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Preparative Isolation of 8-Methoxypsoralen from the Rhizomes of *Pulsatilla chinensis* using High-speed Counter-current Chromatography

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A simple and rapid purification method of 8-methoxy-psoralen from the crude methanol extract of the rhizomes of *Pulsatilla chinenis* was established using high-speed counter-current chromatography (HSCCC). From the crude extract (233.2 mg), 8-methoxypsoralen (10.0 mg) was separated for the first time in one-step, using a two-phase solvent system composed of *n*-hexane/ethyl acetate/methanol/water (5:5:5:5, v/v/v/v). The isolated amount of this compound was found to be equivalent to 89% of the amount observed quantitatively in the crude methanolic extract. The final purity of the 8-methoxy-psoralen was determined to be over 98% by ultra-performance liquid chromatography-UV analysis.

Key words: high-speed counter-current chromatography, 8-methoxypsoralen, *Pulsatilla chinensis*

The rhizomes of *Pulsatilla chinensis* (Bunge) Regel have been used in traditional oriental medicine for the treatment of amoebic diseases, vaginal trichromoniasis, and bacterial infections [Bae, 2000]. Phytochemical investigations of this plant have resulted in the identification of coumarins, lignans, monoterpenes, and triterpenes [Ye *et al*., 1998; Mimaki *et al*., 1999; Mimaki *et al*., 2001; Ye *et al*., 2001].

A naturally occurring linear furocoumarin, 8-methoxy-psoralen (8-MOP), was observed in the preliminary study as one of the major secondary metabolites in the UPLC chromatogram and had not been isolated from this plant so far. 8-MOP is photodynamic drug widely used in photochemotherapy for management of skin diseases such as eczema, mycosis, pompholyx, psoriasis, and vitiligo [Knobler *et al*., 1988; Wollina, 2010]. Clinically, 8-MOP has been introduced for the treatment of cutaneous T-cell lymphoma [Edelson, 1988; Zic, 2003].

High-speed countercurrent chromatography (HSCCC) is one of countercurrent chromatographic methods [Ito and Bowman, 1970; Conway and Ito, 1984; Pauli *et al*., 2008] that rely on distribution of the analytes between two immiscible liquid phases. Currently, HSCCC has been widely used for the separation and purification of various natural and synthetic products [Sutherland and Fisher, 2009]. The purpose of the present study was to isolate 8-MOP and develop simple isolation and purification methods for 8-MOP from the rhizomes of *P. chinensis* using HSCCC.

Preparative HSCCC was carried out with a model TBE-1000A high-speed counter-current chromatography system (Shanghai Tauto Biotech Co. Ltd, Shanghai, China) with three serially connected multilayer coil separation columns (i.d. of tubing=1.6 mm; total volume =1000 mL) and an 80-mL sample loop. The system was equipped with LPLC pumps TBP5002 (Shanghai Tauto Biotech Co. Ltd) and Thermo Finnigan SSI 500 UV Detector (Thermo Electron Co. San Jose, CA), Autochro-WIN software (version 2.0, Younglin-Tech, Seoul, Korea). Ultra-performance liquid chromatography (UPLC) was carried out using a Waters ACQUITY UPLC® System with a PDA Detector. NMR spectral data were obtained on a Varian UNITY 300 NMR system (Varian, Palo Alto, CA). The High Resolution Electrospray Ionization Mass Spectrometer (HRESIMS) were measured on a Waters Q-Tof Premier mass spectrometer.

All solvents (methanol, ethyl acetate, chloroform, *n*-hexane) used for HSCCC experiments were obtained from SK chemicals (Seoul, Korea) and distilled prior to use. All UPLC solvents were purchased from SK

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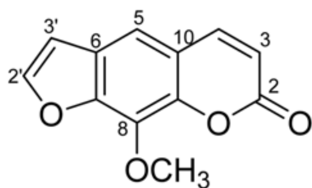

Fig. 1. Structure of 8-methoxypsoralen.

chemicals. Deuterated chloroform ($CDCl_3$) for NMR was purchased from Cambridge Isotope Laboratories (Andover, MA). Deionized water was used in all solutions and for all dilutions.

