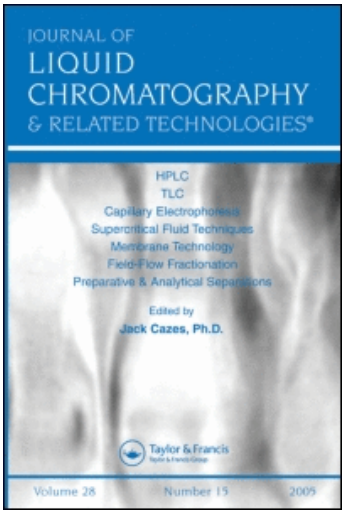

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Countercurrent Chromatographic Separation of Lipophilic Ascorbic Acid Derivatives and Extract from *Kadsura coccinea* Using Hydrophobic Organic-Aqueous Two-Phase Solvent Systems

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Abstract: Countercurrent chromatographic (CCC) separation of lipophilic ascorbic acid derivatives and the crude extract from *Kadsura coccinea* was performed using the type-J multilayer coil planet centrifuge with a hydrophobic organic-aqueous two-phase solvent system composed of *n*-hexane/ethyl acetate/ethanol/aqueous 0.1% trifluoroacetic acid at the volume ratio of 5:5:6:2. The lipophilic ascorbic acid derivatives were separated in the order of L-ascorbyl 2,6-dibutyrate, L-ascorbyl 6-palmitate and L-ascorbyl 6-stearate by eluting the lower phase as the mobile phase, and L-ascorbyl 2,6-dipalmitate was separated by eluting the upper phase at the opposite direction. The above solvent system was then applied to the CCC separation of the extract prepared from *K. coccinea*. With lower phase mobile, the extract was mainly separated into two peaks corresponding to lignans and triterpenoids accordingly. The HPLC analysis of the fractions showed that the former peak contained Kadsulignan N, Schizandrin H and Neokadsuranin as lignans, and the latter peak, Micranoic acid A, Neokadsuranic acid B and beta-Sitosterol as triterpenoids. The overall results indicate that the hydrophobic organic-aqueous two-phase solvent system

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used in the present studies was useful for the CCC separation of lignans and triterpenoids present in the natural products.

Keywords: Countercurrent chromatography, Lignan, Lipophilic ascorbic acid derivative, Natural product, Triterpenoid, Two-phase solvent system

INTRODUCTION

Countercurrent chromatography (CCC) has been increasingly utilized for the separation and purification of various chemical compounds from natural and synthetic products.[1–4] The absence of solid support eliminates serious complications such as loss of samples by adsorption and chemical degradation of compounds.

In order to achieve successful CCC separation, it is necessary to select a suitable two-phase solvent system that provides a proper range of partition coefficients (K) for the target compounds. When the nature of the compounds is unknown or the literature on the CCC separation of the similar compound is not available, a time-consuming trial and error method is generally required for the search of the suitable solvent system. Oka *et. al.* have reported a simple test tube method for systematically selecting suitable organic-aqueous two-phase solvent system in a wide range of hydrophobicity by adjusting the volume ratios of *n*-hexane/ethyl acetate/1-butanol/methanol/water and chloroform/methanol/water systems.[5] Our previous studies also revealed that the polar two-phase solvent systems composed of methyl *t*-butyl ether/acetonitrile/water are available for the CCC separation of water-soluble carboxylic acids.[6,7] Among these solvent systems described above, 1-butanol/water is most hydrophilic and has been applied to the separation of polar compounds such as sugars[8] and water-soluble vitamins.[9,10] In our previous studies, the CCC separation of polar biotic compounds was performed using more hydrophilic organic-aqueous two-phase solvent systems composed of ethanol or acetonitrile and inorganic salts.[11]

This paper describes the CCC separation of lipophilic ascorbic acid derivatives and the extract from *Kadsura coccinea* using hydrophobic organic-aqueous two-phase solvent systems composed of *n*-hexane/ethyl ethyl acetate/ethanol/aqueous 0.1% trifluoroacetic acid (TFA).

EXPERIMENTAL

Apparatus

The type-J multilayer coil planet centrifuge (type-J multilayer CPC) was purchased from Hitachi Tokyo Electronics (presently Renesas Eastern Janan Semiconductors, Inc., Tokyo, Japan).

Preparation of Coiled Columns

The multilayer coil for the type-J multilayer CPC was prepared by tightly winding a piece of 2.0 mm ID and 3.0 mm OD PTFE (polytetrafluoroethylene) tubing (Flon Kogyo, Tokyo, Japan) around the holder hub of 9 cm in diameter, forming tight coiled layers between a pair of flanges spaced 5.6 cm apart. The total column capacity was 108 mL.

