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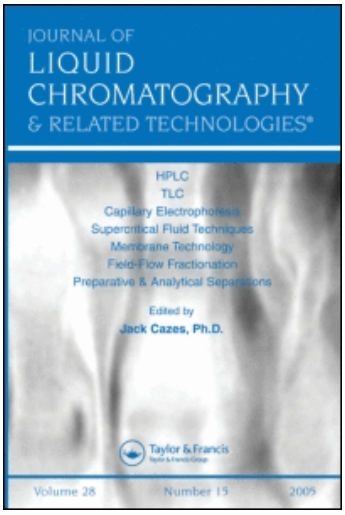

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ABSTRACT

Sugars and their p-nitrophenyl (PNP) derivatives were separated by high-speed countercurrent chromatography using a cross-axis coil planet centrifuge (cross-axis CPC) equipped with a pair of eccentric coil assemblies. A polar two-phase solvent system composed of 1-butanol/acetic acid/water (4:1:5) was used for the separation of sucrose and fucose, and glucuronic acid (lactoid form) and galacturonic acid, while 1-butanol/ethanol/water (4:1:4) was used for the separation of free and lactoid forms of glucuronic acid.

PNP-sugar derivatives such as neutral sugars, uronic acids, and amino sugars were separated with a less polar solvent system composed of n-hexane/ethyl acetate/1-butanol/methanol/water at

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various volume ratios. PNP-glucose derivatives were further separated according to the number of sugar chains, and five PNP-neutral sugars were resolved by adding 0.1 M sodium tetraborate in the two-phase solvent system.

Overall results of experiments revealed that the cross-axis CPC is useful for the separation of polar compounds such as sugars.

INTRODUCTION

Countercurrent chromatography (CCC) belongs to a member of liquid-liquid partition chromatography with one characteristic feature that it uses no solid support in the separation column. By eliminating complications caused by the solid support, CCC has many advantages in the separation and purification of various natural and synthetic compounds.[1-3]

Among CCC systems developed in the past, the cross-axis coil planet centrifuge (cross-axis CPC) has proven especially useful for separation of polar compounds such as proteins.

The cross-axis CPC has a unique mode of planetary motion such that the column holder rotates about its horizontal axis of the centrifuge.[4,5] The centrifugal force field produced by this planetary motion provides stable retention of the stationary phase for polar solvent systems such as aqueous-aqueous polymer phase systems with low interfacial tension and high viscosity.

Recently, an improved model of the cross-axis CPC has been constructed in our laboratory for performing CCC with aqueous polymer phase systems.[6-8] Our previous studies demonstrated that the cross-axis CPC equipped with either a multilayer coil or eccentric coil assembly in the off-center position of the column holder can be effectively applied for the separation of proteins using a polyethylene glycol - potassium phosphate polymer phase systems.[6-8] This apparatus is also extremely useful for the separation of highly polar compounds such as sugars which requires the use of polar two-phase solvent systems.

In the present paper, we report the separation of sugars and their p-nitrophenyl (PNP) derivatives by CCC using the cross-axis CPC. In the past the CCC separation of sugars is reported by only two research groups, Ogihara et al.[9] and Murayama et al.[10] using droplet CCC and centrifugal partition chromatography, respectively. However, these methods are time-consuming and yield relatively low partition efficiency.

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EXPERIMENTAL

Apparatus

The cross-axis CPC employed in the present studies was constructed at the Machining Technology Center of Nihon University, Chiba, Japan. The design of the apparatus was previously described in detail.[6-8]

Preparation of Coiled Columns

The separation columns used in the present study were a pair of eccentric coil assemblies. Each assembly was prepared by winding a 1 mm ID PTFE (polytetrafluoroethylene) tubing (Flon Kogyo, Tokyo, Japan) onto 7.6 cm long, 5 mm OD nylon pipes forming a series of tight left-handed coils. A set of these coil units was arranged symmetrically around the holder hub of 7.6 cm diameter in such a way that the axis of each coil unit is parallel to the axis of the holder. Two sets of coil assemblies were mounted on the rotary frame, one on each side, and serially connected with the flow tube. The separations were performed using two different sets of columns one consisting of 20 coil units and the other 50 coil units with the total column capacity of 26.5 mL and 61.5 mL, respectively.

