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Counter-current chromatographic separation of nucleic acid constituents with an extremely hydrophilic solvent system

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Abstract

Nucleic acid constituents such as nucleobases, nucleosides and nucleotides were separated by counter-current chromatography using a type J coil planet centrifuge. The separation was performed with an extremely hydrophilic solvent system composed of 1-propanol/800 mM potassium phosphate buffer (pH 7.4) (1 : 1) by eluting the lower aqueous phase at a flow-rate of 0.5 ml/min. Eight kinds of nucleic acid constituents, including UMP, AMP, deoxyAMP, uridine, urasile, 2' deoxy uridine, adenosine and adenine were well resolved within 170 min.

1. Introduction

Counter-current chromatography (CCC) is a separation technique based on the partition of solutes between two immiscible liquid phases. CCC is essentially a form of liquid-liquid partition chromatography in which one phase of the equilibrated two-phase solvent system is retained in the column as a stationary phase with the aid of gravity or a centrifugal force field while the other phase is continuously eluted through the column [1–4]. Since CCC performs separations without the solid support matrix, the adsorptive loss and the chemical degradation of solute are eliminated. High-speed CCC (HSCCC) technique [5,6], which utilizes an Archimedean Screw effect in a rotating coiled column, has been applied for both analytical and preparative separations of a wide variety of compounds using a variety of aqueous-organic two-phase solvent systems.

The chromatographic process in HSCCC is based on the partition of a solute between the mobile and stationary phases of an equilibrated two-phase solvent system. The partition coefficient (K) value is therefore the most important parameter in HSCCC. Optimization of the solvent composition to adjust the partition coefficient value of the target analyte is essential for successful CCC separation. Although there are a number of two-phase solvent systems available for separation of a variety of compounds by HSCCC [7], separations of nucleic acid constituents, such as nucleobases, nucleosides and nucleotides have not been reported yet. Therefore, in the present study, HSCCC separation of nucleic acid constituents has been performed using the type-J coil planet centrifuge (J-CPC) with an

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extremely hydrophilic solvent system composed of 1-propanol/ 800 mM potassium phosphate buffer (pH 7.4) (1 : 1).

2. Experimental

2.1. Reagents

Standard compounds of adenine, adenosine, adenosine 5'-monophosphate monohydrate, 2'-deoxyadenosine 5'-monophosphate monohydrate, guanine, guanosine, guanosine 5'-monophosphate disodium salt, cytosine, cytidine, cytidine 5'-monophosphate disodium salt, uracil, uridine 2'-deoxyuridine, and uridine 5'-monophosphate disodium salt were purchased from Sigma-Aldrich Japan Co. (Tokyo, Japan). The chemical structures of these 14 nucleic acid constituents were shown in Fig. 1. 1-Propanol used for preparation of the two-phase solvent system was obtained from Kanto Chemical Co. (Tokyo, Japan). All other chemicals were of reagent grade.

2.2. Measurement of partition coefficient

A two-phase solvent system composed of 1-propanol/800 mM potassium phosphate buffer (pH 7.4) (1 : 1) was selected to measure the partition coefficients (K) of 14 different nucleic acid constituents such as nucleobases, nucleosides and nucleotides prior to their HSCCC separation. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature, and the two phases were separated shortly before use. K values of the above standard compounds in this solvent system were determined spectrophotometrically by the following procedure: 1 ml of each phase was delivered into a test tube to which 1 mg of each test compound was added. The contents were thoroughly mixed and allowed to settle at room temperature. After two clear layers were formed, an aliquot of each phase was diluted with methanol (2 to 100 times) to determine the absorbance at 260 nm using a Jasco V 530 spectrophotometer (Jasco, Tokyo, Japan). The K value was expressed as the solute concentration in the upper phase divided by that in the lower phase.

2.3. HSCCC apparatus

The type-J coil planet centrifuge (J-CPC) (Renesas Eastern Japan Semiconductor Inc, Tokyo, Japan) holds a multilayer coiled separation column and a counter-weight symmetrically at a distance of 10 cm from the central axis of the centrifuge. A separation column was fabricated by winding a single piece of polytetrafluoroethylene (PTFE) tubing (1.0 mm I.D. $\times$ 2.0 mm O.D. $\times$ 50 m length; Tokyo Rikakikai, Tokyo, Japan) directly onto a holder hub, making eight coiled layers (26 turns in each layer) between a pair of flanges ($\beta=0.5-0.6$). The total capacity of the column is 40 ml. The revolution speed of the apparatus was regulated at 1000 rpm with a speed control unit. The coiled column rotates about its own axis as it synchronously revolves around the central axis of the centrifuge in the same direction, producing an efficient mixing of the two phases while retaining a sufficient amount of the stationary phase in the coiled column.

This J-CPC was connected to the Hitachi HPLC instrument (Hitachi, Tokyo, Japan) consisting of a model L-7100 pump, a Rheodyne 7166 sample injector (Rheodyne, CA, USA) and a model L-7455 diode-array detector (Hitachi) and a model 2000ES evaporative light scattering detector (Alltech Associates, IL, USA) as shown in Fig. 2.

