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Short communication

Separation of nucleobases and their derivatives with organic-high ionic strength aqueous phase systems by spiral high-speed counter-current chromatography

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ABSTRACT

A set of nucleic acid constituents were separated with ultra polar two-phase solvent systems by a spiral multilayer coil mounted on the rotary frame of a type-J coil planet centrifuge. These two-phase systems were composed of 1-butanol/ethanol/50% saturated aqueous ammonium sulfate at various volume ratios. Nucleobases including adenine, cytosine, uracil, and thymine; nucleosides including adenosine, guanosine, cytidine, and uridine; and nucleotides including, AMP, GMP, CMP, UMP, and TMP are partitioned in each group with suitable solvent ratios. Adenine derivatives such as adenosine, AMP, ADP, and ATP were well resolved in the most polar solvent system composed of ethanol/50% saturated aqueous ammonium sulfate at a volume ratio of 1:2. It was found that cytosine and cytidine peaks showed some irregular two peaks probably due to their keto and enol isomers, while the separation of AMP forms two peaks especially when TMP was added in the sample solution, the mechanism of which is now under investigation in our laboratory.

1. Introduction

Since 1980, high-speed counter-current chromatography (HSCCC) has been increasingly used for separation of a wide variety of samples in natural and synthetic products [1–5]. Different from other conventional liquid chromatography, it uses two-liquid phases in the column containing no solid support. The retention of the stationary phase is attained by an Archimedean Screw effect produced by the combination of coiled column geometry and rotating centrifugal force field in the planetary centrifuge. In this system, solute separation depends on the partition coefficient (K) which determines the retention volume of each peak. In order to achieve a suitable separation, it is essential to select the two-phase solvent system which provides suitable K values for the target compounds. Applicable solvent systems may be largely divided into two types, namely organic–aqueous and aqueous–aqueous systems. The organic–aqueous solvent systems are most widely used for separation of natural products [6], while aqueous–aqueous systems such as PEG (polyethylene glycol) and dextran are mainly used for separation of biopolymers and cells [7]. The existing organic–aqueous solvent systems can be applied for separations of hydrophobic to moderately polar compounds, but they fail to provide suitable K values for highly polar samples including catecholamines, zwitter ions, sulfonic acids, nucleotides and their constituents, etc. Although aqueous–aqueous polymer phase systems may be used for separation of these compounds, they are not well retained in the conventional multilayer coil separation column and it is not easy to eliminate polymers from the collected fractions. In order to expand the utility of counter-current chromatography, we have recently devised highly polar solvent systems composed of alcohols and high ionic strength aqueous solution which can be used for separation of highly polar compounds [8,9]. The retention of these solvent systems is improved using the spiral column in HSCCC [10,11].

The present paper describes separation of nucleotides and their constituents in a series of polar two-phase solvent systems composed of 1-butanol, ethanol, and 50% saturated ammonium sulfate at various volume ratios.

2. Experimental

2.1. Apparatus

The apparatus used in the present studies was a type-J coil planet centrifuge (Ito Multilayer Coil Separator/Extractor, P.C. Inc. Potomac, MD, USA). It holds the separation column and its counterweight in symmetrical positions at 10 cm from the central axis of the centrifuge. The column holder (spiral tube support) was

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purchased from CCBiotech, Rockville, MD, USA. It was made of Nylon by a laser sintering method for rapid prototyping, and consisted of 16 cm diameter and 5 cm thick with four interwoven spiral grooves to accommodate a long piece of PTFE (polytetrafluoroethylene) tubing (Zeus Industrial Product, Orangeburg, SC, USA) to make multiple spiral layers. The column was made in our laboratory by inserting flat-twisted PTFE tubing [12] of 1.6 mm I.D. to form 10 spiral layers with a total capacity of about 90 mL. Both ends of tubing were connected to flow tubes (PTFE SW 20, 0.85 mm I.D., Zeus Industrial Products) which were first led through the column holder downward and then through the opening of the hollow central shaft of the centrifuge upward to exit the centrifuge. They are tightly supported on the upper plate of the centrifuge with a pair of clamps. These flow tubes are free of twisting during the centrifugation [1]. The solvent was pumped with an HPLC pump (LC-10AD, Shimadzu Corporation, Kyoto, Japan) and the effluent was monitored with a UV detector (Uvicord SII, LKB Instruments, Bromma, Sweden) and a strip-chart recorder (Pharmacia, Stockholm, Sweden).