Reagents

D(+)-Glucose, galactose, D(+)-mannose, D(+)-xylose, L(-)-fucose, D-ribose, L-arabinose, α-L-rhamnose, sucrose, lactose, α-D-galacturonic acid (monohydrate), D-mannuronic acid lactone, D(+)-glucuronolactone, N-acetyl-D(+)-glucosamine, and p-nitrophenyl α-D-glucopyranoside (PNP-Glc) were purchased from Wako Pure Chemicals (Osaka, Japan). D-Glucosamine (hydrochloride salt), D-galactosamine (hydrochloride salt), and N-acetyl-D-galactosamine was obtained from Nacalai Tesque Inc. (Kyoto, Japan). D-Glucuronic acid (sodium salt, monohydrate), p-nitrophenyl β-D-galactopyranoside (PNP-Gal), p-nitrophenyl β-D-mannopyranoside (PNP-Man), p-nitrophenyl β-D-xylopyranoside (PNP-Xyl), p-nitrophenyl α-L-fucopyranoside (PNP-α-Fuc), p-nitrophenyl β-D-fucopyranoside (PNP-β-Fuc), p-nitrophenyl β-L-arabinopyranoside (PNP-Ara), p-nitrophenyl β-D-glucuronide (PNP-GlcUA), p-nitrophenyl β-D-galacturonide (PNP-GalUA), p-nitrophenyl N-acetyl β-D-glucosaminide (PNP-GlcNAc), p-nitrophenyl N-acetyl β-D-galactosaminide (PNP-GalNAc), p-nitrophenyl 2,3,4,6-tetraacetyl β-D-glucosaminide (PNP-GlcN4Ac), p-nitrophenyl β-D-cellobioside (PNP-2Glc),

p-nitrophenyl β-D-cellotrioside (PNP-3Glc), p-nitrophenyl β-D-cellopentaoside (PNP-5Glc) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of reagent grade.

Preparation of Two-Phase Solvent Systems and Sample Solutions

According to the polarity of the analytes, several types of two-phase solvent systems were prepared: 1-butanol/ethanol/water (4:1:4); 1-butanol/acetic acid/water (4:1:5); chloroform/methanol/water (7:13:8); ethyl acetate/1-butanol/0.1 M sodium tetraborate (4:1:5); and n-hexane/ethyl acetate/1-butanol/methanol/water at various volume ratios.

Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated after two clear layers formed. Sample solutions were prepared by dissolving each sugar mixture in 0.5 mL of each phase of the two-phase solvent system used for separation.

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Measurement of Partition Coefficients of Sugar Samples

The partition coefficient (K) of each sugar was determined spectrophotometrically using a simple test tube procedure as follows: Two-milliliters of each phase of the equilibrated two-phase solvent system were delivered into a test tube to which about 1 mg of the sample was added. The contents were thoroughly mixed and allowed to settle at room temperature. After the two clear layers were formed, a 1 mL aliquot of each phase was evaporated and the residue was redissolved with an equal volume of distilled water. To 100 μL of this solution 400 mL of distilled water was added and subjected to the suitable colorimetric reaction, i.e., the phenol-sulfuric acid method[11] for neutral sugars, the carbazole method[12] for uronic acids, Elson-Morgan method[13] for amino sugars, and Morgan-Elson method[14] for N-acetyl amino sugars. The absorbance was measured using a spectrophotometer (Model UV-1600, Shimadzu Corporation, Kyoto, Japan).

The K value was obtained by dividing the absorbance value of the organic phase by that of the aqueous phase. The measurement of K values of PNP-sugar derivatives was carried out by the method described by Oka et al.[15]

CCC Separations of Sugars

For each separation, the coil was completely filled with the organic stationary phase and the sample solution (ca 1 mL) injected into the column

inlet. Then, the aqueous mobile phase was pumped into the column using a reciprocating pump (Model KHU-W-52H, Kyowa Seimitsu Co., Tokyo, Japan) while the column was rotated at a desired speed. The effluent from the column was collected into test tubes at 0.4 mL/tube using a fraction collector (Model SF-200, Advantec Co., Tokyo, Japan).

Analysis of CCC Fractions

Each eluted sugar fraction was evaporated to dryness and redissolved with 1 mL of distilled water. This solution was submitted to the colorimetric reaction described above. The absorbance was measured at 490 nm for neutral sugars and 530 nm for uronic acids, respectively.

To the p-nitrophenyl sugar derivative fraction, an aliquot of methanol was added and the absorbance was determined at 305 nm which gives a maximum absorbance value.

