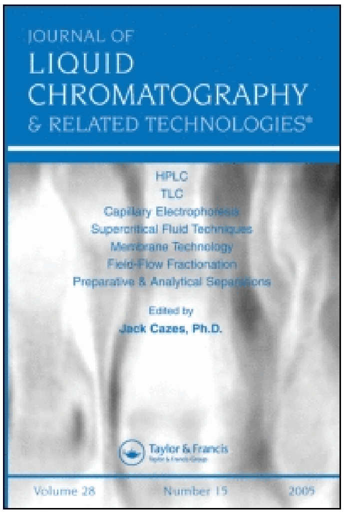

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Kazufusa Shinomiya[a]; Ken Yoshida[a]; Yozo Kabasawa[a]; Yoichiro Ito[b]
[a] College of Pharmacy, Nihon University, Chiba, Japan [b] Laboratory of Biophysical Chemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, U.S.A.

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COUNTERCURRENT CHROMATOGRAPHIC SEPARATION OF WATER-SOLUBLE VITAMINS BY CROSS-AXIS COIL PLANET CENTRIFUGE USING AN ION-PAIR REAGENT WITH POLAR TWO-PHASE SOLVENT SYSTEM

Kazufusa Shinomiya,[1,*] Ken Yoshida,[1] Yozo Kabasawa,[1]
and Yoichiro Ito[2]

[1] College of Pharmacy, Nihon University, 7-7-1,
Narashinodai, Funabashi-shi, Chiba 274-8555, Japan
[2] Laboratory of Biophysical Chemistry, National Heart,
Lung, and Blood Institute, National Institutes of Health,
Building 10, Room 7N-322, Bethesda, MD 20892-1676, USA

ABSTRACT

Countercurrent chromatographic separation of water-soluble vitamins, such as riboflavin sodium phosphate, cyanocobalamin, riboflavin, pyridoxine hydrochloride, and thiamine nitrate was performed using the cross-axis coil planet centrifuge equipped with a pair of eccentric coil assemblies at off-center position. The solvent system used in the present studies was prepared by adding 1.5% of 1-octanesulfonic acid sodium salt, as an ion-pair reagent, to the polar two-phase solvent system composed of 1-butanol and aqueous 0.15 M monobasic potassium phosphate. The resolution

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* Corresponding author. E-mail: kshino@pha.nihon-u.ac.jp

was remarkably improved as compared with that obtained in our previous studies.

Overall experimental results revealed that the cross-axis coil planet centrifuge is useful for the separation of extremely hydrophilic and ionic compounds, such as water-soluble vitamins, by adding a suitable ion-pair reagent to the polar two-phase solvent system.

INTRODUCTION

Countercurrent chromatography (CCC) has been widely used for the separation and purification of various natural and synthetic products.(1-3) Among many CCC devices developed in the past, type-J multilayer coil planet centrifuge and cross-axis coil planet centrifuge (cross-axis CPC) have proven to be the most complete models.

Cross-axis CPC has a unique mode of planetary motion, such that the column holder rotates about its horizontal axis while revolving around the vertical axis of the centrifuge.(4,5) The centrifugal force field produced by this planetary motion provides stable retention of the stationary phase, even for low-interfacial tension two-phase solvent systems, such as aqueous-aqueous polymer phase systems, which gives exhibits retention of the stationary phase when using the type-J multilayer coil planet centrifuge.

Our previous studies have been demonstrated that the cross-axis CPC, equipped with a pair of multilayer coils or eccentric coil assemblies in the off-center position, was useful for the separation of proteins with aqueous-aqueous polymer phase systems by constructing the improved machine in our laboratory.(6-8) This apparatus is also useful for the separation of highly polar compounds, such as sugars,(9) hippuric acid and related compounds,(10) vitamins,(11) and water-soluble organic acids.(12)

Among these studies, the CCC separation for extremely polar vitamins required the further research for suitable solvent systems.(11) The present studies describe the improved CCC separation of water-soluble vitamins by adding an ion-pair reagent to the polar two-phase solvent system.

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 Separation Columns

A pair of eccentric coil assemblies was used in the present study. 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 symmetrically arranged 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 total column capacity is 26.5 mL.

