

High-performance Liquid Chromatography for Analysis of *p*-Aminobenzoic Ethyl Ester-converted D-Allose and D-Altrose on XBridge Column Containing Polyethoxysilane

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Abstract

p-Aminobenzoic ethyl ester (ABEE)-converted four D-aldohexoses including rare sugar D-allose (the C-3 epimer of glucose) were analyzed by high-performance liquid chromatography (HPLC) on two reverse-phase silica columns with a 0.2 M potassium borate buffer (pH 8.9) and acetonitrile (93:7) as the eluent using a detector of fluorescence (excitation of 305 nm and emission of 360 nm). On Cosmosil AR column, the four aldohexoses, which have identical molecular weight, were eluted in the order galactose < mannose < allose < glucose, although mannose, allose, and glucose were insufficiently separated. By contrast, on XBridge column containing polyethoxysilane, the four sugars were completely separated with the sharp peak. Each sugar could be sufficiently detected up to 0.5 nmol (90 ng) with a UV (305 nm) detector and 1 pmol (0.18 ng) with a fluorescence detector. Furthermore, HPLC analysis on XBridge column revealed that rare sugar D-altrose (C-2 and C-3 epimer of glucose) labeled with ABEE was eluted in the position between galactose and mannose. The HPLC analysis described here is simple, reliable, and will be applicable to the identification and quantification of traces of D-allose and D-altrose in biomaterials such as animal blood and plant cells.

Key words: rare sugar; D-allose; D-altrose; *p*-aminobenzoic ethyl ester; HPLC

Introduction

In high-performance liquid chromatography (HPLC) analysis, monosaccharides other than *N*-acetyl amino sugars such as *N*-acetylglucosamine are generally detected by monitoring the refractive index (RI)⁽¹⁾. However, this detection method has low sensitivity in the micromolar range. By contrast, D-aldohexoses with *p*-aminobenzoic ethyl ester (ABEE) coupled at the reducing end (-CHO) by reductive amination (-CH₂-NH-) can be detected by both fluorescence and UV (Fig. 1).⁽²⁾ This simple and highly sensitive precolumn method provides detection in the picomolar range. D-Allose, the C-3 epimer of D-glucose (Glc), is a rare sugar that is produced from D-psicose using an immobilized L-rhamnose isomerase bioreactor.^(3,4) D-Allose is currently interest in the biological activities such as an immunosuppressive effect⁽⁵⁾ and inhibitory effects on plant growth⁽⁶⁾ and on fruiting body formation of *Myxococcus xanthus*.⁽⁷⁾ A novel XBridge C18 column containing polyethoxysilane has recently been developed by Waters Corp., (Milford, Mass) and the HPLC analysis of D-allose in the picomolar range has not previously been established. In this study, therefore, we describe the use of HPLC to separate ABEE-D-allose from ABEE-Glc, -D-man-

nose (Man, C-2 epimer of Glc), and -D-galactose (Gal, C-4 epimer of Glc) (Fig. 2) and its application to the detection of D-allose in animal serum. In addition, ABEE-D-altrose (C-2 and C-3 epimer of Glc) (Fig. 2) was also analyzed by HPLC.

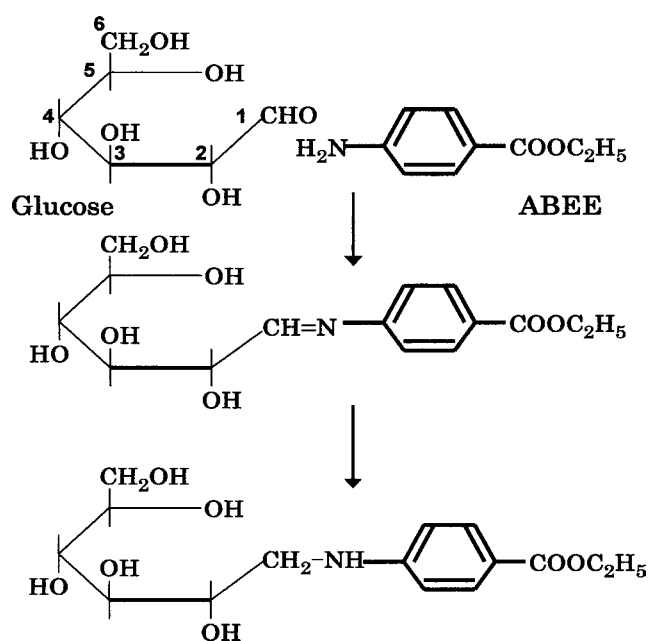


Fig. 1. ABEE labeling of D-glucose at the reducing end by reductive amination.