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RESULTS AND DISCUSSION

CCC Separation of Sugars

Since sugars are extremely hydrophilic, it is difficult to find a two-phase solvent system which distributes the analytes evenly in both phases. In the present studies, three different kinds of solvent systems were tested for partition coefficients of sugars. The results are summarized in Table 1. Most of the neutral sugars were partitioned almost unilaterally into the aqueous phase in all three solvent systems except that fucose was partitioned significantly to the organic phase (K = 0.34 - 0.44). In uronic acids (second group), the lactoid form was partitioned in the organic phase slightly more than the free form. However, all components of both amino sugars (third group) and N-acetyl amino sugars (fourth group) were mostly partitioned into the aqueous phase. These partition data may be useful for predicting the retention time of various sugar samples.

Figure 1 illustrates the CCC separation of sucrose and fucose using cross-axis CPC with eccentric coil assemblies. When using the polar two-phase solvent system composed of 1-butanol/ethanol/water (4:1:4) which was used by Murayama et al.,[10] sucrose and fucose were only partially resolved as shown in Fig. 1A. However, this separation was improved by using the solvent system composed of 1-butanol/acetic acid/water (4:1:5) as shown in Fig. 1B. The separation was further improved using the longer eccentric coil assemblies, as in Fig. 1C, where the resolution between the sucrose and fucose peaks is 1.3.

Table 1

Partition Coefficients of Sugars by Three Kinds of Organic-Aqueous Two-Phase Solvent Systems*

Solvent System	Partition Coefficient (K) Chloroform-Methanol-Water (7:13:8)	1-Butanol-Ethanol-Water (4:1:4)	1-Butanol Acetic Acid-Water (4:1:5)
Neutral Sugar			
D(+)-Glucose	0.06	0.19	0.26
Galactose	0.11	0.25	0.29
D(+)-Mannose	0.08	0.19	0.31
D(+)-Xylose	0.11	0.25	0.26
L(-)-Fucose	0.44	0.40	0.34
D-Ribose	0.14	0.40	0.31
L-Arabinose	0.16	0.21	0.27
α-L-Rhamnose	0.13	0.31	0.31
Sucrose	0.16	0.14	0.19
Lactose	0.09	0.12	0.21
Uronic Acid			
D-Glucuronic acid (sodium salt)	0.11	0.08	0.12
α-D-Galacturonic Acid	0.15	0.16	0.14
D-Mannuronic Acid lactone	0.24	0.22	0.19
D(+)-Glucuronolactone	0.15	0.37	0.33
Amino Sugar			
D-Glucosamine	0.02	0.11	0.07
D-Galactosamine	0.02	0.11	0.07
N-Acetyl Amino Sugar			
N-Acetyl-D(+)-glucosamine	0.08	0.19	0.17
N-Acetyl-D-galactosamine	0.07	0.16	0.13

* Partition coefficients of neutral sugars, uronic acids, amino sugars and N-acetyl amino sugars were calculated from the absorbance of the organic phase divided by that of aqueous phase obtained by the phenol-sulfuric acid method, the carbazole method, Elson-Morgan method and Morgan-Elson method, respectively.

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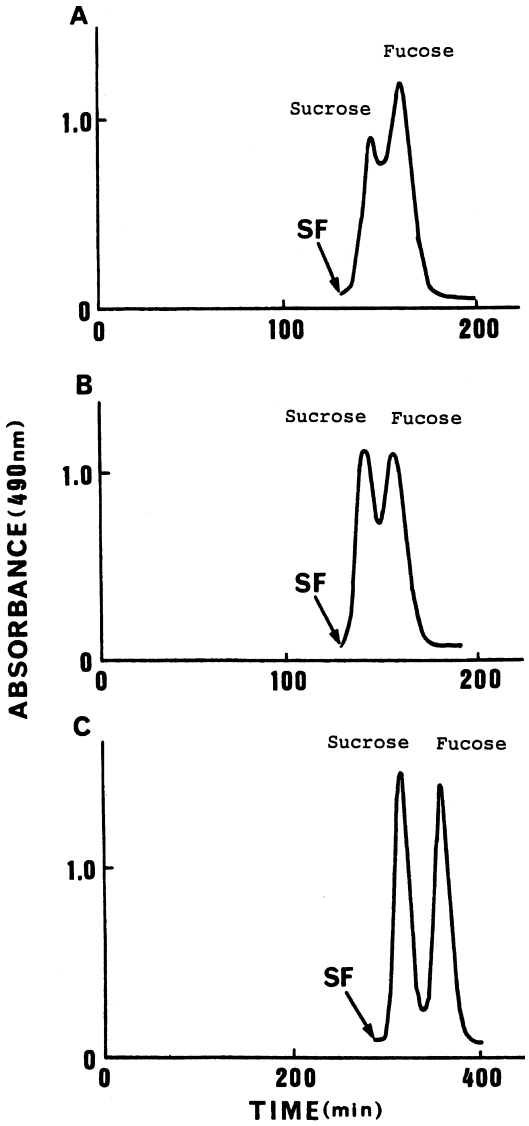

