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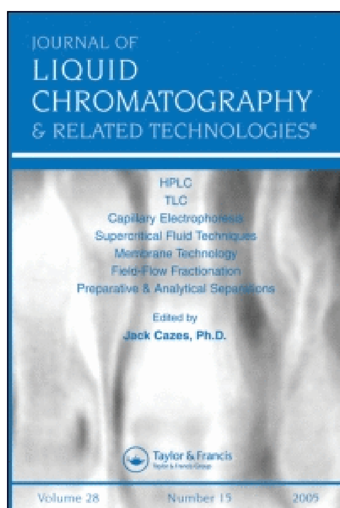

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Simple Preparative Isolation of Salvinorin A from the Hallucinogenic Sage, *Salvia divinorum*, by Centrifugal Partition Chromatography

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Abstract: A simple procedure was developed to isolate salvinorin A from the hallucinogenic sage, *Salvia divinorum*, by means of centrifugal partition chromatography (CPC) using *n*-hexane–dichloromethane–methanol–water (8:8:9:2, v/v) as a two-phase solvent system. The upper phase (organic phase) of the two-phase solvent system was used as the stationary phase for CPC separation. About 100 mg of salvinorin A was obtained from ca. 2.5 g of crude methanol extract by CPC, followed by purification in a silica gel short column (*n*-hexane–ethyl acetate = 1:1, v/v) and recrystallization from methanol. The product was more than 98% pure as determined by high performance liquid chromatography (HPLC).

Keywords: Centrifugal partition chromatography, CPC, Salvinorin A, *Salvia divinorum*, Hallucinogenic sage, Neo-clerodane diterpene

INTRODUCTION

The Mexican hallucinogenic sage, *Salvia divinorum*, which is called "diviner's sage" or "magic mint," contains neo-clerodane diterpene salvinorin A (Figure 1) as the hallucinogenic active constituent.[1,2] Recently, due to the

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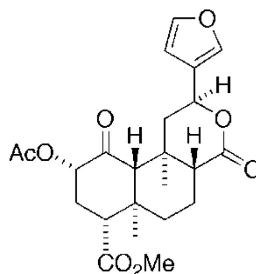

Figure 1. Structure of salvinorin A.

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increased popularity of hallucinogenic sage as a recreational drug, a number of countries have begun to regulate the plant or salvinorin A, or both, as control substances.[3] On the other hand, salvinorin A is a potent naturally occurring non-nitrogenous κ-opioid selective agonist and, hence, it is considered to be a seed for the development of novel therapeutic agents for the treatment of Alzheimer's disease.[4]

Several methods are described in the literature for extraction and isolation of salvinorin A from leaves of *S. divinorum*.[1,2,5] Most of these methods involve extraction by chloroform, ether, or acetone; partition between hexane and aqueous methanol; and column chromatography on silica gel or activated carbon, which are all time consuming steps. In general, silica gel strongly absorbs high polar compounds and sometimes reacts adversely with the sample, whereas activated carbon tightly absorbs aromatic and plane compounds so firmly that elution often fails to flush them out. It was against this background that we attempted to develop a simple method to isolate salvinorin A as a standard compound that can be used in criminal investigations related to the drug and also for pharmacological research.

The technique of centrifugal partition chromatography (CPC) used for the isolation of salvinorin A described in this report is one of the methods to separate a mixture of compounds, using the difference in their partition coefficients in a two-phase solvent system in which the two solvents do not mix mutually.[6] In CPC, the stationary phase is held in partition cells by centrifugal forces, and partition chromatography is performed because the mobile phase takes the form of minute droplets that pass through the stationary phase. Since an absorbent like silica gel is not used in this technique, it is applicable to a broad range of compounds, from high polar compounds to low polar compounds and from compounds of low molecular weight to polymers of high molecular weight. The method does not entail any loss of the sample, does not involve adsorption of compounds, which may be unstable, requires no special pretreatment of the sample, and allows the compound to be separated even from a crude extract. However, a great deal of experience and trial and error are necessary for setting up the condition,

particularly the two-phase solvent system that allows the compounds to be separated.