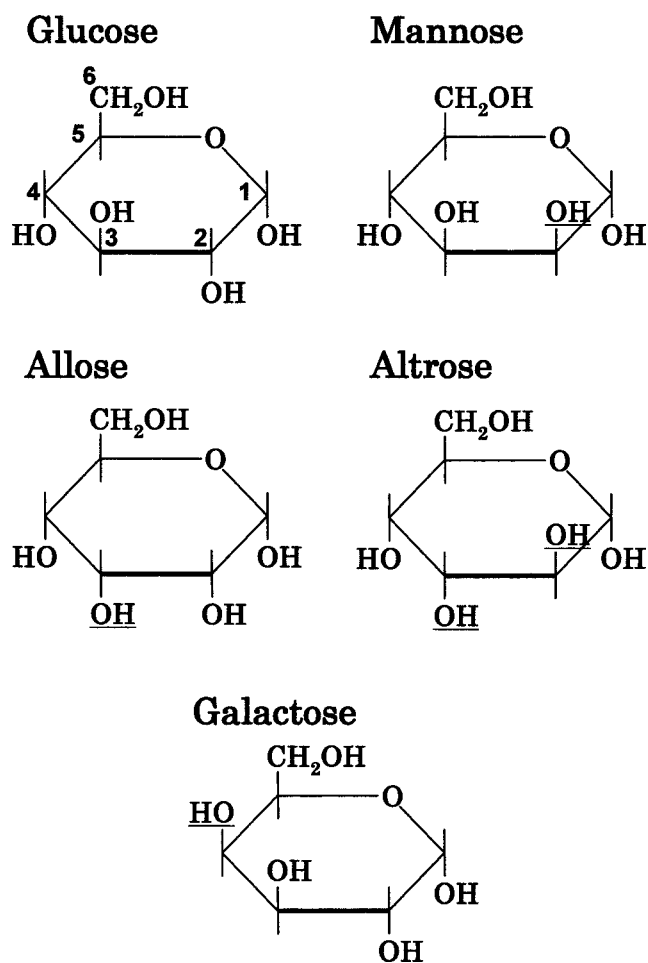


Fig. 2. Structures of D-aldoheptoses.

Materials and Methods

Materials

D-Allose and D-altrose was supplied from the Rare Sugar Research Center, Kagawa University. Three monosaccharides (Glc, Man, and Gal) and acetonitrile (guaranteed grade, purity of 99.5%) were purchased from Wako Pure Chemical Ind., Ltd., (Osaka) and Nakarai Tesque (Kyoto, Japan) respectively. The ABEE labeling kit and solvent set (potassium borate buffer, pH 8.9) were obtained from Seikagaku Kogyo Corp., (Tokyo, Japan).

ABEE labeling method

Monosaccharide solution (100 nmol = 18 μg / 10 μl) was added to 40 μl of ABEE reagent solution in the presence of borane-pyridine complex,²⁾ and the mixture was heated at 80°C for 1 hr. After cooling to room temperature, distilled water (0.2 ml) and chloroform (0.2 ml) were added to the

mixture. The mixture was centrifuged at 3,000 rpm for 5 min and the upper aqueous layer (100 nmol = 18 μg / 200 μl) was obtained.