The roots of *P. chinensis* (Bunge) Regel were purchased from the Plant Extract Bank, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon, Korea. A voucher specimen (FBM028-019) was deposited at KRIBB. The dried rhizomes of *P. chinensis* (16.0 g) were extracted three times by sonication in 200 mL methanol for 30 min at room temperature. The extracts were then combined and evaporated to dryness under reduced pressure. The crude extract (2.2 g) was used for preparative HSCCC separation.

The composition of the two-phase solvent was selected according to the partition coefficient (K) of the target compounds in the crude extract. The partition coefficient for the solvent system was determined by UPLC. The crude extract (1.0 mg) was added into 4.0 mL of each two-phase solvent system. The vial was then shaken vigorously until the two phases were thoroughly equilibrated. After setting, 400 µL of each phase was transferred to new vials and condense under vacuum. The residue was diluted with 200 µL of methanol and analyzed by UPLC. The K values were expressed as the value of the peak area of the target compound in the upper phase, divided by that of the compound in the lower phase. The two-phase solvent system was composed of *n*-hexane/ethyl acetate/methanol/water (HEMW) at various volume ratios. The solvent mixture was equilibrated in a separatory funnel at room temperature, and the two phases were separated shortly before use.

The upper (stationary) and lower phase (mobile) were simultaneously pumped into the multilayer-coiled column at a 3:1 volumetric ratio [Slacanin *et al*., 1989]. When the column was completely filled with the two phases, the apparatus was rotated at 500 rpm; simultaneously, the mobile phase was pumped through the column at 5.0 mL/min. The temperature was set to 25°C. The sample solution (20 mL) containing the crude MeOH extract (233.2 mg) was loaded onto the HSCCC, and the effluent was monitored at 254 nm. Fractions were collected each minute and analyzed by UPLC-UV.

The crude extract of *P. chinenis* and HSCCC peak

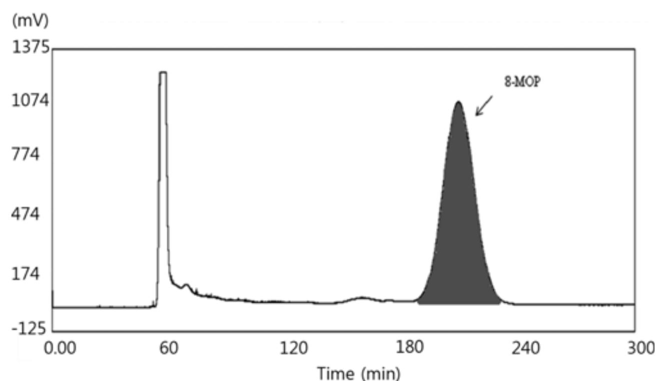

Fig. 2. HSCCC chromatograms of the crude extract from root of *Pulsatilla chinensis*. Solvent system: HEMW (5:5:5:5, v/v/v/v); column volume: 1000 mL; mobile phase: lower phase; flow rate: 5 mL/min; rotation speed: 500 rpm; separation temperature: 25°C; detection wavelength: 254 nm; sample size: 233.2 mg of crude sample dissolved in 14 mL of stationary phase and 6 mL mobile phase; retention percentage of the stationary phase: 69.8%

fractions were analyzed by UPLC. The UPLC conditions were as follows: the column used in this study was an AcQUITY UPLC®BEH C18 (1.7 µm 2.1×50 mm, Waters, Menchester, UK). The mobile phase composed of acetonitrile/water/formic acid with a linear gradient (20:80:0.1-100:0:0.1, v/v/v) was eluted at a flow-rate of 0.4 mL/min. The UV detector was set at MAX Plot, 210, 366 and 254 nm. The column temperature was set at 35°C (Fig. 3).

8-Methoxypsoralen: white needles; HR-ESI-MS *m/z* 217.0584 $[M+H]^+$ (calcd. for $C_{12}H_9O_4$, 217.0501); ^1H-NMR (400 MHz, $CDCl_3$) δ 7.74 (1H, d, J=9.6 Hz, H-4), 7.67 (1H, d, J=2.2 Hz, H-2'), 7.33 (1H, s, H-5), 6.80 (1H, d, J=2.2 Hz, H-3'), 6.35 (1H, d, J=9.6 Hz, H-3), 4.28 (1H, s, 8-OCH_3); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 160.68 (C-2), 147.9 (C-7), 146.9 (C-9), 144.5 (C-2'), 143.2 (C-4), 133.0 (C-8), 126.3 (C-6), 116.7 (C-10), 115.0 (C-3), 113.1 (C-5), 106.9 (C-3'), 61.6 (8-OCH_3).