Reagents

All organic solvents including *n*-hexane, ethyl acetate and ethanol were HPLC grade (Wako Pure Chemicals, Osaka, Japan). L-Ascorbyl 6-palmitate was purchased from Aldrich (St. Louis, MO, USA), and L-ascorbyl 2,6-dibutyrate, L-ascorbyl 2,6-dipalmitate, and L-ascorbyl 6-stearate were obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). All other reagents were of reagent grade. Rhizomes of *K. coccinea* were collected in Guangxi Province, People's Republic of China, in April 2004 and were identified by Dr. Bao-Lin Guo, Peking Union Medical College, Beijing, China. Voucher specimens were deposited in the Department of Pharmacognosy, College of Pharmacy, Nihon University, Japan.[12–14]

Preparation of Extract Sample for CCC Separation from *Kadsura coccinea*

The extract sample was prepared as follows:[12–14] The powdery dried rhizomes of *K. coccinea* (1.75 kg) were extracted three times with 80% acetone. The extracts were combined and dried in vacuum. The residue was dissolved and suspended in water, and extracted in turn with *n*-hexane, chloroform, ethyl acetate and 1-butanol. The *n*-hexane extract (12.2 g) and chloroform extract (32.5 g) were mixed and then loaded on a Silica gel chromatography column and subjected to a linear gradient elution of *n*-hexane/ethyl acetate at the volume ratio from (98:2) to (30:70). The effluents were fractionated into 8 fractions, and the 5th fraction was applied to the CCC separation.

Preparation of Two-Phase Solvent Systems and Sample Solution

A set of two-phase solvent systems was prepared from *n*-hexane, ethyl acetate, ethanol and aqueous 0.1% TFA at various volume ratios. Each solvent mixture was thoroughly equilibrated in a separatory funnel at

room temperature, and the two phases were separated after two clear layers were formed. The sample solution for CCC separation was prepared by dissolving each sample mixture in 1.0 mL of each phase of the two-phase solvent system used for separation.

Measurement of Partition Coefficients of Samples

Successful CCC separation highly depends upon the choice of the solvent system, which provides suitable K values for a set of analytes. In the present study, the K value of each standard sample was determined spectrophotometrically using a simple test tube method described by Oka et al.[5] as follows: Two milliliters of each phase of an equilibrated 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 clear layers were formed, a 1 mL aliquot of each phase was diluted with 2 mL of ethanol and the absorbance was measured at a desired wavelength 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.

Separation Procedure

Each separation was initiated by completely filling the column with the stationary phase, followed by injection of the sample solution into the column inlet. Then, the mobile phase was pumped into the column using a reciprocating pump (Model LC-10ADVP, Shimadzu), 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 (1.0 mL/tube) using a fraction collector (Model SF-160, Advantec Co., Tokyo, Japan).

Analysis of CCC Fractions

Each collected fraction was diluted with 2 mL of ethanol and the absorbance was measured using a spectrophotometer (Model UV-1600, Shimadzu).

An aliquot of each CCC fraction of the *K. coccinea* extract was also analyzed by high-performance liquid chromatography (HPLC). The HPLC equipment included a reciprocating pump (Model LC-10ATVP, Shimadzu), a UV detector (Model SPD-10AVP, Shimadzu), and a variable input recorder (Model C-R8A, Shimadzu).

Analytical conditions for HPLC method were as follows: column: ODS-silica (YMC-Pack Pro C18, 4.6 mm I.D. × 250 mm, YMC Corporation, Kyoto, Japan); column temperature: room temperature, eluent: acetonitrile–water (95:5); flow rate: 1.0 mL/min; detection: 210 nm.

RESULTS AND DISCUSSION

CCC Separation of Lipophilic Ascorbic Acid Derivatives

Figure 1 illustrates the chemical structures of lipophilic ascorbic acid derivatives used in the present studies. Each structure contains the same hydrophilic part of ascorbic acid coupled with different hydrophobic fatty acids. The search on the suitable *K* value for CCC separation was first examined by the simple test tube method using the solvent system composed of *n*-hexane/ethyl acetate/methanol/water by varying their volume ratios. It was found that the volume ratios of (9:1:5:5) and (8:2:5:5) tended to form emulsion in the upper phase of the solvent system. Then, more hydrophobic solvent system composed of *n*-hexane/ethyl acetate/ethanol/aqueous 0.1% TFA was examined. Table 1 summarizes the *K* values at four different volumes ratios (8:2:6:2), (7:3:6:2), (6:4:6:2), and (5:5:6:2) in an order of increasing polarity in the upper phase. At the volume ratio of (8:2:6:2), three derivatives including L-ascorbyl 2,6-dibutyrate, L-ascorbyl 6-palmitate and L-ascorbyl 6-stearate were mostly distributed into the lower aqueous phase, but they were gradually partitioned in the upper organic phase as the polarity of