HPLC analysis

The HPLC system consisted of a three-line degasser, two pumps, a mixer, a sample injector, a UV/VIS detector, a fluorescence detector and a data processor linked to a PC (Japan Spectroscopic Co, Ltd., Osaka). The diluted solution (20 μl) of ABEE-labeled monosaccharides was injected and analyzed by HPLC under the following conditions: columns (4.6 mm I.D. \times 250 mm, 5 mm), Cosmosil C18 AR column from Nakarai Tesque and XBridge C18 column from Waters Corp.; solvent, A 0.2M potassium borate buffer (pH 8.9)/acetonitrile (93/7), B 0.02% trifluoroacetic acid/acetonitrile (50/50); program, 0 to 50 min (solvent A for separation), 50 to 70 min (solvent B for washing) and then 70 to 85 min (solvent A for equilibration); flow rate, 1.0 ml/min; temp., 30°C; detection, UV (305 nm) or fluorescence (excitation of 305 nm and emission of 360 nm, setting up gain of 100).

Results and Discussion

Separation of ABEE-D-allose from ABEE-Glc, -Man, and Gal by HPLC

To determine whether ABEE-D-allose can be separated from ABEE-D-Glc, -Man, and -Gal, a mixture containing 0.5 nmol (90 ng) of each of the four ABEE-labeled monosaccharides was analyzed by HPLC on two reverse-phase columns with a fluorescence detector (set up gain of 100). On Cosmosil C18 AR column (Fig. 3A), the four monosaccharides, which have identical molecular weight, were eluted in the order Gal < Man < allose < Glc, although ABEE-Man, -allose, and -Glc were insufficiently separated. By contrast, the four sugars were completely separated on XBridge C18 column containing polyethoxysilane and their peaks were sharp (Fig. 3B). The retention times were 22.24 min for Gal, 28.50 min for Man, 30.42 min for allose, and 32.58 min for Glc at 7% of acetonitrile concentration. When various amounts of each ABEE-labeled sugars were mixed and analyzed by HPLC on a XBridge C18 column at 7% acetonitrile, each sugar could be sufficiently detected up to 0.5 nmol (90 ng) and 1 pmol (0.18 ng) with a detector of UV and fluorescence (set up gain of 1,000) respectively (data not shown). Furthermore, the effect of the concentration of acetonitrile on the separation was examined. ABEE-labeled Man and allose were eluted close

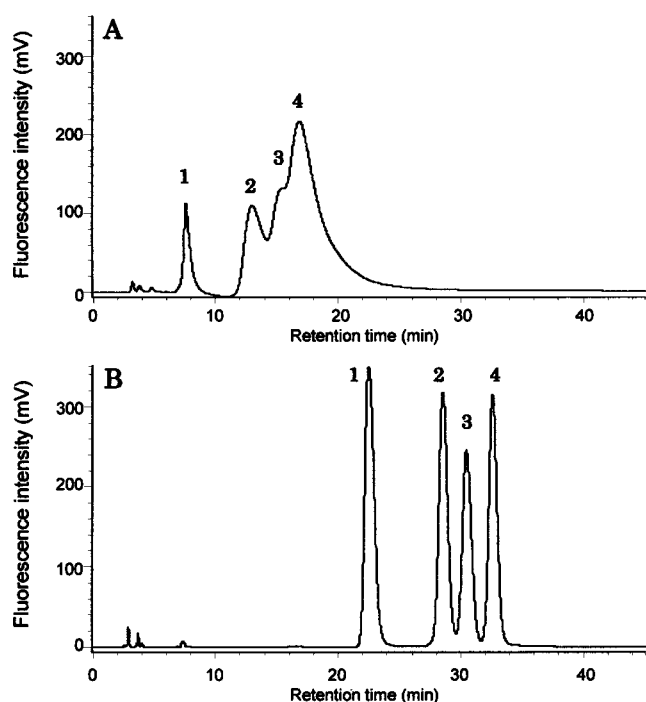


Fig. 3. Separation of ABEE-converted monosaccharides by HPLC on two reverse-phase columns.