EXPERIMENTAL

Reagents and Materials

All solvents used for preparation of the crude samples, CPC separation, silica gel short columns, and recrystallization were of the first grade (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Acetonitrile used for HPLC was of HPLC grade (Wako Pure Chemical Industries, Ltd.) and deionized water used was prepared by Milli-Q (Millipore, USA). Silica gel short column chromatography was performed on Silica gel 60 (Merck KGaA, Germany). TLC was conducted on precoated silica gel 60 F_{254} (Merck) and RP-18 F_{254s} (Merck), and the spots were detected by heating after spraying with vanillin-phosphoric acid reagent.

Dried *Salvia divinorum* leaves were purchased in June 2005 from Ethnogens.com (KS, USA). Voucher specimens were deposited at the Medicinal Herbarium, Faculty of Pharmaceutical Sciences at Kagawa campus, Tokushima Bunri University (specimen # 050601-001).

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Apparatus

The CPC instrument employed in this study was Model LLB-M (Sanki Engineering Inc., Kyoto, Japan) equipped with a RB-012 rotor that had a total of 2136 partition cells (total cell volume was 240 mL) and with a 10 mL sample loop. An SSC-3220 pump (Senshu Scientific Co., Ltd., Tokyo, Japan) was used to pump the two-phase solvent system.

The HPLC equipment consisted of a LaChrom Elite HPLC system (Hitachi High-Technologies Corp., Tokyo Japan) including two L-2130 pumps, an L-2450 PDA detector, an L-2200 auto sampler, an L-2300 column oven, and an EZChrom Elite workstation and a Prominence HPLC system (Shimadzu Corp., Kyoto, Japan) consisting of an LC-20AB pump system, an SPD-M20A PDA detector, an SIL-20AC auto sampler, a CTO-20AC column oven, and an LCsolution M-PDA workstation.

The melting point was determined on a MP-J3 micro melting point apparatus (Yanaco LID Co., Ltd., Kyoto, Japan) and was uncorrected. Optical rotation was measured with a P-1030 polarimeter (JASCO Corp., Tokyo Japan). UV, CD, and IR spectra were obtained with a Jasco V-560 UV/VIS spectrophotometer, a Jasco J-820 spectropolarimeter, and a Jasco FT/IR-6300 spectrometer with ATR option, respectively. 1D and 2D ^{1}H- and ^{13}C-NMR spectra were recorded on a Unity Inova 500 spectrometer (Varian Inc., USA) at 300 K using Varian standard pulse sequences.

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ESI-TOFMS and HR-ESI-TOFMS spectra were obtained on a Micromass Q-TOF micro mass spectrometer (Waters Corp., USA).

Selection of the Two-Phase Solvent System

The two-phase solvent system for separation of salvinorin A from crude extract by CPC was selected on the basis of the partition coefficient (P). The P value was determined by HPLC as follows: 5 mL each of the upper phase and the lower phase of the two-phase solvent system were taken into a partition funnel, 2 mg of salvinorin A was added, and the contents of the funnel were mixed well. The two phases were separated and concentrated. Each concentrate was dissolved in 5 mL of acetonitrile and analyzed using HPLC. Partition coefficient P was obtained by the following equation: P = [quantity dissolved in the mobile phase (aqueous phase)]/[quantity dissolved in the stationary phase (organic phase)].

HPLC Analysis

HPLC analysis for determining the partition coefficient (P) and the purity of isolated salvinorin A was carried out on an Inertsil Prep ODS column (6.0 mm I.D. $\times$ 250 mm; 5 μm particle size) from GL Science (Tokyo, Japan). An isocratic system of 45% acetonitrile in water as the mobile phase and a flow rate of 1 mL/min with 10 μL injection volume at 35°C was used for separation. Run time was 30 min at a detection wavelength of 210 nm. The column was washed with 100% acetonitrile for 5 min and then re-equilibrated for 10 min. Under this condition, the retention time of salvinorin A was 25.2 min.

Preparation of Sample Solution

Dried *S. divinorum* leaves (ca. 50 g) were mixed with methanol (500 mL) three times using a blender. The methanol solution was filtered and its volume reduced to ca. 100 mL *in vacuo* less than 40°C. The concentrated methanol extract was centrifuged, and the supernatant evaporated less than 40°C to yield a methanol extract (ca. 3.5–4 g). This methanol extract was dissolved in the stationary phase (ca. 7–10 mL), and ca. 5 mL of the sample solution (ca. 2–3 g of the methanol extract) was injected to the CPC in each run.