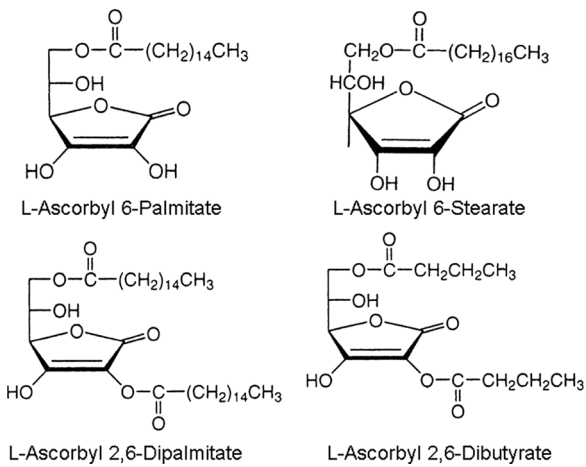

Figure 1. Chemical structures of lipophilic ascorbic acid derivatives used in the present studies.

Table 1. Distribution of lipophilic ascorbic acid derivatives and the extract sample prepared from *Kadsura coccinea*

Two-phase solvent system		Volume ratio			
n-hexane		8	7	6	5
EtOAc		2	3	4	5
EtOH		6	6	6	6
Aqueous 0.1% TFA		2	2	2	2
Sample	Measured wavelength (nm)	Partition coefficient ($K(C_U/C_L)$)			
L-Ascorbyl 6-palmitate	245	0.01	0.20	0.30	0.53
L-Ascorbyl 2,6-dipalmitate	230	5.30	7.18	4.31	4.06
L-Ascorbyl 6-stearate	245	0.08	0.27	0.29	0.75
L-Ascorbyl 2,6-dibutyrate	230	0.05	0.06	0.04	0.11
		Distribution ratio ($D(C_U/C_L)$)			
The extract sample	210	0.99	0.93	0.98	1.04
	280	0.69	1.33	1.32	1.26

the solvent system is increased whereas L-ascorbyl 2,6-dipalmitate was unilaterally partitioned in the upper phase in all the volume ratios. The ratio (5:5:6:2) showed the average K value of around 1 so that this solvent system was selected for the CCC separation of these test samples. Figure 2 illustrates the CCC chromatogram of ascorbic acid derivatives obtained using the type-J multilayer CPC with the above solvent system. With the lower phase mobile till 100 min from the start of operation, L-ascorbyl 2,6-dibutyrate, L-ascorbyl 6-palmitate and L-ascorbyl 6-stearate were eluted in the order of their K values measured by the simple test tube method, while L-ascorbyl 6-palmitate and L-ascorbyl 6-stearate were only partially resolved in this elution. After 100 min, L-ascorbyl 2,6-dipalmitate was eluted from the column by changing the mobile phase to the upper phase and switching the elution direction. The result suggests that these derivatives were separated by the difference of the hydrophobicity of fatty acid present in each structure. The derivatives having shorter number of carbons in fatty acid are hydrophilic and eluted earlier.

CCC Separation of Extract Sample from *Kadsura coccinea*

A series of above experiments was revealed that the solvent system composed of *n*-hexane/ethyl acetate/ethanol/aqueous 0.1% TFA is

