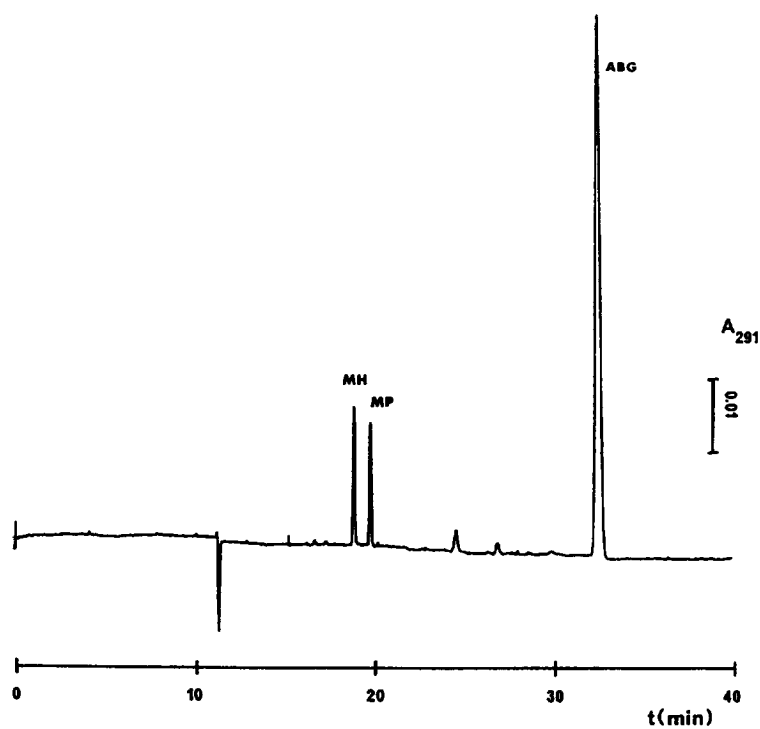


Fig. 4. Separation of maltopentaose and maltohexaose derivatized with ABG. Electrophoretic conditions: voltage, 10 kV; electrokinetic injection, 5 s (5 kV); 100 mM sodium borate buffer (pH 10.0); uncoated capillary.

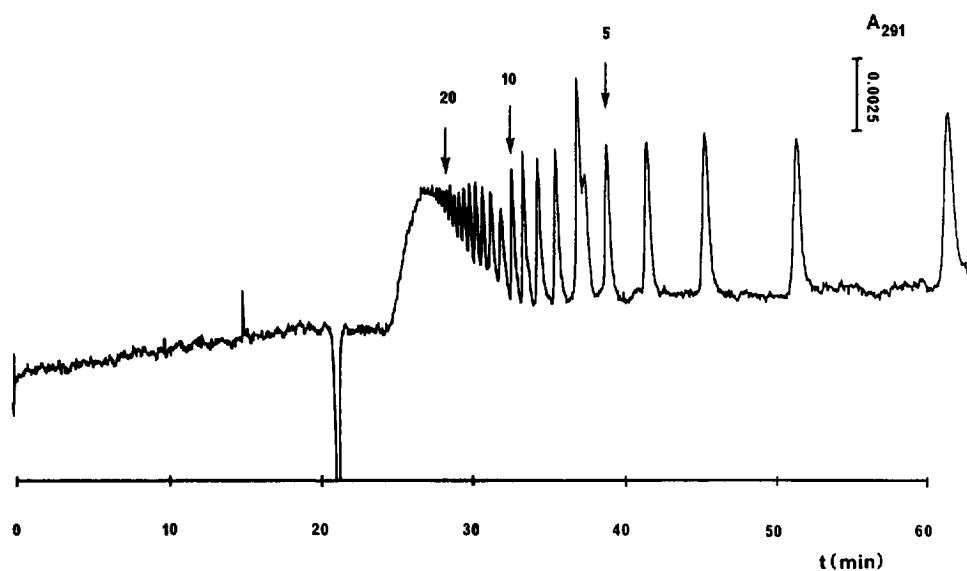


Fig. 5. Separation of the ABG-derivatized oligosaccharides from a partially hydrolyzed dextran, in an uncoated capillary. Electrophoretic conditions: applied voltage, 6 kV (40 μ A); electrokinetic injection, 5 s (5 kV); 0.2 M sodium borate buffer (pH 10.0); uncoated capillary.

