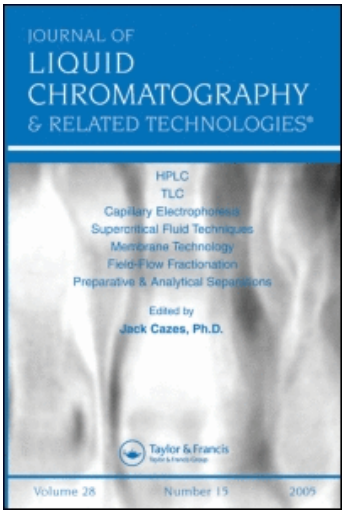

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Preparative Separation of Tripdiolide from Chinese Traditional Herb by Multidimensional CCC

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Abstract: Multidimensional countercurrent chromatography (CCC) was used for isolation and purification of tripdiolide from *Tripterygium wilfordii* Hook F. with a pair of two-phase solvent systems composed of n-hexane-dichloromethane-methanol-water (3:22:17:8, v/v/v/v) and chloroform-methanol-water (4:3:2, v/v/v), both of which were selected by analytical high-speed CCC. About 1 g of crude extract was separated using two preparative units of the HSCCC centrifuge, yielding 7.8 mg (0.78% w/w) of tripdiolide with a purity over 98%.

Keywords: Multidimensional countercurrent chromatography, Isolation and purification, Tripdiolide, *Tripterygium wilfordii* Hook F

INTRODUCTION

Tripterygium wilfordii Hook F. (*lei gong teng*) is a traditional Chinese medicinal herb, which has also long been used as an insecticide in China. Traditionally, lei gong teng dispels wind and dampness, and is usually used to treat the illness called *bi zheng* (a painful obstruction syndrome). It also

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relieves pain and reduces swelling in patients who have swollen joints, polyarthritis, and difficulty moving. It can be used alone or with other anti-rheumatic herbs. Recently, this plant has been used in some Chinese clinics to treat cancer, rheumatic arthritis, and various skin diseases. It is of interest that *Tripterygium* showed significant antifertility activities. The active principles in *Tripterygium* are found to be diterpenoid containing triepoxides, which exhibit anti-inflammatory, immunosuppressive, and antifertile actions.[1]

Crude extracts derived from the roots of *Tripterygium wilfordii* Hook F. were found to have immunosuppressive properties and used as anti-rheumatic therapy in Chinese traditional medicine. Although these extracts contain a variety of components, the precise nature of the compound(s) responsible for this therapeutic effect has not been established with certainty. An aqueous extract of *Tripterygium wilfordii* Hook F. was purified to yield several compounds. They were identified with a mixed lymphocyte reaction (MLR) and chemically characterized by NMR spectroscopy and mass spectrometry. Two major components of immunosuppressive activity were identified. These were the closely related diterpenoid triepoxides, triptolide and tripdiolide. No other immunosuppressive compounds were identified using MLR as the biologic screening assay. Therefore, triptolide and tripdiolide may be responsible for the anti-rheumatic properties of crude aqueous extracts of *Tripterygium wilfordii* Hook and represent a novel class of immunosuppressive drugs with potential clinical utility.[2]

Tripterygium wilfordii Hook is also known as *qi bu si*, literally, "seven steps to death," implying that it is extremely toxic. Toxic signs include local irritation of the gastrointestinal tract, damage to the central nervous system, internal bleeding, and necrosis of the organs.[3] Other symptoms include dizziness, dry mouth, palpitations, necrosis of mucous membranes, and irregular menstruation. However, these side-effects are minimal when this herb is prescribed following the proper dosage and preparation. According to some research reports, terpenoids have a lower toxicity than the crude extract of *Tripterygium wilfordi* Hook F. So, it is a worthy goal to be able to extract and purify the terpenoids contained in the crude extract. High purity terpenoids will allow for an accurate quantitation of terpenoid doses used in treating patients, greatly reducing all side-effects.

The separation of active compounds from natural sources may encounter various problems. For example, the compound of interest is often present only as a minor component in an extremely complex mixture. Tripdiolide is scarce among diterpenoid triepoxides in crude extract from *Tripterygium wilfordii* Hook F. High-speed countercurrent chromatography (HSCCC) being a support-free liquid-liquid partition chromatographic technique, eliminates irreversible adsorption of the sample onto the solid support,[4,5] and has been widely used in preparative separation of natural products.[6]

The present paper describes the successful preparative separation and purification of tripdiolide from *Tripterygium wilfordii* Hook F. by multidimensional countercurrent chromatography (MDCCC).