Figure 1. CCC separation of sucrose and fucose by cross-axis CPC. Experimental conditions: apparatus: cross-axis CPC equipped with a pair of eccentric coil assemblies, capacity 26.5 mL (A) and (B), 61.5 mL (C); sample: sucrose (2.5 mg) and fucose (10 mg); solvent system: (A) 1-butanol/ethanol/water (4:1:4), (B) and (C) 1-butanol/acetic acid/water (4:1:5); mobile phase: lower phase; flow rate: (A) and (B), 0.2 mL/min and (C) 0.1 mL/min; revolution: 800 rpm. SF = solvent front.

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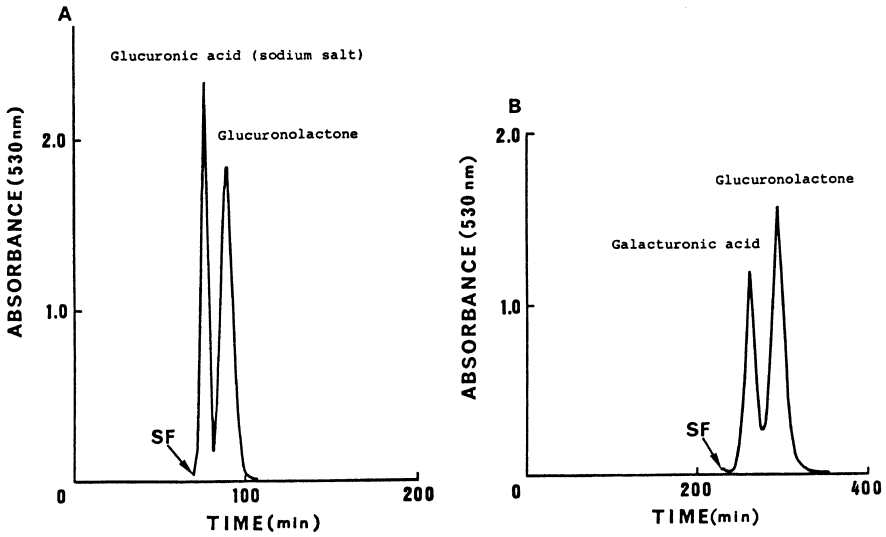

Figure 2. CCC separation of uronic acids by cross-axis CPC. Experimental conditions: apparatus: cross-axis CPC equipped with a pair of eccentric coil assemblies, (A) 26.5 mL capacity and (B) 61.5 mL capacity; sample: (A) glucuronic acid (sodium salt) (2.5 mg) and glucuronolactone (2.5 mg), (B) galacturonic acid (2.5 mg) and glucuronolactone (2.5 mg); solvent system: (A) 1-butanol/ethanol/water (4:1:4) and (B) 1-butanol/acetic acid/water (4:1:5); mobile phase: lower phase; flow rate: (A) 0.2 mL/min and (B) 0.1 mL/min; revolution: (A) 800 rpm and (B) 870 rpm. SF = solvent front.

Figure 2 shows the CCC separation of uronic acids. In Fig. 2A the free form (sodium salt) and the lactoid form (glucuronolactone) of glucuronic acid were well resolved using the solvent system composed of 1-butanol/ethanol/water (4:1:4). The resolution between these peaks is 1.2.

When 1-butanol/acetic acid/water (4:1:5) was used as the solvent system, this separation was not successful, because both free and lactoid forms of glucuronic acid were eluted at similar retention times. However, as shown in Fig. 2B, the separation between galacturonic acid and glucuronolactone was successfully performed with the latter solvent system at the peak resolution of 1.3.