The mixture ($20 \mu\text{l}$), containing 90 ng (0.5 nmol) of each sugar, was injected onto the Cosmosil column (A) and the XBridge column (B). The columns were eluted with 0.2 M potassium borate buffer ($\text{pH } 8.9$)/acetonitrile ($93/7$) at a flow rate of 1.0 ml/min and the ABEE-labeled sugars were detected with fluorescence (excitation of 305 nm and emission of 360 nm , setting up gain of 100). The numbered peaks represent the following sugars: 1, Gal; 2, Man; 3, allose; 4, Glc.

together as the acetonitrile concentration increased and could not be separated each other at 10% of acetonitrile concentration (Fig. 4). These results indicate that ABEE-D-allose can be completely separated from ABEE-D-Glc, -Man, and -Gal and sufficiently detected in the picomolar range by HPLC using the XBridge C18 column whose hybrid particles contain both inorganic (silica) and organic (organosiloxanes) components.

Detection of D-allose in mouse serum

There is currently great interest in the biological activities of rare sugars. When ovalbumin was conjugated to D-allose by the Maillard reaction, an enhanced effect on antioxidant activity was reported.⁸⁾ If D-allose is used as a food supplement, it is important to establish that orally taken D-allose can be absorbed from the intestine and circulate in the blood at biologically significant concentrations.⁹⁾ Therefore, mouse serum ($5 \mu\text{l}$) mixed with $18 \mu\text{g}$ of D-allose ($5 \mu\text{l}$) was labeled

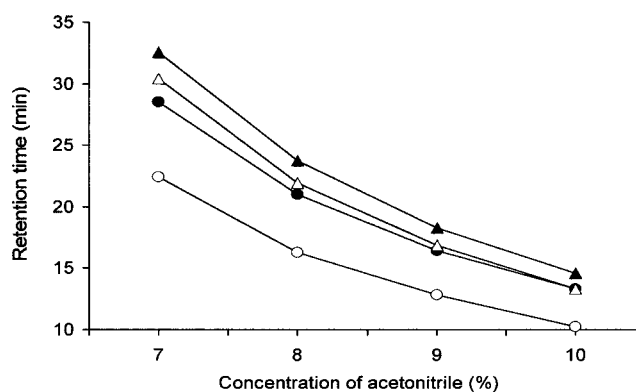


Fig. 4. Effect of acetonitrile concentration on separation of ABEE-converted monosaccharides by HPLC.

The mixture ($20 \mu\text{l}$) of each sugar (90 ng) was injected onto the XBridge column, the column was eluted with 0.2 M potassium borate buffer ($\text{pH } 8.9$) containing various acetonitrile concentrations, and then the ABEE-Gal (\circ), -Man (\bullet), -allose (\triangle), and Glc (\blacktriangle) were detected with fluorescence.

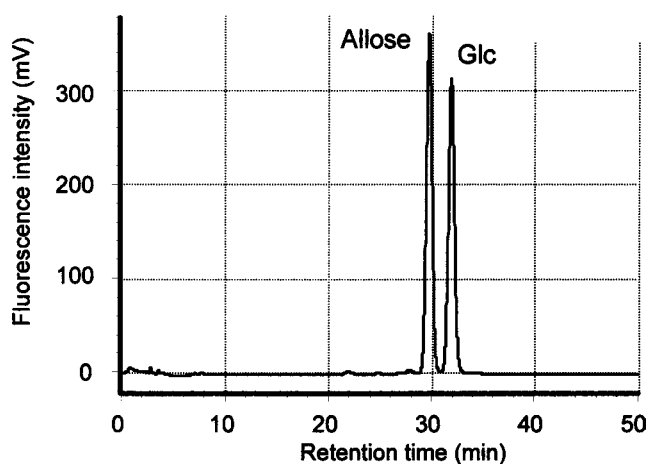


Fig. 5. Detection of ABEE-converted D-allose and D-Glc in mouse serum by HPLC.

Mouse serum mixed with D-allose was labeled with ABEE, and a 20-fold diluted solution containing 90 ng of D-allose was detected with fluorescence.

with ABEE and the diluted solution containing D-allose (90 ng) was analyzed by HPLC on a XBridge C18 column with a fluorescence detector. The preliminary examination showed that the added D-allose and serum D-Glc were completely separated with the same peak intensity (Fig. 5). As such, the HPLC analysis described here is simple, reliable, and will be applicable to the identification and quantification of traces of D-allose in biomaterials such as animal blood and plant cells.