CPC Separation Procedure

The upper phase (stationary phase: organic phase) of the two-phase solvent system was pumped into partition cells using a descending mode at a flow

rate of 15 mL/min and a rotor speed of 300 rpm. When all the cells were totally filled with the stationary phase, the lower phase (mobile phase: aqueous phase) was pumped at a flow rate of 2 mL/min and a rotor speed of 1600 rpm. After the mobile phase began to emerge from the outlet of the CPC system, ca. 5 mL of the sample solution (ca. 2–3 g of the methanol extract) was injected into the CPC system. The effluent from the outlet was collected (10 mL/fraction but 20 mL for the first fraction). Each fraction was analyzed by silica gel TLC (*n*-hexane–ethyl acetate = 1:1, v/v) to identify those that contained salvinorin A. After salvinorin A was eluted out, the separation mode was changed to an ascending mode for inversed elution and the cell washed with methanol.

Purification Step

The CPC derived fractions that contained salvinorin A were pooled and concentrated *in vacuo* under 40°C. The resulting concentrate was dissolved in a small amount of dichloromethane and then passed through a silica gel short column (20 mm I.D. × ca. 50–70 mm), which was eluted with *n*-hexane–ethyl acetate (1:1, v/v), in order to remove a dark spot appearing at the starting point on the TLC. The effluent was evaporated under 40°C, dissolved again in a small amount of dichloromethane, and recrystallized with methanol to afford salvinorin A as tiny, colorless, needle like crystals (ca. 100 mg). The yield of salvinorin A in each run was ca. 0.25–0.3% (w/w) from the dried leaves.

Structure Identification

The chemical structure of salvinorin A was identified according to its ^{1}H- and ^{13}C-NMR, MS spectral, and other physical data compared with their published data.[1,2]

Salvinorin A: Tiny colorless needle like crystals, m.p. 236–238; $[\alpha]_D^{23}$ -42.6 (*c* 0.355, $CHCl_3$); UV λ_{max} (MeOH) nm (log ε): 206 (3.67); CD λ_{max} (MeOH) nm ($\Delta\varepsilon$): 292 (−1.6), 220 (−4.7); IR (ATR) cm^{-1}: 2950, 1725, 1439, 1379, 1227, 1157, 1052, 875, 600; ^{1}H-NMR ($CDCl_3$, 500 MHz): 7.41 (1H, dd, $J = 0.9$, 1.6; H-16), 7.39 (1H, t-like, $J = 1.7$; H-15), 6.38 (1H, dd, $J = 0.9$, 1.8; H-14), 5.53 (1H, dd, $J = 5.1$, 11.7; H-12), 5.15 (1H, t-like, $J = 10.2$; H-2), 3.73 (3H, s; $COOCH_3$), 2.75 (1H, m; H-4), 2.51 (1H, dd, $J = 5.1$, 13.4; H-11α), 2.31 (2H, m; H-3), 2.18 (1H, m; H-10), 2.17 (3H, s; $OCOCH_3$), 2.16 (1H, m; H-7β), 2.08 (1H, dd, $J = 2.8$, 11.8; H-8), 1.80 (1H, dt-like, $J = 3.1$, 13.2; H-6α), 1.66 (1H, m; H-7α), 1.61 (1H, m; H-11β), 1.58 (1H, m; H-6β), 1.45 (3H, s; H-20), 1.12 (3H, s; H-19); ^{13}C NMR ($CDCl_3$, 125 MHz): 202.0 (C-1), 171.6 (C-18), 171.1 (C-17), 170.0 ($O\underline{C}OCH_3$), 143.7 (C-15), 139.4 (C-16), 125.2 (C-13), 108.4 (C-14), 75.0

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(C-2), 72.1 (C-12), 64.1 (C-10), 53.6 (C-4), 52.0 (COO$\underline{C}$H$_3$), 51.4 (C-8), 43.4 (C-11), 42.1 (C-5), 38.2 (C-6), 35.5 (C-9), 30.8 (C-3), 20.6 (O$\underline{C}$OCH$_3$), 18.1 (C-7), 16.4 (C-19), 15.2 (C-20); ESI-TOFMS m/z: 886.9 (7, $[2M + Na]^+$), 455.2 (100, $[M + Na]^+$), 433.3 (4, $[M + H]^+$). HRESI-TOFMS m/z: 455.1672 (Calcd for $C_{23}H_{28}O_8Na$, 455.1682).