2.2. Reagents

1-Butanol of HPLC grade was purchased from Fisher Scientific Co., Park Lawn, NJ, USA and anhydrous ethanol from Warner Graham Co., Cockeysville, MD, USA. Ammonium sulfate was obtained from Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA. Test samples including, adenine, guanine, cytosine, uracil, thymine, adenosine, guanosine, cytidine, uridine, thymidine, adenosine 5′-monophosphate (AMP), adenosine 5′-diphosphate (ADP), adenosine 5′-triphosphate, (ATP), guanosine 5′-monophosphate (GMP), cytidine 5′-monophosphate (CMP), uridine 5′-monophosphate (UMP), and thymidine 5′-monophosphate (TMP) were all purchased from Sigma Chemicals, St. Louis, MO, USA.

2.3. Preparation of two-phase solvent systems and sample solutions

First, saturated ammonium sulfate stock solution was prepared as follows: ammonium sulfate was added to a one gallon glass bottle containing water placed on a magnetic mixer until the water became cloudy. The mixing was continued for a few hours until the solvent returned to room temperature. (At this time if the solution became clear, one must add more ammonium sulfate to repeat the above process.) Then, the bottle was left overnight to form a clear supernatant. This saturated ammonium sulfate solution is about 4 M and has a density of 1.26 g/cm^3, and it is diluted 50% (v/v) with distilled water for solvent preparation. A set of two-phase solvent systems listed in Table 1 was prepared by adding the solvent mixture in a separatory funnel followed by vigorous shaking and degassing several times. After two clear layers were formed, two phases were separated. Sample solutions were prepared by dissolving a sample mixture in the upper phase of the two-phase solvent system used for separation. In order to ensure formation of two-phases in the separation column, a small amount of the lower phase was added to the sample solution until it became cloudy. When the sample is dissolved in one phase, either upper or lower phase, the phase composition is altered especially with high sample concentration which may form a single phase when mixed with the other phase in the column. This would result in substantial loss of the stationary phase from the column. This complication can be avoided by saturating the sample solution with the other phase before injection.

2.4. Partition coefficient measurement

The partition coefficient (K) for each test sample was determined with a spectrophotometer (Genesys 10 UV, Thermo Spectronic, Rochester, NY, USA). In each measurement, a small amount (approximately 100 μg) of sample was added to a test tube containing about 4 mL of solvent consisting of equal volumes of each phase from an equilibrated two-phase phase solvent system. The contents were mixed with a vortex mixer until the sample was completely dissolved and partitioned into both phases. Then, an equal amount of each phase (usually 20 μL) was diluted with 2 mL of water to determine the absorbance at 254 nm with a spectrophotometer. When the solvent containing 1-butanol was diluted with water, the upper phase solution became cloudy. In this case, 50% ethanol was used to dilute each phase. The K value was expressed as the UV absorbance in the upper phase divided by that in the lower phase.

Table 1
K values of nucleobases, nucleosides and nucleotides in key ultra-polar solvent systems.