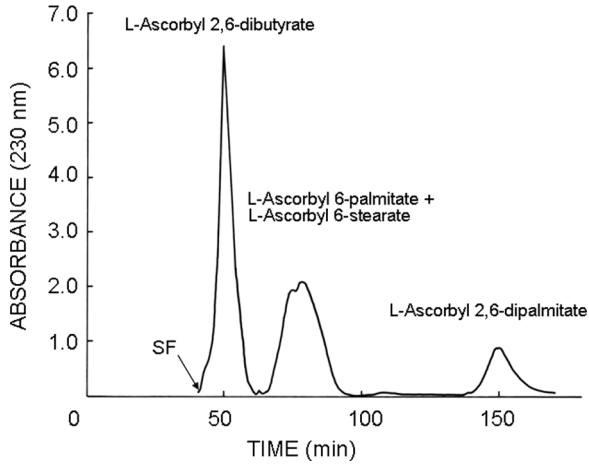

Figure 2. CCC Chromatogram of lipophilic ascorbic acid derivatives using the type-J multilayer CPC. Experimental conditions: apparatus: type-J multilayer CPC with a multilayer coil assembly with 2 mm I.D. and 108 mL capacity; sample: 5 mg each of L-ascorbyl 2,6-dibutyrate, L-ascorbyl 6-palmitate, L-ascorbyl 6-stearate, and L-ascorbyl 2,6-dipalmitate; solvent system: n-hexane/ethyl acetate/ethanol/aqueous 0.1% TFA (5:5:6:2); mobile phase: lower phase (till 100 min) and upper phase (after 100 min); flow rate: 1 mL/min; revolution: 800 rpm (counterclockwise direction). SF = solvent front.

useful for the separation of lipophilic ascorbic acid derivatives without forming emulsion. Then, the present solvent system was applied to the separation of the extract sample prepared from *K. coccinea*. Table 1

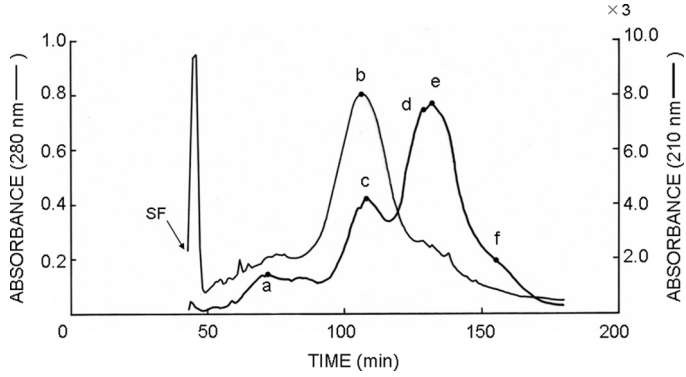

Figure 3. CCC Chromatogram of the extract sample prepared from *K. coccinea* using the type-J multilayer CPC. Experimental conditions: sample: the extract sample prepared from Kadsura coccinea (40 mg); mobile phase: lower phase. Other experimental conditions are same as those described in Figure 2.

(bottom) summarizes the distribution ratio (D) of the extract sample in the solvent system at the above four different volume ratios. Among these, the ratio of (5:5:6:2) showed the D value of around 1, which was similar to that used for the separation of lipophilic ascorbic acid derivatives. Figure 3 illustrates the CCC chromatogram obtained by 40 mg of the extract sample using the present solvent system as the lower mobile phase. The large single peak was observed after the solvent front by measuring the absorbance at 280 nm, while the two peaks were found at the detection with 210 nm. Figure 4 illustrates the HPLC chromatogram of the extract sample prepared from *K. coccinea*. Among numerous peaks observed in the chromatogram, six peaks have already been identified as Kadsulignan N (**1**), Schizandrin H (**2**), Micranoic acid A (**3**), Neokadsuranin (**4**), Neokadsuranic acid B (**5**) and beta-Sitosterol (**6**) in our previous studies.[12–14] Figure 5 illustrates these chemical structures. Figure 6

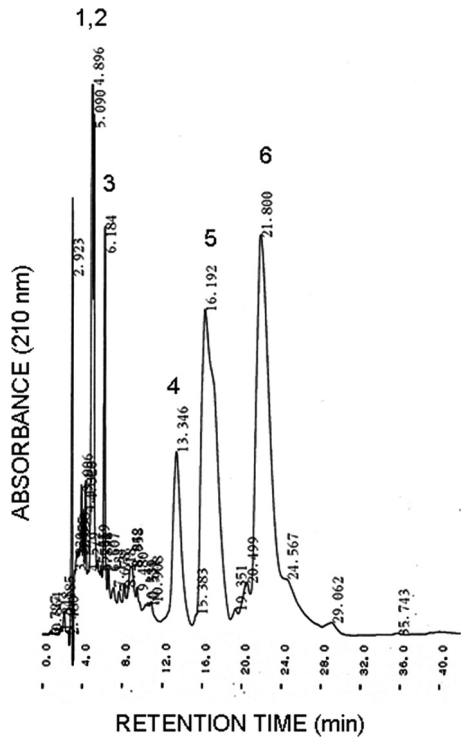

Figure 4. HPLC Chromatogram of the extract sample prepared from *K. coccinea*. Experimental conditions: column: YMC-Pack Pro C18 (4.6 mm I.D. x 250 mm); column temperature: room temperature: eluent: acetonitrile–water (95:5); flow rate: 1.0 mL/min; detection: 210 nm.