2.4. High-speed counter-current chromatography

The separation of selected eight nucleic acid constituents was performed with the two-phase solvent system composed of 1-propanol/800 mM potassium phosphate buffer (pH 7.4) (1 : 1) on a reversed-phase partition mode (mobile phase: aqueous lower phase; stationary phase: organic upper phase). For the separation of the mixture of standard compounds, the coiled

PTFE column (capacity: 40 ml) mounted on the type J-CPC was first entirely filled with the organic upper phase as a stationary phase. Then, the column was rotated at 1000 rpm while the aqueous lower mobile phase was pumped into the column at a flow-rate of 0.5 ml/min. After the hydrodynamic mixing between the two phases reached a state of equilibrium in the column, a sample solution containing a set of the standard compounds (each 0.5 mg) in a 0.5 ml of two-phase solvent system was injected and separated with the lower mobile phase for 100 min. Then, the upper phase was used as the mobile phase for the elution of the components still retained column. During HSCCC separation, UV absorbance of the effluent was monitored by a diode-array detector and an evaporative light scattering detector.

3. Results and discussion

3. 1. Partition coefficients of nucleic acid constituents

Since the HSCCC process is based on a solute partitioning between mobile and stationary phases, the partition coefficient value is one of the most important parameters for successful separation. Table 1 lists the K values of 14 kinds of nucleic acid constituents, such as nucleobases, nucleosides and nucleotides in the two-phase solvent system composed of 1-propanol/800 mM potassium phosphate buffer (pH 7.4) (1 : 1) that were determined by a simple test tube method.. Most of these nucleic acid constituents were more partitioned in the lower aqueous phase except for adenine, adenosine and 2'-deoxyuridine. In the present sets of nucleic acid constituents, the K values of nucleobases were greater than those of nucleosides with sugars bonded to the nucleobases. The similar relationship was observed between nucleoside and nucleotide where the K values of the nucleosides were greater than those of the nucleotides which have an additional phosphate group. Among the nucleosides, the K value of uridine is smaller than that of 2'-deoxyuridine suggesting that uridine bonded to sugar is more hydrophilic than deoxysugar bonded 2'-deoxyuridine. In the nucleotide, the K value of AMP is smaller than that of dAMP which has a deoxysugar component.

3. 2. Separation of eight nucleic acid constituents by HSCCC

Capability of the type-J CPC was demonstrated on the separation of selected eight nucleic acid constituents containing nucleobases, nucleosides and nucleotides using an extremely hydrophilic solvent system composed of 1-propanol/800 mM potassium phosphate buffer (pH 7.4) (1 : 1). As described earlier, the separation column was first entirely filled with the stationary upper organic phase, then the column was rotated at 1000 rpm while an aqueous lower mobile phase was pumped into the column at a flow-rate of 0.5 ml/min. After the hydrodynamic equilibrium was established in the column, a sample solution containing UMP, AMP, dAMP, uridine, uracil 2'-deoxyuridine, adenosine and adenine (each 0.5 mg/ 500 μ l solvent system) was injected through the sample port.

Fig. 3 shows a comprehensive separation of these eight nucleic acid constituents having different K values. The number of each elution peak corresponds to the number on the test compounds as shown in Fig. 1. UMP, AMP, dAMP, uridine, uracil and 2'-deoxyuridine were eluted from the column in an increasing order of their K values. After six kinds of polar compounds were eluted from the column, the upper phase, which was initially used as the stationary phase, was eluted through the column to facilitate a rapid elution of adenosine and adenine still retained in the column. The upper stationary phase retained in the column before switching the mobile phase was estimated as 42.5% of the total column capacity (40 ml). The separation was completed within 160 min.

Conclusion

The overall results of our studies indicate that the present method is capable of separating highly polar compounds, such as nucleic acid constituents using the new extremely hydrophilic solvent system composed of 1-propanol/800mM potassium phosphate (1:1, v/v) by HSCCC. This solvent system would also be useful for the separation of other hydrophilic compounds by the type J coil planet centrifuge.

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1. adenine

2. adenosine

3. AMP

4. 2'-deoxy AMP

5. guanine

6. guanosine

7. GMP

8. cytosine

9. cytidine

10. CMP

11. uracil

12. uridine

13. 2'-deoxy uridine

14. UMP

Fig. 1.
Chemical structures of 14 nucleic acid constituents

Fig. 2.
Analytical HSCCC system connected to the type J coil planet centrifuge. J-CPC: type J coil planet centrifuge; DAD: diode-array detector; ELSD: evaporative light scattering detector; UP: upper phase; LP: lower phase.

Fig. 3. Separation of 8 kinds of nucleic acid constituents by HSCCC using extremely hydrophilic solvent system. Dimension of PTFE multilayer coiled column: 1.0 mm I.D. $\times$ 50 m (40 ml capacity); solvent system: 1-propanol/800 mM potassium phosphate buffer (pH 7.4) (1 : 1); stationary phase: upper phase; mobile phase: lower phase; flow rate: 0.5 ml/min; revolution: 1000 rpm; UP = the point when the mobile phase was switched from lower phase to upper phase.

Table 1

Partition coefficient values of several nucleic acid constituents

nucleic acid constituents	compounds	$K_{UP/LP}$
nucleobase	adenine	3.24
	guanine	0.75
	cytosine	0.63
	uracil	0.81
nucleoside	adenosine	2.11
	guanosine	0.36
	cytidine	0.35
	uridine	0.43
	2'-deoxyuridine	1.13
nucleotide	adenosine 5'-monophosphate (AMP)	0.17
	2'-deoxyadenosine 5'-monophosphate (dAMP)	0.32
	guanosine 5'-monophosphate (GMP)	0.01
	cytidine 5'-monophosphate (CMP)	0.01
	uridine 5'-monophosphate (UMP)	0.02