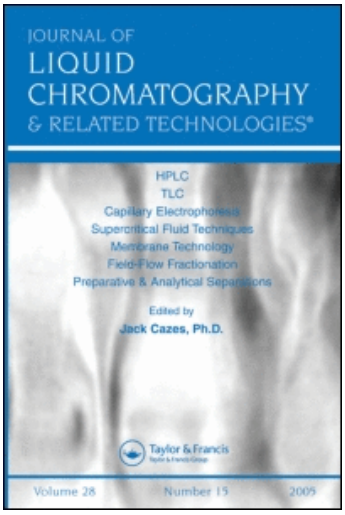

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High Performance Liquid Chromatography of Mono- and Oligosaccharides Derivatized with *P*-Aminobenzoic Ethyl Ester on a C_{18}-Bonded Silica Column

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF MONO- AND OLIGOSACCHARIDES DERIVATIZED WITH *P*-AMINOBENZOIC ETHYL ESTER ON A C_{18}-BONDED SILICA COLUMN

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ABSTRACT

This report examines a behavior of mono- and oligosaccharide-ABEE derivatives in RP-HPLC. Sugar-ABEE derivatives are saparated by reverse-phase partition chromatography rather than normal-phase partition chromatography. Reducing sugars are derivatized with *p*-aminobenzoic ethyl ester(ABEE) for the UV detection at 254 nm. C18-bonded silica column is used for the separation of sugar-ABEE derivatives in an isocratic mode. RP-HPLC is performed by using ternary mixture as a mobile phase and column temperature is maintained at 45°C. Sugar-ABEE derivatives are separated well within a short run time(*ca.* 25 min) by reverse-phase partition chromatographic mode, and C18-bonded silica column is very stable during the analysis. The (1→6)-linkage type of dihexose-ABEE derivatives has shorter retention time than (1→4)-linkage type. β-Anomeric configuration of [Glc-β-(1→4)Glc]-oligomer has shorter retention time than α-anomer in RP-HPLC. Column

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temperature and ionic strength slightly affect the elution behavior of sugar-ABEE derivatives.

INTRODUCTION

Saccharides are very abundant in nature and important in biology, medicine, pharmacology, and food industry. Traditionally, paper[1] and thin-layer chromatography[2] have been used to separate the saccharides, but these techniques have poor resolution and are not always quantitative[3]. Although gel filtration[4] and ion-exchange column chromatography[5] have been used to obtain large amount of sugars, they are not adequate for microanalysis and require very long analysis time. Gas-liquid chromatography with flame ionization detector or mass spectrometer has been used for the analysis of saccharides. This methodology is very powerful for the structural analysis, but it needs very tedious derivatization steps before the analysis and is not applicable to the purification of the sample because of the destructive nature of the method [6].

Nowadays, high-performance liquid chromatography has been used as a powerful technique to separate the saccharides. For the separation, various stationary phase have been used include silica gel[7], amine-bonded silica[3,8-11], polyfunctional amine modified silica[12-14], polystyrene-based anion-[15] and cation-exchange[16] resin, cyano-bonded silica[17], and C18-bonded silica [18-24]. Although the breakthrough has been achieved with the introduction of amine-bonded phase for the seaparation of saccharides, there are many disadvantages; their short life time because of the formation of Shiff's bases between the sugars and the amine-bonded phase[13,19,21], the limited saccharides solubility in mobile phase due to higher organic solvent contents demand as an eluent [13,22], and their low efficiencies. Ion-exchange resin is very useful for the separation of saccharides, but its throughput is low and it gives poor resolution for the higher oligosaccharides[22].

There is another problem in saccharides separation with high-performance liquid chromatography because saccharides have no chromophores or fluorophores. Although underivatized saccharides have been detected by refractive index detector[6,11,14], it is insensitive, requires pulseless pump for low noise levels and can not be used for gradient mode[6]. Many researchers have derivatized the saccharides by using pre- or post-column derivatization for more sensitive detection[18,23-27].

In this report, mono- and oligosaccharides are derivatized with ABEE in the presence of sodium cyanoborohydride for the detection and separated by C18-bonded silica column[18]. The seperation behavior of mono- and oligosaccharide-ABEE derivatives is studied by varying mobile-phase composition, column temperature, and ionic strength in RP-HPLC. All sugar-ABEE derivatives are saparated well by reverse-phase partition chromatographic mode rather than normal-phase partition chromatographic mode.