Sample	Solvent systems			
	$BuOH/H_2O$	I[a]	II[b]	III[c]
1. Adenine	1.71	1.82	2.51	2.37
2. Guanine	0.91	0.33	1.12	1.01
3. Cytosine	0.26	0.24	0.59	1.51
4. Uracil	0.32	0.51	1.08	1.97
5. Thymine	1.38	2.31	2.78	2.93
6. Adenosine	0.64	1.72	2.94	3.38
7. Guanosine	0.21	0.11	0.46	1.42
8. Cytidine	0.15	0.08	0.31	0.91
9. Uridine	0.19	0.34	0.77	1.66
10. Thymidine	0.63	2.57	3.64	4.34
11. AMP			0.35	1.27
12. ADP			0.04	0.58
13. ATP			0.03	0.38
14. GMP			0.03	0.32
15. CMP			0.02	0.31
16. UMP			0.06	0.45
17. TMP			0.28	1.38

[a] 1-Butanol/50% saturated aqueous ammonium sulfate (1:1, v/v).
[b] 1-Butanol/ethanol/50% saturated aqueous ammonium sulfate (1:1:2, v/v).
[c] Ethanol/50% saturated aqueous ammonium sulfate (1:2, v/v).

2.5. High-speed CCC separation

In each separation, the column was first completely filled with the upper stationary phase followed by sample injection with a syringe. Then the column was rotated at 750 or 800 rpm while the mobile phase was pumped into the internal head terminal of the spiral column at a desired flow rate of 0.5 or 1 mL/min. The effluent from the outlet of the column was monitored with a UV detector at 280 nm and the chromatogram was traced with a strip-chart recorder. The effluent was collected into a graduated cylinder to measure the volume of stationary phase displaced with the mobile phase to compute the retention of the stationary phase in the column.

2.6. Computation of partition efficiency

The efficiency of separation was expressed in terms of theoretical plates (TP or N) using a conventional formula:

$$N = \left(\frac{4R}{W}\right)^2 \quad (1)$$

where R is the retention volume and W, the peak width.

2.7. Analysis of HSCCC fractions

LC/MS analysis employed a Waters LC-MS system (Waters, Milford, MA) that included an Acquity UPLC system coupled to the Waters Q-Tof Premier high resolution mass spectrometer.

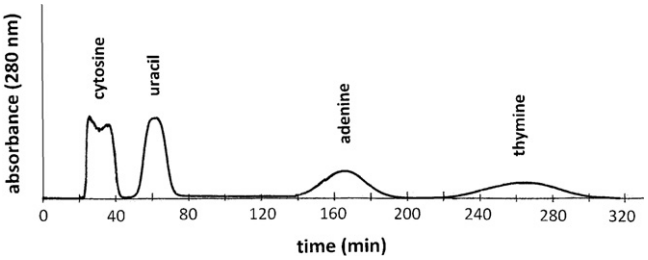

Fig. 1. Separation of nucleobases by spiral high-speed CCC. Experimental conditions: apparatus: type-J CCC centrifuge equipped with a spiral column containing 1.6 mm ID, flat-twisted PTFE tubing with a total capacity of 90 mL; sample: a set of nucleobases including adenine, cytosine, uracil, and thymine each 0.3 mg in 1 mL upper phase; solvent system: 1-butanol/50% saturated aqueous ammonium sulfate (1:1, v/v) (solvent system I); mobile phase: lower phase; elution mode; internal head to external tail of the spiral column; flow rate: 1 mL/min; revolution: 800 rpm; retention of stationary phase: 80%.

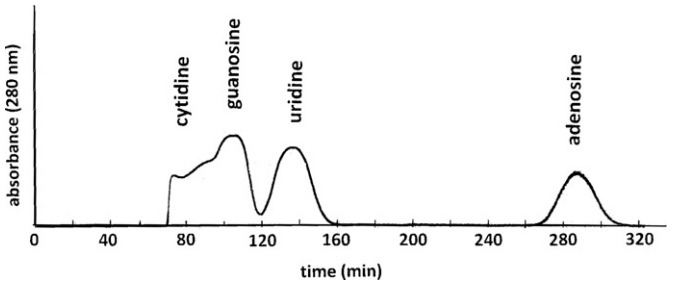

Fig. 2. Separation of nucleosides by spiral high-speed CCC. Experimental conditions: sample: a set of nucleosides including adenosine, guanosine, cytidine and uridine each 1 mg in 1.5 mL solvent: solvent system: 1-butanol/ethanol/50% saturated aqueous ammonium sulfate (0.8:1:2, v/v) (solvent systems II:III = 4:1); flow rate: 0.5 mL/min; revolution: 750 rpm; retention of stationary phase: 71%. Other conditions are described in Fig. 1 caption.