RESULTS AND DISCUSSION

A series of experiments were carried out to determine the optimal two-phase solvent system for the CPC separation. First, to dissolve as much of the crude sample as possible, dichloromethane was chosen as one of the solvents in the two-phase solvent system. Both chlorophylls, which are found in large amounts in the crude extract, and salvinorin A readily dissolved in dichloromethane. Methanol, which can solve the crude extract as well as dichloromethane, was chosen as the mutual solvent, while water, which cannot mix with dichloromethane, served for the second phase to make up a two-phase solvent system. Partition coefficient (P) is the ratio of solute distributed between the mutually equilibrated two solvent phases, and is the most important factor in the separation of target compounds. Several solvents were tested to find out the best modifier solvent for salvinorin A to obtain an optimum partition coefficient and, finally, *n*-hexane was chosen. Various two-solvent systems comprised of dichloromethane–methanol–water–*n*-hexane were examined for determining the P value (Table 1). Values of R_f for salvinorin A on silica gel TLC for the organic phase and on ODS TLC for the aqueous phase were checked. However, these efforts provided little useful information for optimizing the condition. Some of the two-phase solvent systems that had a P value of about 1.0 were employed for CPC separation of salvinorin A from the crude methanol extract of *Salvia divinorum* leaves. Because the crude methanol extract of *S. divinorum* leaves contains chlorophylls in large amounts, an organic phase was chosen for the stationary phase to retain them in the partition cells, whereas an aqueous phase was chosen for the target compound to elute it in the mobile phase. Although, the speed of the CPC rotor consisting of partition cells is an important factor in retention of the stationary phase by centrifugal forces, the pressure limit of the cell assembly and tubing is below $50\,kg/cm^3$, which means that the rotation speed has to be different for each solvent system. Five mL of sample solution (ca. 2–3 g of the crude methanol extract) was injected for each run of CPC separation, and the flow rate of the mobile phase was 2.0 mL/min. These explorations finally led to a two-phase solvent system of *n*-hexane–dichloromethane–methanol–water (8:8:9:2, v/v). The organic phase, which consisted of dichloromethane, was the upper phase in this two-phase solvent system; therefore, the descending mode

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Table 1. The P and R_f values of salvinorin A in several solvent systems

n-Hexane–dichloromethane–methanol–water (v/v)[a]	R_f		
	Si[b]	ODS[c]	P
8:8:9:2	0.52	0.67	1.051
8:6:6:1	0.44	0.87	1.942
7:6:6:1	0.33	0.84	1.678
6:6:5:1	0.39	0.70	1.456
6:5:5:1	0.27	0.83	0.695
5:6:6:1.5	0.36	0.66	0.958
5:6:5:1	0.53	0.77	1.063
5:4:4:1	0.22	0.79	1.100
4:5:5:2	0.42	0.39	0.186
4:5:5:1.5	0.47	0.54	0.945
4:5:5:1	0.49	0.81	0.716
4:4:4:1	0.33	0.70	0.660
3:6:6:2	0.51	0.44	0.178
3:6:5:2	0.64	0.33	0.079

[a]The upper phases were the organic phase and the lower phases were the aqueous phase, except for the 3:6:5:2 solvent system.

[b]Values obtained from silica gel TLC with organic phase as the developing solvent.

[c]Values obtained from ODS TLC with aqueous phase as the developing solvent.