MATERIALS AND METHODS

Materials

Mono- and oligosaccharide standards, ABEE, sodium cyanoborohydride, sodium chloride and sodium acetate trihydrates were obtained from Sigma Chemical Co. (St. Louis, MO). HPLC-grade acetonitrile and methanol were purchased from Baxter Healthcare Corp. (Muskegon, MI). HPLC-grade distilled, deionized water (Millipore, Bedford, MA) was used. All other chemicals and solvents were of analytical reagent or HPLC grade.

Derivatization of mono- and oligosaccharides with p-aminobenzoic ethyl ester

The procedure employed for derivatization of mono- and oligosaccharides at their reducing end with ABEE was done by the method of Wang *et al* . [27]. The sample solution was made by dissolving 100 μmole sugars in 10 ml water. The ABEE reagent was made by mixing 165mg ABEE, 35 mg sodium cyanoborohydride, 41 μl glacial acetic acid, and 0.35 ml warm methanol. 40 μl of the ABEE-reagent was added to a 6 x 50 mm glass tube (Corning 9820, Corning, NY) containing 10 μl of sample solution and reacted at 80°C. After 1 h, the reaction mixture was cooled to room temperature. The distilled water (0.2 ml) and equal volume (0.2 ml) of chloroform were added to the reaction mixture. After vigorous vortexing, it was centrifuged for 1 min. The upper aqueous layer was subjected to HPLC analysis.

Reverse-phase high-performance liquid chromatography of sugar-ABEE derivatives

A Hewlett-Packard HP 1050 Series system (Hewlett-Packard, Avondale, PA) was used. Detection was performed with a Hewlett-Packard HP 1050 variable wave length detector at 254 nm. The Rheodyne injector was used with

a 10 μl sample loop and the data was collected by using a Hewlett-Packard HPLC ChemStation system. A 3.9 x 150 mm Pico · Tag column (Waters, Milford, MA) was used.

Samples were injected onto a Pico · Tag column (3.9 x 150 mm, Waters) at 45°C, if not specified otherwise. Elution (1.2 ml/min) was performed isocratically with Solvent A(Sol A) (50 mM sodium acetate buffer, pH 4.5 with glacier acetic acid) and Solvent B(Sol B) (50 mM Sodium acetate buffer, pH 4.5 with glacial acetic acid / acetonitrile / methanol = 40 / 40 / 20) in a ratio of Sol A / Sol B = 80 / 20 (%,v/v), if not specified otherwise.

RESULTS AND DISCUSSION

High-performance liquid chromatography has been used as a powerful technique to separate the saccharides. In this report we have described a behavior of mono- and oligosaccharide-ABEE derivatives in RP-HPLC. We have derivatized the sugars with ABEE in the presence of sodium cyanoborohydride for the UV detection [18,27]. This procedure is simple and only chloroform extraction is required for removal of excess ABEE. Although underivatized sugars can be detected by refractive index detector, sugar-ABEE derivatives are detected more sensitively and separated with high resolution [18].

To study the behavior of sugar-ABEE derivatives on a C18-bonded silica column, ternary mixture of aqueous buffer, methanol, and acetonitrile was used as a mobile phase. When we used the optimized condition for resolving of sugar-ABEE derivatives, many sugars were separated very well within a short run time (*ca.* 25 min) with high reproducibility (Figure 1). As shown in Figure 1A, the blank chromatogram had a very good baseline with few side products formed during the derivatization procedure. Many amino- and neutral saccharide-ABEE derivatives were separated in a single chromatography (Figure 1B and 1C) and Gal, Glu, and Man were separated each other when Sol A / Sol B = 90 / 10 (%,v/v) was used as a mobile phase [18]. The shape of peaks was very sharp and was not a doublet which was usually found in the separation of underivatized sugars [8,21]. There were many advantages in derivatization of saccharides with ABEE in the presence sodium cyanoborohydride as shown in Figure 1; few side products in background(Figure 1A), higher sensitivity(*ca.* 50 pmol) [18], and elimination of the possible doublet, which could be formed by