As described above, it is suggested that the proposed two-phase solvent system composed of 1-butanol/acetic acid/water (4:1:5) is also useful for separating sugars by cross-axis CPC, which this solvent system has been often used in the separation of peptides by other types of CCC apparatus.

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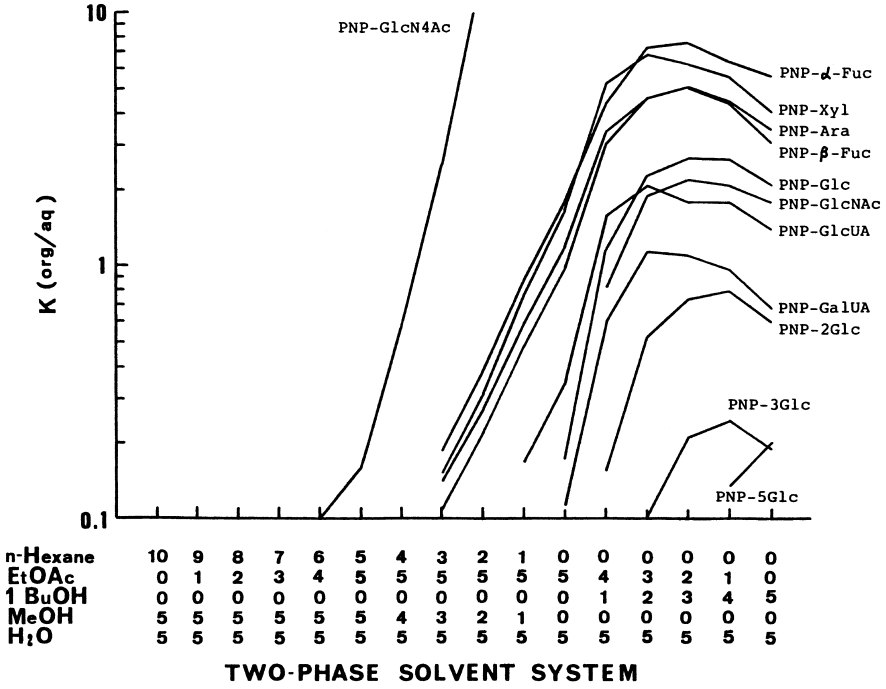

Figure 3. Partition coefficients ($K = C_{org}/C_{aq}$) of PNP-sugar derivatives in 1-hexane/ethyl acetate/1-butanol/methanol/water system. EtOAc = ethyl acetate; 1-BuOH = 1-butanol; MeOH = methanol.

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CCC Separation of PNP-Sugar Derivatives

The analysis of natural sugars generally requires complicated colorimetric reaction for detection because it has no chromophore. Its PNP-derivatives, however, can be directly subjected to the spectrophotometric analysis at the maximum absorbance of 305 nm.

Figure 3 summarizes the K values of main PNP-sugar derivatives in the solvent system composed of n-hexane/ethyl acetate/1-butanol/methanol/water. This solvent system described by Oka et al. provides a wide range of hydrophobicity by adjusting the volume ratios and therefore it is also useful for estimating the polarity of the analytes.[15] Among PNP-sugar derivatives, the hydrophobicity decreases in the order of PNP-GlcN4Ac, PNP-pentose derivatives (PNP-α-Fuc, PNP-Xyl, PNP-β-Fuc, PNP-Ara), PNP-hexose derivatives (PNP-Glc, PNP-Gal, PNP-Man) (among those only PNP-Glc is shown in Figure 3 because all three sugars showed very similar K values), and