Reagents

Thiamine nitrate, thiamine hydrochloride, riboflavin, riboflavin sodium phosphate, pyridoxine hydrochloride, cyanocobalamin, L-ascorbic acid, nicotinamide, 1-butanesulfonic acid sodium salt, and 1-octanesulfonic acid sodium salt were purchased from Wako Pure Chemicals (Osaka, Japan). 1-Pentanesulfonic acid sodium salt and 1-dodecanesulfonic acid sodium salt were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). All other reagents were of reagent grade.

Preparation of Two-Phase Solvent Systems and Sample Solutions

A set of two-phase solvent systems used in the present studies was prepared by dissolving an ion-pair reagent, at a desired concentration, in the two-phase solvent system composed of 1-butanol and aqueous 0.15 M monobasic potassium phosphate. Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature, and the two phases separated after two clear layers were formed.

The sample solutions were prepared by dissolving each vitamin mixture in 0.5 mL of each phase of the two-phase solvent system used for separation.

Measurement of Partition Coefficients of Vitamin Samples

The partition coefficient (K) of each water-soluble vitamin 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 the organic phase was diluted with 2 mL of methanol, while the aqueous phase was diluted with distilled water. The absorbance was measured at 260 nm using a spectrophotometer (Model UV-1600, Shimadzu Corporation, Kyoto, Japan). The K value was obtained by dividing the absorbance value of the upper organic phase by that of the lower aqueous phase.

CCC Separations of Water-Soluble Vitamins

Each separation was initiated by completely filling the column with the upper stationary phase, followed by injection of the sample solution (ca. 1 mL) into the column inlet. Then, the lower mobile phase was pumped into the column at a flow rate of 0.2 mL/min using a reciprocating pump (Model KHU-W-52H, Kyowa Seimitsu Co., Tokyo, Japan), while the column was rotated at 800 rpm in a counterclockwise direction. The effluent from the outlet of the column was collected in test tubes (0.4 mL/tube) using a fraction collector (Model SF-200, Advantec Co., Tokyo, Japan).

Analysis of CCC Fractions

Each collected fraction was diluted with 2 mL of distilled water and the absorbance was measured at 260 nm using a spectrophotometer.

RESULTS AND DISCUSSION

In order to improve the separation of each water-soluble vitamin by cross-axis CPC, the partition coefficient (K) value was measured by adding an ion-pair reagent to a polar two-phase solvent system composed of 1-butanol and aqueous 0.15 M monobasic potassium phosphate. As described in the experimental section, four kinds of ion-pair reagents were examined in the present studies according to their polarities, based on the carbon number of each molecule.

Table 1 summarizes the K values of water-soluble vitamins obtained using the simple test tube methods. Most of the K values increased by increasing the concentration and carbon number of ion-pair reagents. However, it was found that the solvent system made by using 1-dodecanesulfonic acid sodium salt at the concentration of over 1.5% did not form clear two phases. These results suggest, that 1-octanesulfonic acid sodium salt may be most suitable ion-pair reagent for the separation of water-soluble vitamins by CCC.

Figure 1 illustrates the K values of these vitamins obtained by adding 1-octanesulfonic acid sodium salt in the above two-phase solvent system. K values

Table 1. Effect of Ion-Pair Reagents on Partition Coefficients of Water-Soluble Vitamins in 1-Butanol-Aqueous 0.15M KH_2PO_4 Solvent Systems

Ion-Pair Reagent	1-Butane Sulfonic Acid		1-Pentane Sulfonic Acid		1-Octane Sulfonic Acid
Concentration (%)	1.5	2.5	1.5	2.5	1.5
Thiamine Nitrate (M. W. 327.36)	0.18	0.29	0.76	1.01	2.45
Thiamine Hydrochloride (M.W. 327.27)	0.17	0.25	0.73	0.97	2.42
Riboflavin (M.W. 376.37)	0.58	0.70	0.57	0.95	1.07
Riboflavin Sodium Phosphate (M.W. 478.33)	0.04	0.29	0.08	0.20	0.13
Pyridoxine Hydrochloride (M.W. 205.64)	0.57	0.73	0.77	0.86	1.63
Cyanocobalamin (M.W. 1355.38)	0.08	0.44	0.37	0.35	0.47
L-Ascorbic Acid (M.W. 176.12)	0.04	0.13	0.09	0.09	0.09
Nicotinamide (MW. 122.13)	1.32	0.78	1.46	1.39	1.49

Partition coefficients were calculated from the absorbance of the upper phase divided by that of lower phase.

of thiamine nitrate, thiamine hydrochloride, pyridoxine hydrochloride, and cyanocobalamin increased with the increased concentration of this ion-pair reagent, while riboflavin sodium phosphate and L-ascorbic acid were almost unilaterally partitioned in the aqueous phase. Riboflavin and nicotinaminde showed almost constant values regardless of the concentration change. From the above results, the concentration at 1.5% of the ion-pair reagent in the two-phase solvent system was selected for CCC separation using the cross-axis CPC.