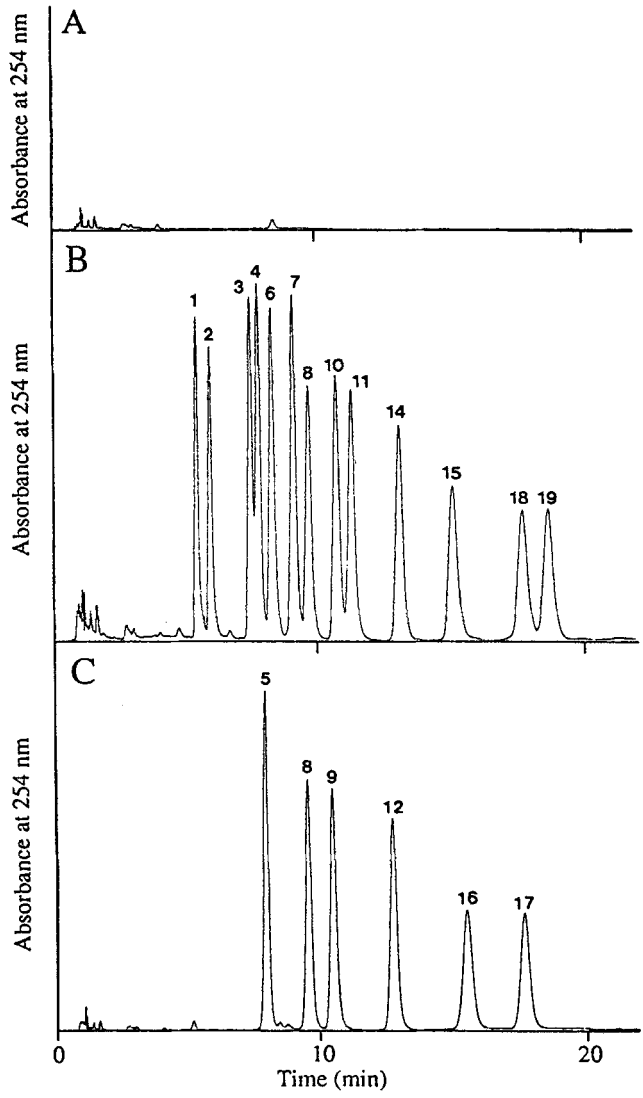

FIGURE 1. Separation of mono- and disaccharide-ABEE derivatives by RP-HPLC. Conditions for RP-HPLC are described under Experimental. (A) Chromatogram of blank reaction. Derivatization step was performed without sugar component. (B) and (C) Chromatogram of mono- and disaccharide-ABEE derivatives. The identity of sugars was as follows; 1. glucosamine(GlcN); 2. galactosamine(GalN); 3. meliobiose(Mel); 4. lactose(Lac); 5.isomaltose(Iso); 6. gentiobiose(Gen); 7. cellobiose(Cel); 8. maltose(Mal); 9. galactose(Gal); 10. glucose(Glc); 11. mannose(Man); 12. arabinose(Ara); 13. ribose(Rib); 14. xylose (Xyl); 15. *N*-acetylglucosamine(GlcNAc); 16. *N,N'*-diacetylchitobiose(Chi); 17. *N*-acetylgalactos amine; 18. fucose(Fuc); 19. rhamnose(Rha); 20. 2-deoxyglucose(DeGlc). The amount of each sugar was 5.0 nmole.

TABLE I
RETENTION TIME OF MONO- AND DISACCHARIDE-ABEE DERIVATIVES

ABEE derivatives	Structure	Retention time (min)	
		SolA/SolB=80/20	SolA/SolB=75/25
Monoaminohexose-ABEE			
1. Glucosamine	GlcN	5.3	3.7
2. Galactosamine	GalN	5.8	4.0
Dihexose-ABEE			
3. Meliobiose	Galα1-6Glc	7.4	4.7
4. Lactose	Galβ1-4Glc	7.8	4.9
5. Isomaltose	Glcα1-6Glc	7.9	5.0
6. Gentiobiose	Glcβ1-6Glc	8.2	5.1
7. Cellobiose	Glcβ1-4Glc	9.0	5.0
8. Maltose	Glcα1-4Glc	9.7	6.0
Monohexose-ABEE			
9. Galactose	Gal	10.7	6.8
10. Glucose	Glc	10.8	6.8
11. Mannose	Man	11.4	7.2
Monopentose-ABEE			
12. Arabinose	Ara	12.8	8.1
13. Ribose	Rib	13.0	8.1
14. Xylose	Xyl	13.2	8.2
Mono- and Diacetylatedamino hexose-ABEE			
15. *N*-acetylglucosamine	GlcNAc	15.0	8.8
16. *N,N'*-diacetylchitobiose	GlcNAcβ1-4GlcNAc	15.6	8.2
17. *N*-acetylgalactosamine	GalNAc	17.9	10.2
Deoxyhexose-ABEE			
18. Fucose	Fuc	18.0	10.8
19. Rhamnose	Rha	18.6	11.3
20. 2-deoxyglusose	2DeGlc	22.0	13.0