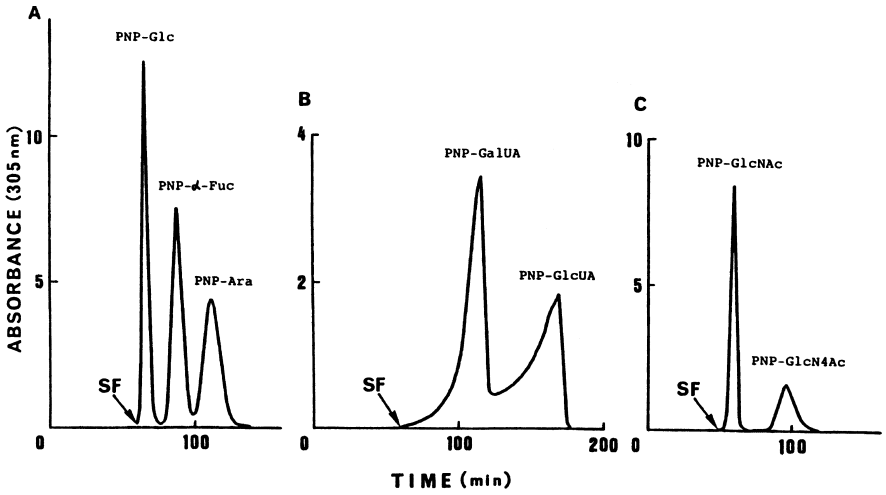

Figure 4. CCC separation of PNP-sugar derivatives by cross-axis CPC. Experimental conditions: apparatus: cross-axis CPC equipped with a pair of eccentric coil assemblies, 1 mm ID and 26.5 mL capacity; sample: (A) PNP-Glc (2.5 mg) + PNP-α-Fuc (2.5 mg) + PNP-Ara (2.5 mg), (B) PNP-GalUA (2.5 mg) + PNP-GlcUA (2.5 mg), and (C) PNP-GlcNAc (2.5 mg) + PNP-GlcN4Ac (2.5 mg); solvent system: (A) n-hexane/ethyl acetate/methanol/water (1:5:1:5), (B) ethyl acetate/1-butanol/water (4:1:5) and (C) n-hexane/ethyl acetate/ methanol/water (4:5:4:5); mobile phase: lower phase; flow rate: 0.2 mL/min; revolution: 870 rpm. SF = solvent front.

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PNP-oligoglucose derivatives (PNP-2Glc, PNP-3Glc and PNP-5Glc). These results suggest that their K values are closely correlated with the number of hydroxyl groups in the sugar. Among PNP-sugar derivatives examined, GlcN4Ac is most hydrophobic because all hydroxyl groups are esterified by the less polar acetyl groups.

Figure 4 illustrates the CCC chromatograms of PNP-sugars obtained with two-phase solvent systems selected from a set of partition studies shown in Figure 3. PNP-Glc, PNP-α-Fuc and PNP-Ara were well resolved in each other with a solvent system composed of n-hexane/ethyl acetate/methanol/water (1:5:1:5) as shown in Fig. 4A. This indicates that PNP-hexose and PNP-pentose are readily separated in this solvent system. When a more polar solvent system such as ethyl acetate/1-butanol/water (4:1:5) was used, PNP-GalUA and PNP-GlcUA were satisfactorily resolved (Fig. 4B). The peak skewing observed in this chromatogram is probably due to non-linear isotherm of these acidic compounds. Fig. 4C shows the separation of PNP-GlcNAc and PNP-GlcN4Ac using a more hydrophobic two-phase solvent system composed of n-hexane/ethyl acetate/methanol/water (4:5:4:5).

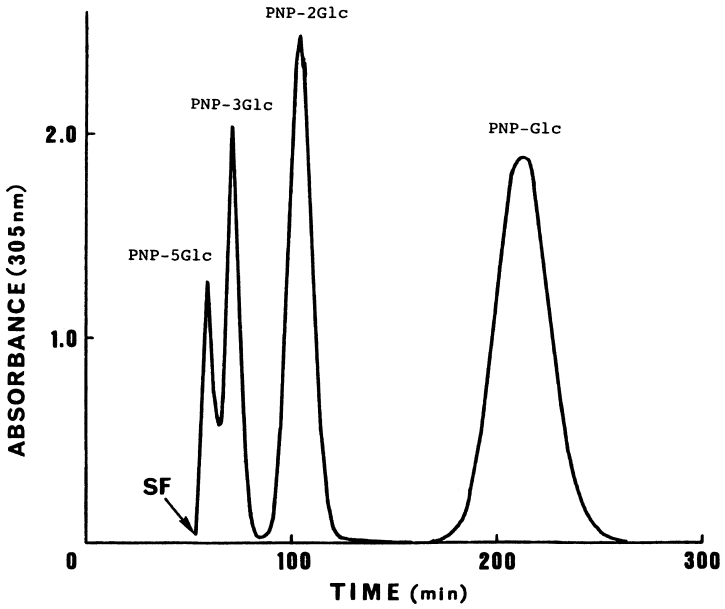

Figure 5. CCC separation of PNP-glucose derivatives by cross-axis CPC. Experimental conditions: sample: PNP-5Glc (1.4 mg) + PNP-3Glc (1.3 mg) + PNP-2Glc (2.5 mg) + PNP-Glc (2.5 mg); solvent system: ethyl acetate/1-butanol/water (1:4:5). For other experimental conditions, see the Fig. 4 caption. SF = solvent front.