Separation of ABEE-D-altrose by HPLC

All possible 8 D-aldohehexoses were divided into 2 epimers

(representative Glc type and Gal type) with respect to the configuration of a hydroxyl group at C-4 position (Fig. 2). We also labeled rare sugar D-altrose (C-2 and C-3 epimer of Glc) with ABEE and analyzed by HPLC on a XBridge C18 column. As shown in Fig. 6A, four sugars (Gal, Man, allose, and Glc) were eluted in same order in Fig. 3B, but their retention times were faster than those in Fig. 3B. In this examination, we used acetonitrile for HPLC grade (purity of 99.8%, Wako Pure Chemical Ind., Ltd.). Therefore, the differences in the retention times observed for four sugars in Fig. 3B and Fig. 6A are probably due to the concentration of acetonitrile in the eluent. When the four sugars were mixed with altrose (Fig. 6B), altrose was eluted in the position between Gal and Man. From the elution pattern, the relationship between the polarity and structures of ABEE-labeled D-aldohexoses could be proposed. ABEE-Gal (C-4 epimer of Glc) is more polar than ABEE-Glc type. The four sugars of ABEE-Glc type are, in increasing order of their polarity, Glc, C-3 (allose), C-2 (Man), and C-2, 3 (altrose) epimer.

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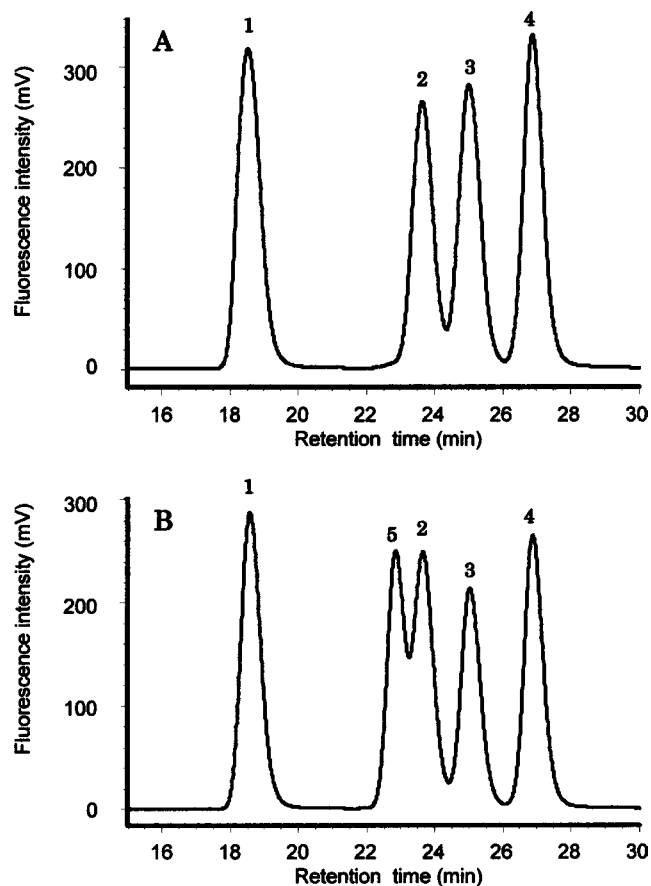


Fig. 6. Separation of ABEE-D-altrose by HPLC.

The mixture (20 μ l), containing 90 ng (0.5 nmol) of each sugar, was injected onto the XBridge column. The columns were eluted with 0.2 M potassium borate buffer (pH 8.9)/acetonitrile (93/7) at a flow rate of 1.0 ml/min and the ABEE-labeled sugars were detected with fluorescence. (A) The mixture contained Gal, Man, allose, and Glc. (B) The mixture contained Gal, Man, allose, Glc, and altrose. The numbered peaks represent the following sugars: 1, Gal; 2, Man; 3, allose; 4, Glc; 5, altrose.