mutarotation of free reducing end, by using the reductive amination of reducing end with ABEE.

To study the behavior of sugar-ABEE derivatives in RP-HPLC, mobile phase composition was changed (Table I). Although many amine- and C18-bonded silica columns were used for sugar separation, most of them were performed by normal-phase partition chromatographic mode[4,11-14,24-26]. As shown in Table I, all sugar-ABEE derivatives were eluted more rapidly as increasing organic solvent (Sol B). These behaviors mean that the separation of sugar-ABEE derivatives was performed by reverse-phase partition chromatography[18,24,25] rather than normal-phase partition chromatography.

Figure 2 showed the RP-HPLC elution profiles of mono- and oligo saccharide-ABEE derivatives separated at different column temperature. The retention time of sugar-ABEE derivatives was decreased as increasing column temperature. As shown in Figure 2, the retention times of sugar-ABEE derivatives were decreased as increasing the column temperature, but the elution profile of sugar-ABEE derivatives was scarcely affected by varying the column temperature in RP-HPLC. Although the elution time of derivatives was decreased as increasing the column temperature, many sugar-ABEE derivatives were separated well because the column efficiency was improved by increasing the rate of mass transfer of sugar-ABEE derivatives into C18-bonded stationary phase.

The retention times of mono- and disaccharide-ABEE derivatives separated with two mobile phase were summarized in Table I. When we examined the elution profile of sugar-ABEE derivatives, we found out the elution patterns of sugar-ABEE derivatives according to the basic structure of sugar moieties. Monoaminohexose-ABEE (GlcN and GalN) derivatives generally exhibited shorter retention times than dihexose-ABEE (Mel, Lac, Iso, and Gen) derivatives and were eluted in following monohexose-ABEE (Gal, Glc, and Man), monopentose-ABEE (Ara, Rib, and Xyl), *N*-acetylamionohexose-ABEE (GlcNAc, Chi, and GalNAc), and deoxyhexose-ABEE (Fuc, Rha, and DeGlc) derivatives. When we examined the elution profiles of dihexose-ABEE derivatives, we could find that (1→6)-linkage in the structure had shorter retention time than (1→4)-linkage and these results were comparable to the oligosaccharide separation profile on an amine-bonded silica column[8-11]. When amine-bonded silica column was used for separation, the (1→6)-linkage in structure had longer retention time than the (1→4)-linkage because sugar-ABEE derivatives[9]. When we examined the elution profile of

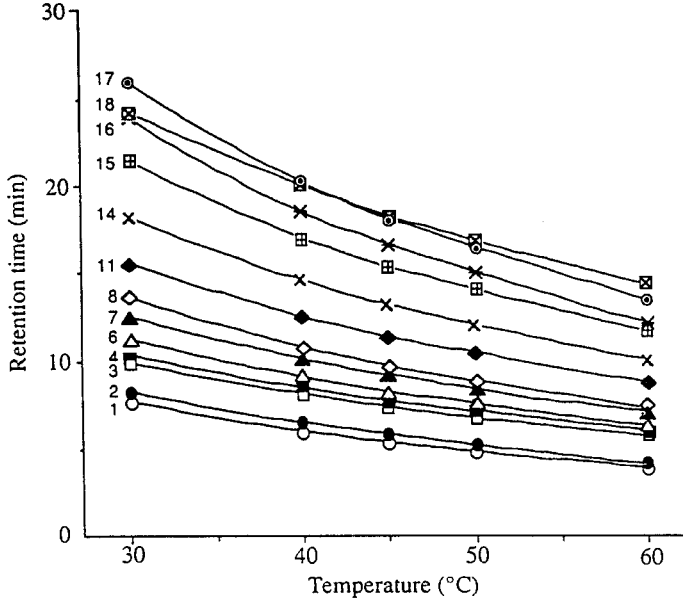

FIGURE 2. Effect of column temperature on retention time. Conditions for RP-HPLC are described under Experimental. The identities of the sugars are the same as those of FIGURE 1.