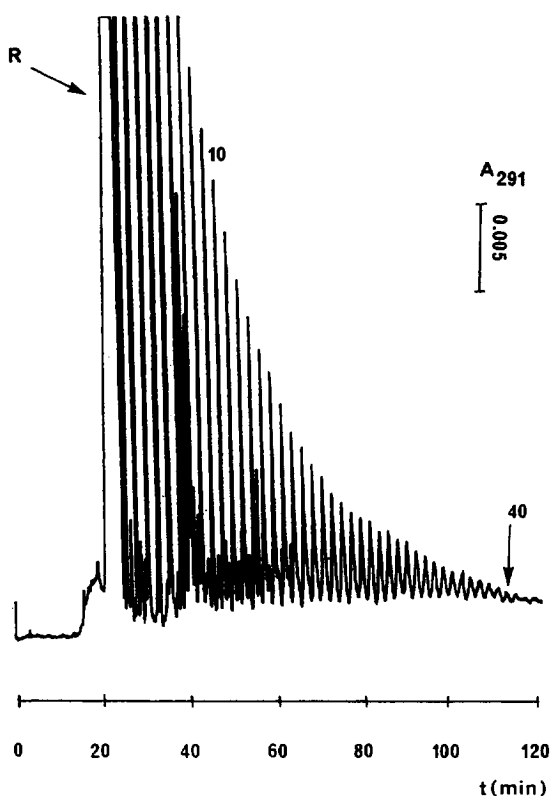


Fig. 6. Separation of the ABG-derivatized oligosaccharides from a partially hydrolyzed dextran sample (same as in Fig. 5), in an HPC-coated capillary. CE conditions: coated capillary; effective length, 50 cm; total length, 70 cm; 100 mM Tris–borate buffer adjusted with NaOH solution to pH 8.8; hydrodynamic injection, 6 s (30-cm reservoir height); R=reagent peak.

Tris–borate buffer of a lower concentration (0.1 M) and pH (8.8) was employed. The sole “irregular” peak between the oligosaccharide peaks in Fig. 5 could be easily identified as an impurity through comparison with electropherogram of the derivatization mixture blank. There were no other peaks present. However, several additional peaks occurred after separation in the coated capillary. Once again, one peak (the highest among the “irregular” peaks of the oligosaccharides of $n > 3$) could be identified as impurity by comparison with the blank. Observation of the additional peaks in Fig. 6 is attributed to occurrence of isomers due to the improvement of separation conditions over those reflected in Fig. 5. It is known that strictly linear dextrans with α -D-glucopyranosyl residues linked together by (1–6)-linkages

rarely exist in nature. The dextran from *Leuconostoc mesenteroides* exhibits some degree of branching that includes a small number of single D-glucosyl or isomaltosyl sidechains attached through (1–3)-linkages [24]. It is also known that borate solutions are complex mixtures of various polyborate anions whose composition depends on pH and ionic strength [25]. Such solutions possess variable complexation power. While glucose is only weakly complexed in 50 mM borate (in contrast to mannose with coplanar *cis*-hydroxyls) [26], the borate–glucose complex of high electrophoretic mobility is formed at high borate concentration (0.2 M) and high pH (10.0) [5]. Thus, it seems that the borate complexation efficiency in uncoated capillary contributes to “unifying” the electrophoretic behavior of oligosaccharides with the same number of glucose units without respect to the actual structural organization of the molecule. In a coated capillary, the higher resolving power and a weaker or uneven complexation of the branched structures may cause their different migration behavior. To characterize the dextran hydrolyzate more in detail, we have utilized the approach described by Offord [27] that was applied recently to the characterization of ANTS derivatives of maltooligosaccharides [10]. The dependence of electrophoretic mobility of the homologous, rod-like molecules on their molecular mass was found to fit the following equation:

$$\mu_{cp} = Cq(M_r)^{-2/3}$$

where C is a constant and q is an electrical charge. The main components in the dextran hydrolyzate are homologues (more or less linear oligosaccharides) formed from α -D-glucopyranosyl residues and linked together by (1–6)-linkages without additional branching. After calculating the electrophoretic mobilities of the major “regular” peaks and plotting them against the logarithm of the glucose residues and against $M_r^{-2/3}$, linear dependencies were shown (Fig. 7), as is also demonstrated for the maltooligosaccharide homologues derivatized with ANTS. The deviations of smaller oligosaccharides from linearity can be explained by a significant influence of the “foreign” part of the molecule, a highly charged tag, ABG. However, beginning with the oligosaccharides of five and more glucose units,

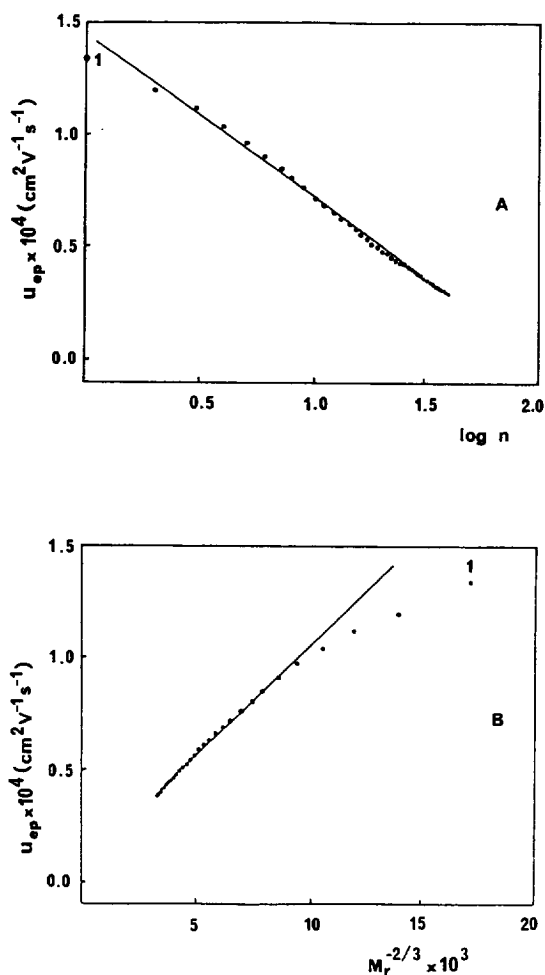


Fig. 7. The dependence of electrophoretic mobilities of ABG-derivatized isomaltooligosaccharides from Fig. 6 on (A) logarithm of glucose residues, $\log n$, and (B) their molecular mass expressed as $M_r^{-2/3}$.

this influence vanishes and the dependence becomes fairly linear.

4. Conclusions

Highly efficient separations of oligosaccharides derivatized with a UV-absorbing tag, ABG, were demonstrated. This derivatization technique can be used for a simple, sensitive and inexpensive characterization of complex oligosaccharide mixtures. However, different carbohydrate composition, degree

of branching and the different ability of borate complexation will have to be taken into account in a complete characterization of more complex molecules.

The described method shares a procedural simplicity of the other approaches using benzoic acid derivatives [17–19] while increasing the electrophoretic mobilities. The advantages of a charged tag were already pointed out [11] through comparison between aminopyridylated sugars and those labeled with ANTS. Compared to ANTS [10], UV detection at a higher wavelength, as demonstrated with ABG, is an additional asset. Yet another potentially important aspect is a suitability for mass spectrometry; sulfonated tags are known to behave poorly in this regard.

Acknowledgments

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