Figure 2 illustrates the CCC chromatogram of water-soluble vitamins obtained with the above solvent system composed of 1-butanol and aqueous 0.15 M monobasic potassium phosphate, including 1.5% of 1-octanesulfonic acid sodium salt. Riboflavin sodium phosphate, cyanocobalamin, riboflavin, pyridoxine hydrochloride, and thiamine nitrate were separated using the lower phase as a mobile phase. Riboflavin was found as an impurity contained in the riboflavin

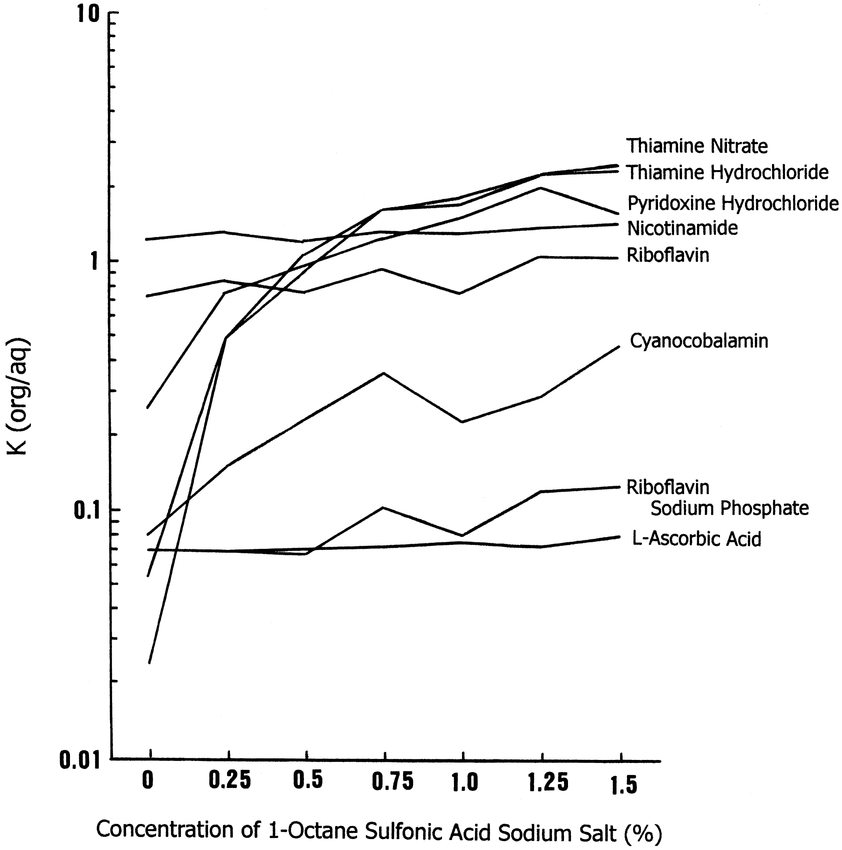

***Figure 1**.* Partition coefficients (K = Corg/Caq) of water-soluble vitamins in the polar two-phase solvent system, composed of 1-butanol and aqueous 0.15 M monobasic potassium phosphate containing 1-octanesulfonic acid sodium salt at various concentrations.

sodium phosphate sample used in this experiment. The retention of the organic stationary phase was 45.3%. Table 2 illustrates the analytical values obtained in the present studies. The resolution between each vitamin peak was 0.8, 2.3, 1.2, and 0.9, respectively, which was improved compared to what was obtained in our previous studies.(11)

The overall results indicate that the cross-axis CPC, equipped with a pair of eccentric coil assemblies at off-center position, can resolve extremely hydrophilic