The measurement of K values of PNP-sugar derivatives shown in Fig. 3 revealed that the hydrophobicity of the compounds is decreased with the number of hydroxyl groups in the sugar molecule. Figure 5 illustrates the CCC separation of PNP-glucose derivatives obtained by the solvent system composed of ethyl acetate/1-butanol/water (1:4:5). PNP-glucose derivatives eluted in the order of the number of glucose chain in the sugar molecule. It so indicates that PNP-monosugars elute in the order of their molecular weight.

It is known that sugars form a complex with borate in an aqueous solution. Figure 6 illustrates that the CCC separation of PNP-neutral sugars with a solvent system composed of ethyl acetate/1-butanol/0.1 M sodium tetraborate aqueous solution (4:1:5). The separation was remarkably improved and five PNP-sugars were resolved by adding sodium tetraborate in the solvent system.

The aqueous-aqueous polymer phase systems composed of polyethylene glycol (PEG) 1000 and dibasic potassium phosphate were also tested for the partitioning of PNP-sugar derivatives. It was found, however, that these solvent

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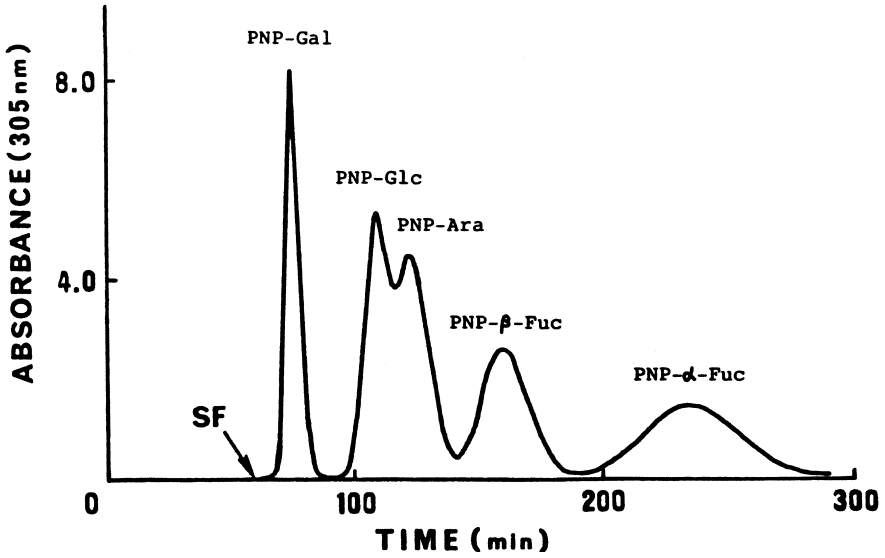

Figure 6. CCC separation of PNP-neutral sugar derivatives by cross-axis CPC. Experimental conditions: sample: PNP-Gal (2.5 mg) + PNP-Glc (2.5 mg) + PNP-Ara (2.5 mg) + PNP-β-Fuc (2.5 mg) + PNP-α-Fuc (2.5 mg); solvent system: ethyl acetate/1-butanol/0.1 M sodium tetraborate aqueous solution (4:1:5). For other experimental conditions, see the Fig. 4 caption. SF = solvent front.

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systems were not suitable for separating PNP-sugar derivatives because these compounds were almost exclusively partitioned into the PEG-rich upper phase due to relatively high hydrophobicity of their molecules and their K values were only slightly altered by the addition of sodium tetraborate to the solvent system.

CONCLUSIONS

The present studies demonstrate that sugars and p-nitrophenyl derivatives can be separated by the cross-axis CPC equipped with eccentric coil assemblies.

Overall results indicate that the method can provide a stable retention of polar two-phase solvent systems such as 1-butanol/acetic acid/water (4:1:5) and 1-butanol/ethanol/water (4:1:4) which may produce emulsification in the standard J-type high-speed CCC centrifuge. The partition efficiency for the separation of sugars can be improved by derivatization that increases the hydrophobicity of the molecule.

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