An Acquity BEH Shield RP18 column (150 mm × 2.1 mm) was employed for chromatography. Elution was achieved with isocratic 2 mM ammonium formate, 0.1% formic acid, and 5% CH_3CN at 0.35 mL/min. The entire column eluate was introduced into the Q-Tof mass spectrometer. Ion detection was achieved in an ESI mode using a source capillary voltage of 3.5 kV, source temperature of 110 °C, desolvation temperature of 200 °C, cone gas flow of 50 L/h (N_2), and desolvation gas flow of 700 L/h (N_2).

3. Results and discussion

3.1. Partition coefficient of test samples

All test samples were subjected to partition coefficient measurement mostly in three key solvent systems, the results of which are summarized in Table 1.

3.2. Separations of nucleobases and their derivatives

Fig. 1 shows a spiral counter-current chromatogram of a set of nucleobases including adenine, cytosine, uracil, and thymine each 0.3 mg with a solvent system (system I) composed of 1-butanol/50% saturated aqueous ammonium sulfate (1:1, v/v). Guanine is not well dissolved in this solvent system. The separation was performed by eluting with the lower phase at a flow-rate of 1 mL/min at 800 rpm. The effluent was monitored with a UV detector at 280 nm. As shown in the chromatogram, cytosine (K = 0.14) was eluted near the solvent front with two peaks. It is well known that cytosine has two isomers of enol and keto forms [13] with each different K values. The partition efficiencies range from 150 to 200 TP. The retention of the stationary phase was over 80% of the total column capacity. The maximum column pressure during the separation was about 100 psi.

Chromatogram of four nucleosides including adenosine guanosine, cytidine and uridine each 1 mg was illustrated in Fig. 2. We have found that between solvents I and II and between solvents II and III, K values of solutes are linearly shifted. Therefore, solute K values between these two key solvents can be determined using a graphic method by drawing a straight line between the K values in these two key solvent systems [14]. Separation was performed with a two-phase solvent system consisting of 1-butanol/ethanol/50% saturated aqueous ammonium sulfate at a volume ratio of 0.8:1:2 determined according to the above graphic method by eluting the lower phase at a flow rate of 0.5 mL/min at 800 rpm. Thymidine was not included in the sample mixture since it is not separable from adenosine as indicated by their K values shown in Table 1. After 74 mL of the lower phase was eluted, the mobile phase was switched to the upper phase (Elution Extrusion CCC) [15] to elute adenosine still retained in the column. As mentioned above, the cytidine peak again showed two components which overlapped with the guanosine peak. The efficiency of separation estimated by the uridine peak was 230 TP while the retention of the stationary phase was 71%.

Finally the separations of nucleotides are illustrated in Figs. 3 and 4. Fig. 3A shows separation of 5 nucleotides including AMP, DMP, UMP, CMP and TMP in a most polar two-phase solvent system composed of ethanol/50% saturated aqueous ammonium sulfate at a volume ratio of 1:2 (system III) using the lower phase as the mobile phase at a flow rate of 0.5 mL/min at 750 rpm. As shown in the chromatogram, the AMP peak is split into two (analyzed by LC/MS) and the second peak is overlapping with the TMP peak. Fig. 3B shows the similar separation of 4 nucleotides (without TMP) where AMP forms almost a single peak with the same retention time of the second AMP peak in Fig. 3A. These results were quite reproducible, and indicate some interaction between the AMP and TMP in solvent system III resulting in the first AMP peak in Fig. 3A. Also it is interesting that the K value of TMP in solvent system II