References

- (1) Okazaki, K., Kato, F., Watanabe, N., Yasuda, S., Matsui, Y., and Hayakawa, S.: Purification and properties of two chitinases from *Streptomyces* sp. J-13-3. *Biosci. Biotechnol. Biochem.*, **59**, 1586-1587 (1995).
- (2) Yasuno, S., Murata, T., Kokubo, K., Yamaguchi, T., and Kamei, M.: Two-mode analysis by high-performance liquid chromatography of *p*-aminobenzoic ethyl ester-derivatized monosaccharides. *Biosci. Biotechnol. Biochem.*, **61**, 1944-1946 (1997).
- (3) Izumori, K.: Bioproduction strategy for rare hexose sugars. *Naturwissenschaften*, **89**, 120-124 (2002).
- (4) Menavuvu, B. T., Poonperm, W., Leang, K., Noguchi, N., Okada, H., Morimoto, K., Granstrom, T.B., Takada, G., and Izumori, K.: Efficient biosynthesis of D-allose from D-psicose by cross-linked recombinant L-rhamnose isomerase: separation of product by ethanol crystallization. *J. Biosci. Bioeng.*, **101**, 340-345 (2006).
- (5) Hossain, M. A., Wakabayashi, H., Goda, F., Kobayashi, S., Maeta, T., and Maeta, H.: Effect of the immunosuppressants FK506 and D-allose on allogenic orthotopic liver transplantation in rats. *Transplant. Proc.*, **32**, 2021-2023 (2000).

- (6) Afach, G., Kawanami, Y., Kato-Noguchi, H., and Izumori, K.: Practical production of 6-*O*-octanoyl-D-allose and its biological activity on plant growth. *Biosci. Biotechnol. Biochem.*, **70**, 2010-2012 (2006).
- (7) Chavira, M., Cao, N., Le, K., Riar, T., Moradshahi, N., McBride, M., Lux, R., and Shi, W.: β -D-allose inhibits fruiting body formation and sporulation in *Myxococcus xanthus*. *J. Bacteriol.*, **189**, 169-178 (2007).
- (8) Sun, Y., Hayakawa, S., Chuamanochan, M., Fujimoto, M., Innun, A., and Izumori, K.: Antioxidant effects of Mailard reaction products obtained from ovalbumin and different D-aldohehexoses. *Biosci. Biotechnol. Biochem.*, **70**, 598-605 (2006).
- (9) Hirose, Y., Murosaki, S., Yamamoto, Y., Ikematsu, H., and Nomoto, K.: Nigeroooligosaccharides augments mitogen-induced proliferation and suppresses activation-induced apoptosis of human peripheral blood mononuclear cells. *Immunopharmacol. Immunotoxicol.*, **26**, 387-399 (2004).

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ポリエトキシシランを含むXBridgeカラムを用いた*p*-アミノ安息香酸
メチルエステル化D-アロースとD-アルトロース分析のための
高速液体クロマトグラフィー

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要 約

希少糖であるD-アロース（グルコースのC-3エピマー）を含む4種のアルドヘキソースを*p*-アミノ安息香酸メチルエステル（ABEE）化し，2種の逆相シリカカラムを用いて0.2Mホウ酸カリウム緩衝液（pH 8.9）とアセトニトリル（93:7）を溶出液とした高速液体クロマトグラフィー（HPLC）で蛍光検出器（励起波長 305 nm，測定波長 360 nm）を用いて分析した．Cosmosil ARカラムでは，分子量が同一な4種のアルドヘキソースはガラクトース，マンノース，アロース，グルコースの順に溶出したが，マンノース，アロースとグルコースの相互の分離は不完全であった．それに対してポリエトキシシランを含むXBridgeカラムを用いると，4種の糖は相互にシャープなピークとして完全に分離した．各々の糖はUV検出器（305 nm）では0.5 nmol（90 ng）以上，蛍光検出器では1 pmol（0.18 ng）以上が十分検出できた．さらに，XBridgeカラムを用いたHPLC分析でABEE標識した希少糖であるD-アルトロース（グルコースのC-2とC-3エピマー）はガラクトースとマンノースの間の位置に溶出した．ここで示したHPLC分析は簡単に信頼性があるので，動物血清や植物細胞のような生体試料中での微量なD-アロースやD-アルトロースの同定や定量に応用可能である．