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of separation was chosen. The rotor speed was set to 1600 rpm to keep the pressure of the solvent pumping unit around 25 kg/cm^3. Figure 2 shows a typical TLC pattern of the separation of salvinorin A by CPC with this two-phase solvent system. In Figure 2, salvinorin A appears between fraction numbers 16 and 19 as an almost sole spot. By switching to the ascending mode, inverse elution was started from fraction number 41, so that the chlorophylls retained in large amounts were eluted out. Fractions that contained salvinorin A were then pooled and concentrated *in vacuo*, a dark color substance (probably comprising some polymers) was removed by the silica gel short column (*n*-hexane–ethyl acetate = 1:1, v/v), and finally recrystallized from methanol to yield pure salvinorin A as colorless, tiny, needle like crystals (ca. 100 mg or about 0.25–0.3% w/w of dried leaves). The retention volume (eluted fraction numbers) and the yield of salvinorin A were consistent in each run, which indicated that the process was reliably reproducible. The structure of isolated salvinorin A was confirmed by means of 1D and 2D NMR and ESI-MS spectral analyses; the purity was checked by HPLC, and found to be more than 98%. A typical experimental procedure established in this study is shown in Figure 3.

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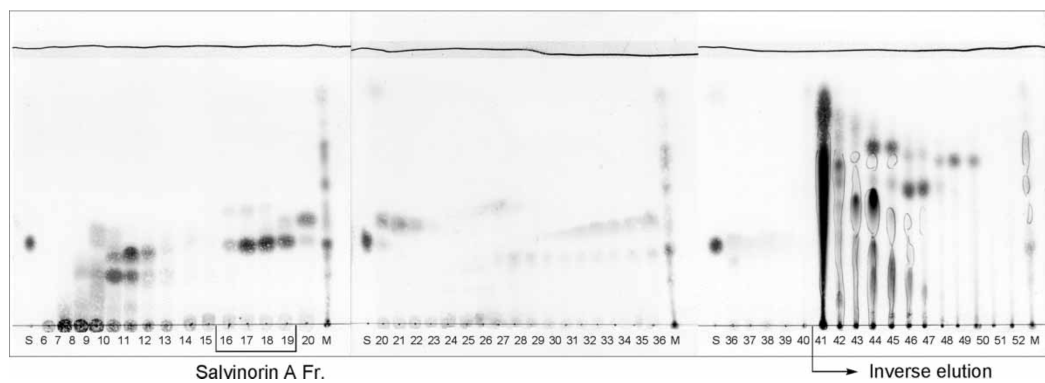

Figure 2. TLC monitoring of fractions from CPC separation of the methanol extract of *Salvia divinorum* leaves. Spots were detected by heating after spraying with vanillin-phosphoric acid reagent. S: salvinorin A; M: methanol extract of *Salvia divinorum* leaves.

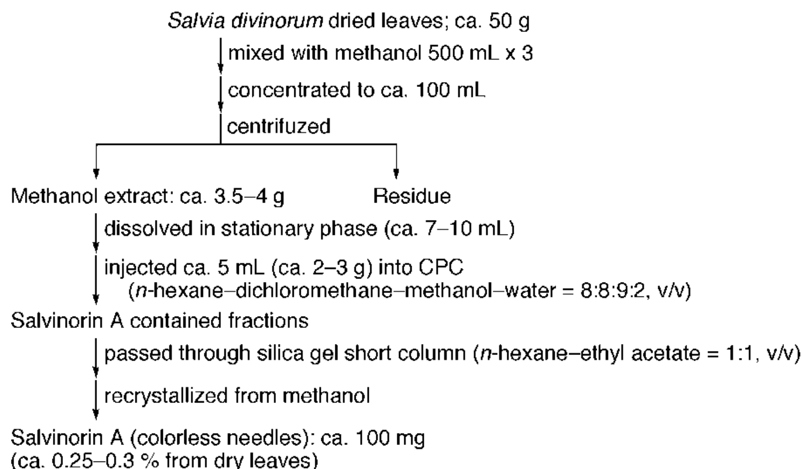

Figure 3. Typical experimental procedure for isolation of salvinorin A from the hallucinogenic sage, *Salvia divinorum*.

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CONCLUSION

CPC was successfully applied to isolate salvinorin A from methanol extracts of the hallucinogenic sage, *Salvia divinorum*. Salvinorin A, ca. 100 mg, was obtained from ca. 2–3 g of the crude methanol extract by CPC, using *n*-hexane–dichloromethane–methanol–water (8:8:9:2, v/v) as the two-phase solvent system, followed by the silica gel short column and recrystallization. The yield, 0.25–0.3% w/w of dried leaves, was consistent enough to assume that the procedure is reliable. Thus, a simple procedure has been developed to isolate salvinorin A from the hallucinogenic sage, *Salvia divinorum*.

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