dihexose-ABEE derivatives, the effect of an anomeric configuration of the linkage was not clear. To obtain the information about the effect of anomeric configuration on separation of sugar-ABEE derivatives, we used malto oligosaccharide([Glc-α-(1→4)Glc])-ABEE and cellooligosaccharide([Glc-β-(1→4)Glc])-ABEE derivatives as a sample (Figure 3). The Glc-ABEE (DP=1) was eluted at the same retention time but cellooligosaccharide-ABEE (DP≥2) derivatives were eluted more rapidly than maltooligosaccharide-ABEE derivatives. In the ABEE derivatives of glucooligomers polymerized through (1→4)-linkage, the β-anomeric configuration of [Glc-β-(1→4)Glc]-oligomer was eluted more rapidly than α-anomer. These results confirmed that the linkage type and the anomeric configuration of the oligosaccharides were very important factors on separation [3, 9, 20].

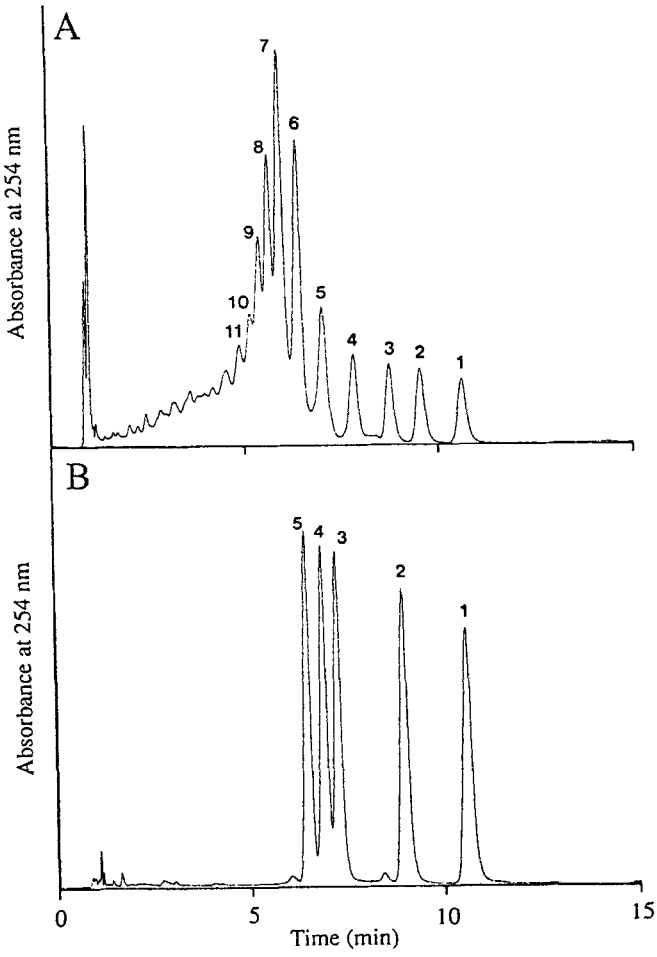

FIGURE 3. Separation of oligosaccharide-ABEE derivatives by RP-HPLC. Conditions for RP-HPLC are described under Experimental. (A) Maltooligosaccharide-ABEE derivatives. (B) Cellooligosaccharide-ABEE derivatives. The number on chromatogram indicate degree of polymerization (DP).