The standard solutions (200, 120, 100, 80, 60, 40, 20, 10, 5, 2, 1.5, and 1 µg/mL) for the calibration curve were filtered through a 0.22-µm membrane prior to injection. Each standard solution was injected six times consecutively. The calibration curve was achieved by plotting the ration of peak area versus concentration (µg/mL). The limit of detection (LOD) was calculated as signal-to-noise (S/N) ratio of 3, whereas the limit of quantification (LOQ) was defined as S/N ratio of 10 for the column.

The precision of this UPLC method was obtained by intra- and inter-day variations. The intraday precision was evaluated by analyzing the results of six consecutive injections of sequential standard solutions of 8-MOP (1-120 µg/mL) as described above, whereas inter-day

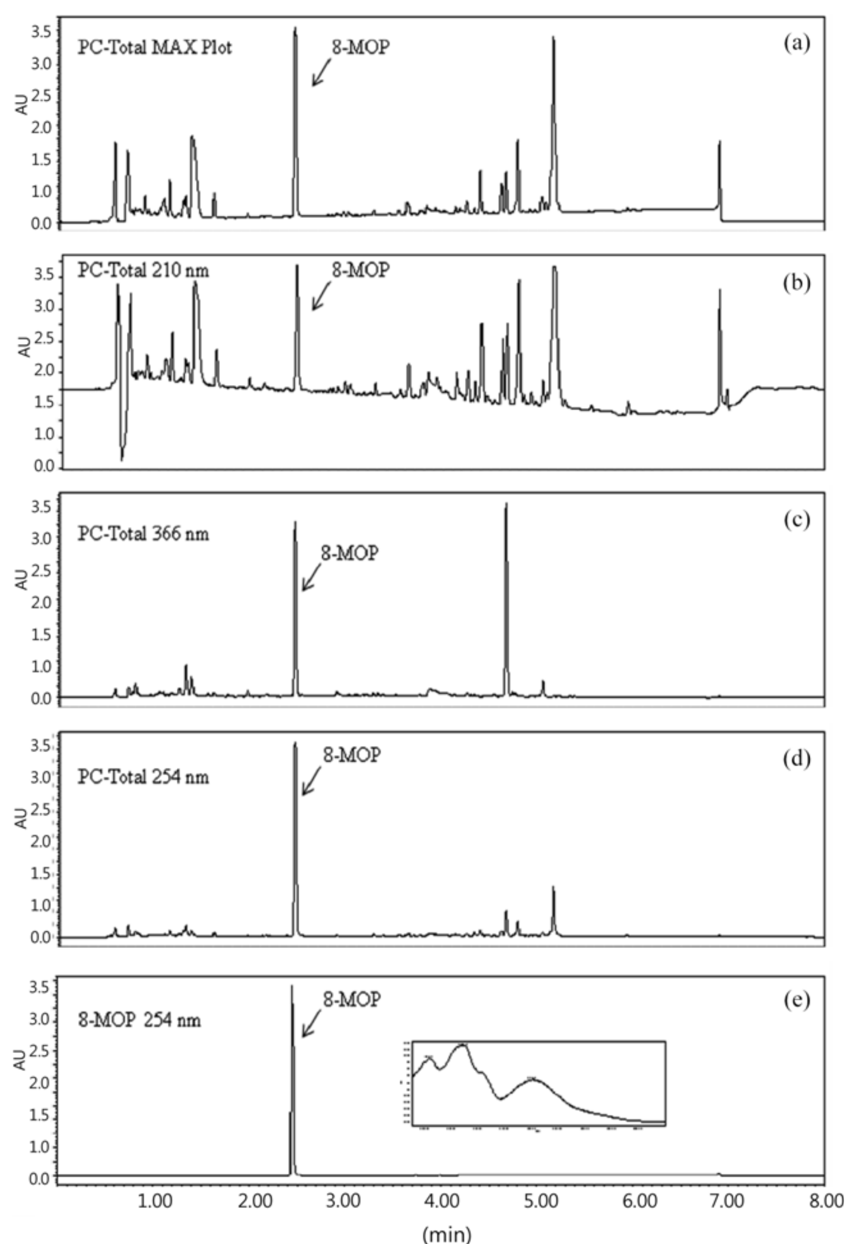

Fig. 3. UPLC chromatograms of the crude extract from the root of *P. chinensis* [UV wavelength MAX Plot (a), 210 nm (b), 366 nm (c), and 254 nm (d)], and 8-methoxypsoralen (8-MOP) [UV wavelength 254 nm (e)] separated from HSCCC. Column: AcQUITY UPLC®BEH C18 1.7 μm 2.1×50 mm; mobile phase: acetonitrile-water-formic acid (20:80:0.1-100:0:0.1, gradient); flow rate: 0.4 mL/min; sample volume: 2 μL; column temperature: 35°C.

variation was performed by six consecutive injections of sequential standard solutions of 8-MOP at three different days. The precision expressed by the calculation of relative standard deviations [RSD=(SD/mean)×100%]. The accuracy of the UPLC method was achieved by the performing recovery test. Six concentration levels (1, 1.5, 2, 5, and 10 μg/mL) of 8-MOP were spiked directly to the methanol extract of sample solutions (540 and 720 μg/mL) and analyzed as described above. The recoveries were calculated as: Recovery (%)=$(C_1-C_0)\times100/C_a$, where C_0 and C_1 are the measurements before and after addition of 8-MOP standard, respectively, and C_a is the amount of added 8-MOP.