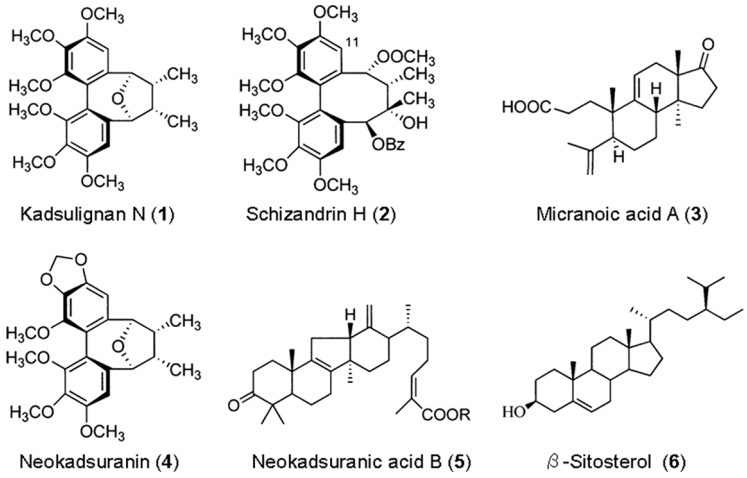

Figure 5. Chemical compounds isolated from the extract sample of *K. coccinea*. The number in the parenthesis corresponds to that of the peak in the HPLC chromatogram shown in Figure 4.

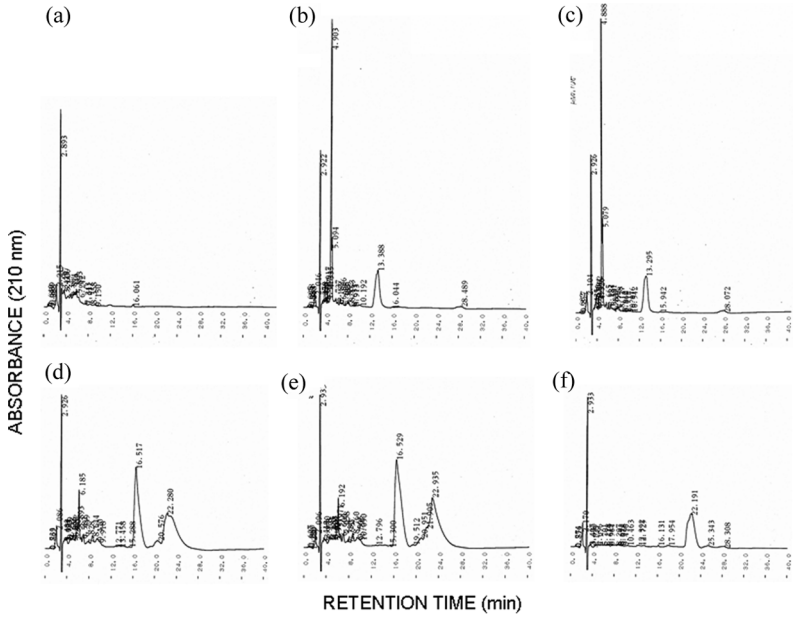

Figure 6. HPLC Chromatograms of CCC fractions. Experimental conditions are same as those described in Figure 4. The number of each HPLC chromatogram corresponds to that described in Figure 3.

illustrates a set of HPLC chromatograms obtained by the CCC fractions shown in Figure 3. Fractions b and c showed similar HPLC chromatograms including lignans **1, 2**, and **4**, while no identical peak was found in the fraction a. Fractions d and e also showed similar HPLC chromatograms including triterpenoids **3, 5**, and **6**. Compound **6** was also found in fraction f. These results indicate that the present CCC method can separate lignans and triterpenoids while the HPLC system separates triterpenoid **3** between lignans **2** and **4**.

CONCLUSION

The separation of lipophilic ascorbic acid derivatives was performed using the type-J multilayer CPC with an organic-aqueous solvent system composed of *n*-hexane/ethyl acetate/ethanol/aqueous 0.1% TFA at a volume ratio of 5:5:6:2. This solvent system was also successfully applied to the CCC separation of lignans and triterpenoids in the crude extract from *K. coccinea*. The overall results suggest that the solvent system used in the present studies is available for the CCC separation of hydrophobic compounds present in the natural and synthetic products.

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