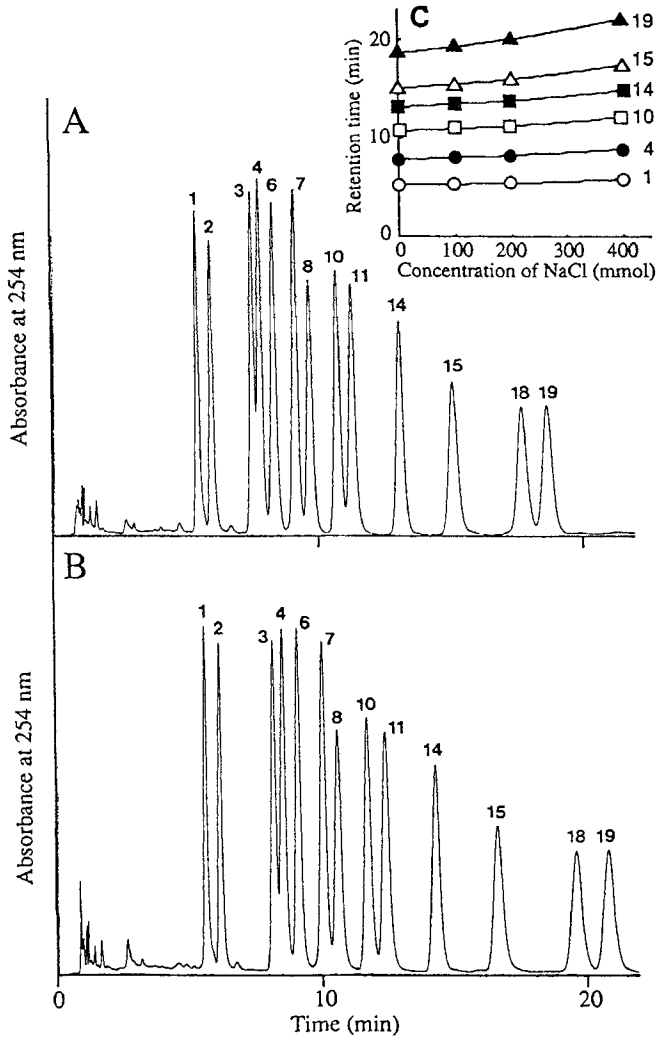

FIGURE 4. Effect of salt concentration on retention time. Conditions for RP-HPLC are described under Experimental. (A) 0 mmol NaCl in mobile phase. (B) 200 mmol NaCl in mobile phase. (C) Retention time profile of sugar-ABEE derivatives in proportion to increasing salt concentration. The identities of the sugars are the same as those of FIGURE 1.

C18-bonded silica column has the advantage of being pressure stable and readily available in suitable particle size and properties. Although there were lots of success of silica based packings for chromatography, there has been a number of occasions on which they were noted to fail dismally in achieving a separation of basic amino group containing compounds and all the problems of chromatography were assigned to the unbonded silanol groups. We examined the silanol group effect on separation by varying ionic strength because sugar-ABEE derivatives have a positive charges at pH=4.5, but the effect of ionic strength on retention time and peak shapes was not critical (Figure 4A and 4B). Although the pK_a of silanol groups is around 7.1[28], unbonded silanol groups are partilally ionized at pH=4.5[29]. If there were unexpected ionic interactions between unbonded silanol groups and positively charged sugar-ABEE derivatives, the retention time of positively charged molecules should be decreased, and then increased as increasing the salt concentration[29-31]. Figure 4C showed that the retention times of sugar-ABEE derivatives were only slightly increased because the solubility of sugar-ABEE in mobile phase was decreased due to the salting out effect[30], and unexpected silanol group effects on separation had to be eliminated by using sodium acetate buffer as a mobile phase component because sodium cations in mobile phase were powerful suppressors of free silanol groups[31].

CONCLUSIONS

This report has demonstrated behaviors of mono- and oligosaccharide-ABEE derivatives on a C18-bonded silica column. All sugar-ABEE derivatives were separated by reverse-phase partition mode rather than normal-phase partition condition. Sugar-ABEE derivatives were resolved well with a simple isocratic mode within a short run time(*ca.* 25 min). C18-bonded silica column is very stable for analysis of saccharides in contrast to widely used amine-bonded silica column[13, 19, 21]. The linkage type and the anomeric configuration of oligomers were important factors on separation of oligosaccharide-ABEE derivatives in RP-HPLC. The (1→6)-linkage type of dihexose-ABEE derivatives has shorter retention time than (1→4)-linkage type. β-Anomeric configuration of [Glc-β-(1→4)Glc]-oligomer has shorter retention time than α-anomer. Column temperature and ionic strength slightly affected the elution behavior of sugar-ABEE derivatives in RP-HPLC.

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