UPLC analysis of the methanol extract [UV wavelength: MAX Plot (**a**), 210 nm (**b**), 366 nm (**c**) and 254 nm (**d**)] revealed that 8-MOP (**e**) was one of the major secondary metabolites (Fig. 3). However, there has been no report on 8-methoxypsoralen isolated from *P. chinensis* in the previous studies. In order to isolate and develop a simple and rapid isolation and purification method using HSCCC, key conditions were closely studied. The *K* values of 8-MOP were tested in several

Table 1. Partition coefficient (*K*) values of 8-MOP in different two-phase solvent systems

Two-phase solvent system	Volume ratio (v/v)	*K* value
chloroform-ethyl acetate-methanol-water	3:1:3:2	0.05
chloroform-ethyl acetate-methanol-water	2.4:1.6:3:2	0.25
ethyl acetate-butanol-water	4:2:3	20.74
ethyl acetate-butanol-water	2:1:3	35.40
n-hexane-ethyl acetate-methanol-water	7:3:5:5	0.38
n-hexane-ethyl acetate-methanol-water	5:6:6:6	0.56
n-hexane-ethyl acetate-methanol-water	6:5:4.5:5.5	0.88
n-hexane-ethyl acetate-methanol-water	5:5:5:5	1.00

Table 2. Results of intra- and interday variations (n=6)

Retention time (min)	Amount taken (μg/mL)	Amount found (μg/mL)	RSD (%)	Accuracy[a]
2.44	60	61.3	1.6	102.1
2.44	80	81.4	1.8	101.8
2.44	100	95.1	1.6	95.1
2.44	120	118.3	0.9	98.6

[a](Amount found/Amount taken)×100%

Table 3. Recovery by the standard added to the extract

Retention time (min)	Amount of extract (μg/mL)	Amount of 8-MOP added (μg/mL)	Recovery (%)[a]	RSD (%)
2.44	540	1	102.8	1.3
2.44	540	1.5	98.7	0.8
2.44	540	2	97.0	1.8
2.44	720	2	100.5	0.8
2.44	720	5	102.0	2.6
2.44	720	10	100.3	6.7

[a]n=3

two-phase solvent systems (Table 1), among which HEMW 5:5:5:5 (v/v/v/v) was found to be suitable. (K= 1.01, $0.5 < K < 2$) The rhizomes of *P. chinensis* (233.2 mg) were injected, and 8-MOP (t_R 187-232 min, 10.0 mg) was successfully isolated (Fig. 2). Identification of peak fraction was performed by interpretation and comparison of previously published ^{1}H-NMR spectroscopic data [Abu-Mustafa *et al.*, 1967]. The purity of the 8-MOP was determined to be over 98% by UPLC-UV (Fig. 3**e**).