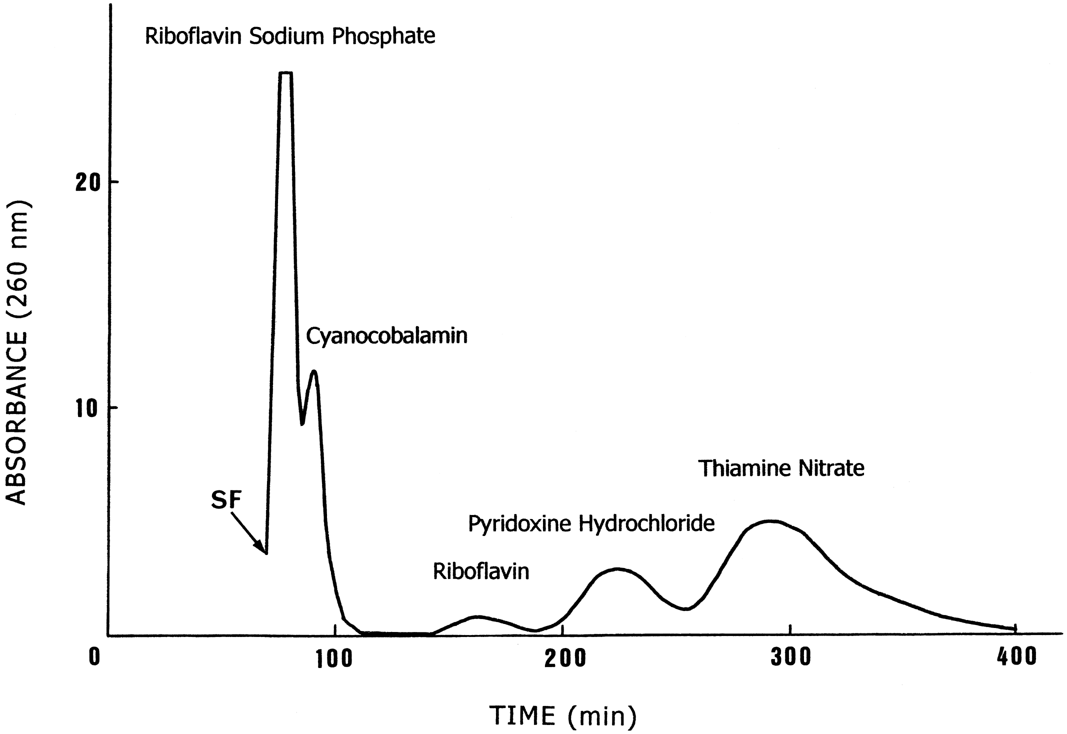

Figure 2. CCC separation of water-soluble vitamins by cross-axis CPC. Experimental conditions: apparatus: cross-axis CPC, equipped with a pair of eccentric coil assemblies, 1 mm ID and 26.5 mL capacity; sample: riboflavin sodium phosphate (2.5 mg), cyanocobalamin (2.5 mg), pyridoxine hydrochloride (2.5 mg) and thiamine nitrate (2.5 mg); solvent system: 1-butanol and aqueous 0.15 M monobasic potassium phosphate containing 1.5% of 1-octanesulfonic acid sodium salt; mobile phase: lower phase; flow rate: 0.2 mL/min; revolution: 800 rpm. SF = solvent front.

Table 2. Analytical Values Obtained from Present CCC Separation Studies

Water-Soluble Vitamin	Partition Coefficient*	Separation Factor (α)	Resolution (Rs)
Riboflavin Sodium Phosphate	0.10 (0.13)		
		3.20 (3.62)	0.8
Cyanocobalamin	0.32 (0.47)		
		4.59 (2.28)	2.3
Riboflavin	1.47 (1.07)		
		1.49 (1.52)	1.2
Pyridoxine Hydrochloride	2.43 (1.63)		
		1.43 (1.50)	0.9
Thiamine Nitrate	3.48 (2.45)		

*Partition coefficients were calculated according to the conventional formula: $K = (V_r - V_{sf}) / (V_c - V_{sf})$, where V_r, V_c and V_{sf} indicate the retention volume of the solute peak, the column capacity and the retention volume of the solvent front (volume of the mobile phase in the column), respectively. The values in the parentheses were obtained from the experimental results using the test tube method.

and ionic compounds by adding a suitable ion-pair reagent to the polar two-phase solvent system.

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