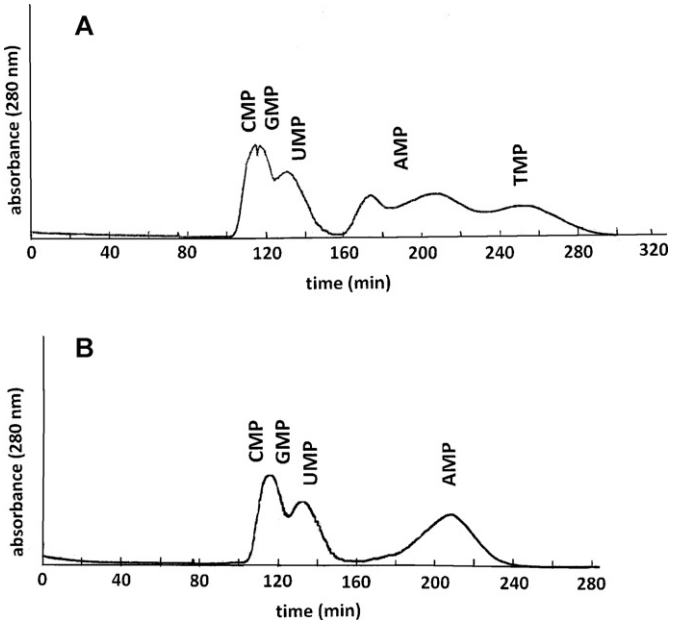

Fig. 3. (A and B) Separations of a set of nucleotides. Experimental conditions: Sample: (A) AMP, GMP, CMP, UMP, and TMP each 1 mg in 2 mL of solvent, (B) AMP, GMP, CMP and UMP each 1 mg in 2 mL of solvent: solvent system: ethanol/50% saturated aqueous ammonium sulfate (1:2, v/v) (solvent system III); flow rate: 0.5 mL/min; revolution 750 rpm; retention of stationary phase: 60%. Other conditions are described in Fig. 1 caption.

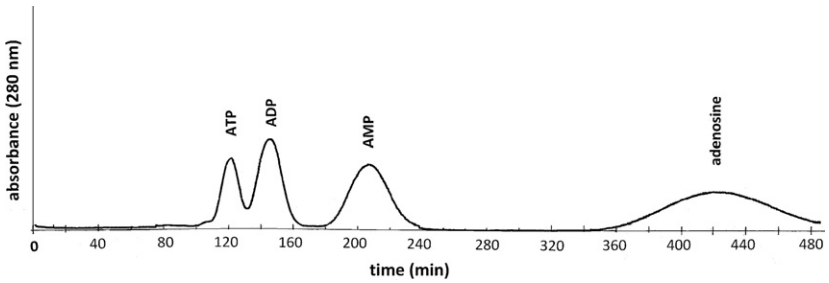

Fig. 4. Separation of adenosine and its three nucleotides. Experimental conditions: sample: adenosine, AMP, AMP, ADP and ATP, each 0.5 mg in 0.5 mL upper phase; solvent system: ethanol/50% saturated aqueous ammonium sulfate (1:2, v/v) (solvent system III); flow rate: 0.5 mL/min; revolution: 750 rpm; retention of stationary phase: 60%. Other conditions are described in Fig. 1 caption.

measured with the test tube procedure is 0.28 while the *K* value estimated from the chromatogram obtained in the same solvent system is about 0.5 (chromatogram not shown). The mechanisms involved in these unexpected findings are now under investigation in our laboratory. Fig. 4 shows separation of ATP, ADP, AMP and adenosine indicating that polarity of the compounds increases with the number of phosphate moieties in the molecule. The partition efficiencies in this separation range from 160 to 500 TP. The retention of the stationary phase was slightly over 60%

4. Conclusions

Nucleotides and their constituents were separated in each group with a novel series of two-phase solvent systems composed of 1-butanol/ethanol/50% saturated aqueous ammonium sulfate at various volume ratios. Most of these separations are unlikely to be possible with the conventional organic–aqueous solvent systems due to their high polarity. In addition successful separations were achieved by HSCCC using a spiral column which provided satisfactory stationary phase retention in the column. The present method may be widely applied to separations of other highly polar compounds.

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