To analyze the amount of 8-MOP present in the crude extract, quantitative method was developed and validated. A linear calibration curve was obtained Y=27895X+ 11349 in the range of 1-120 μg/mL, where Y is the peak area, and X (μg/mL) is the amount of 8-MOP, and correlation coefficient was 0.9990. The limit of detection (LOD) and limit of quantitaion (LOQ) in this method were 0.004 and 0.01 μg/mL, respectively.

The precision of this analytical method was evaluated as the standard deviation (SD) of replicated measurements of the sample of brand. Both intra- and inter-day repeatability of the analytical method were assessed on two parameters: retention time and peak area repeatability. For the intra-day variability test, the brandy sample was analyzed with three replicates (Table 2).The accuracy of the methods is given in Table 3. The recoveries of 8-MOP were in the range of 96.99-102.82% [RSD 0.81-6.74%]. Using the established method, the MeOH extract was found to contained 4.79% (w/w) of 8-MOP.

In the present study, a simple and rapid HSCCC separation method was established for the preparative isolation and purification of 8-MOP from the methanol extract of *P. chinensis*. 8-MOP (10.0 mg), with purity over 98%, was successfully purified from the extract (233.2 mg) using HSCCC run with a solvent system composed of HEMW (5:5:5:5, v/v/v/v). This amount is equivalent to 89.5% of theoretical content of 8-MOP calculated from the quantitative analysis for the MeOH extract. The present study is the first report on the isolation of 8-MOP from *P. chinensis*, as a good source of 8-MOP, and demonstrates that HSCCC application could be applied to the preparative purification of 8-MOP from *P. chinensis*.

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References

Abu-Mustafa EA and Fayez MBE (1967) Natural coumarins. VI. Nuclear magnetic resonance spectra of some coumarin and coumarilic acid derivatives. *Can J Chem* **45**, 325-327.

Bae KH (2000) The Medicinal Plants of Korea, p.139, Kyo-Hak, Seoul, Korea.

Conway WD and Ito Y (1984) Development of countercurrent chromatography. *Anal Chem* **56**, 534-554.

Edelson RL (1988) Light-activated drugs. *Sci Am* **259**, 68-75.

Ito J and Bowman RL (1970) Countercurrent chromatography:

Liquid-liquid chromatography without solid support. *Science* **167**, 281-283.

Knobler RM, Honigsmann H, and Edelson RL (1988) Psoralen Phototherapies, In *Psoralen DNA Photobiology*, **II**, pp. 117-134, CRC Press, Boca Roton, FL, USA.

Mimaki Y, Kuroda M, Asano T, and Sashida Y (1999) Triterpene saponins and lignans from the roots of *Pulsatilla chinensis* and their cytotoxic activity against HL-60 cells. *J Nat Prod* **62**, 1279-1283.

Mimaki Y, Yokosuka A, Kuroda M, Hamanaka M, Sakuma C, and Sashida Y (2001) New bisdesnosidic triterpene saponins from the roots of *Pulsatilla chinensis*. *J Nat Prod* **64**, 1226-1229.

Pauli GF, Pro SM, and Friesen JB (2008) Countercurrent separation of natural products. *J Nat Prod* **71**, 1489-1508.

Slacanin I, Marston A, and Hostettmann K (1989) Modifications to a high-speed counter-current chromatography for improved separation capability. *J Chromatogr A* **482**, 234-239.

Sutherland IA and Fisher D (2009) Role of counter-current chromatography in the modernization of Chinese herbal medicines. *J Chromatogr A* **1216**, 740-753.

Wollina U (2010) Pompholyx: A review of clinical features, differential diagnosis, and management. *Am J Clin Dermatol* **11**, 305-314.

Ye WC, He A, Zhao SX, and Che CT (1998) Pulsatilloside C from the roots of *Pulsatilla chinensis. J Nat Prod* **61**, 658-659.

Ye WC, Zhang, QW, Wendy WL, Hsiao, Hsiao Wendy WL, Zhao SX, and Che CT (2001) New lupane glycosides from *Pulsatilla chinensis. Planta Med* **68**, 183-186.

Zic JA (2003) The treatment of cutaneous T-cell lymphoma with photopheresis. *Dermatol Ther* **16**, 337-346.