EXPERIMENTAL

Apparatus

The preparative HSCCC instrument employed in the present study is a Model GS 10 countercurrent chromatograph designed and constructed in the Beijing Institute of New Technology Application (Beijing, China). The apparatus holds a pair of column holders symmetrically placed on the rotary frame at a distance of 5 cm from the central axis of the centrifuge. The multilayer coil separation column was prepared by winding a 110 m long, 1.6 mm I.D. PTFE (polytetrafluoroethylene) tube directly onto the holder hub, forming multiple coiled layers with a total capacity of 230 mL. The β value varied from 0.5 at the internal terminal to 0.8 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft, and R, the revolution radius or the distance between the holder axis and central axis of the centrifuge). Although the revolution speed of the apparatus could be regulated with a speed controller in the range between 0 to 1000 rpm, an optimum speed of 800 rpm was always used in the present studies. A manual sample injection valve with a 20 mL loop was used.

Multi dimensional CCC (MDCCC) was done associating two units of these centrifuges (as set up by Dr. Fuquan Yang[7,8] in our Beijing laboratory), and shown by Figure 1. Two Model NS-1007 constant-flow pumps (Beijing Institute of New Technology Application, Beijing, China) were used to elute the mobile phase, while continuous monitoring of the effluent was achieved with two Model 8823A-UV detectors (Beijing Institute of New Technology Application) at 214 nm. Two manual six-port valves, one with a 20 mL loop used as the injection valve and the other without loop used as the switching valve (Tianjin High-New Science & Technology Company, Tianjin, China), were used to introduce the sample into the column. Two portable recorders (Yokogawa Model 3057, Sichuan Instrument Factory, Chongqing, China) were used to draw the chromatogram.

A rotary evaporator (Model RE-90, Beijing Institute of New Technology Application) was also used.

The high performance liquid chromatography (HPLC) equipment used was a Shimadzu LC-10AVP system including two LC-10ATVP solvent delivery units, a SPD-M10AVP UV-VIS photodiode array detector, a Model 7726 injection valve with a 20 μL loop, a SCL-10AVP system controller, a CTO-10ASVP column oven, a DGU-12A degasser, and a Class-VP-LC workstation (Shimadzu, Kyoto, Japan).

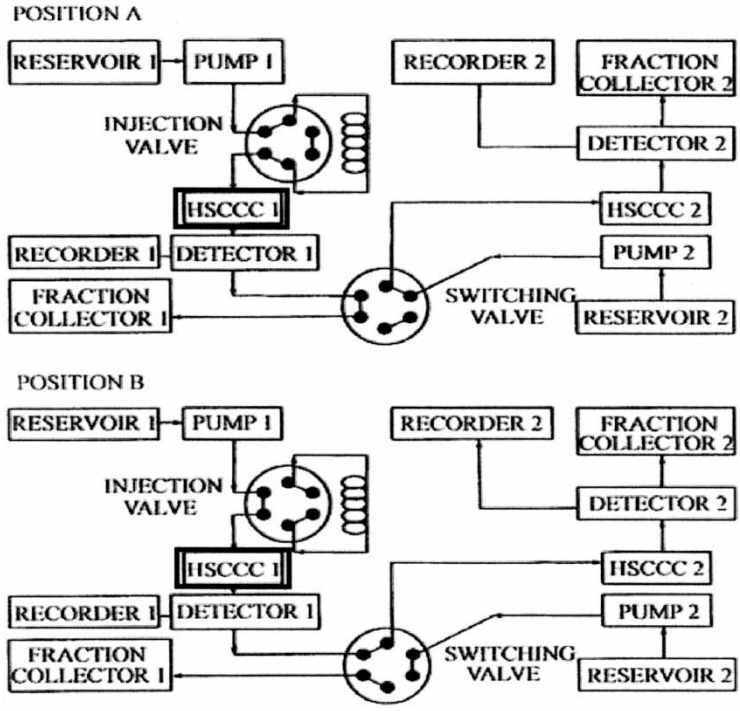

Figure 1. Schematic diagram of the multidimensional countercurrent chromatography (MDCCC) system with two sets of high-speed countercurrent chromatography (HSCCC) system, a six port injection valve and a six port switching valve. Adapted from Figure 8.1, Ref. 8.

Reagents

All organic solvents used for HSCCC were of analytical grade and purchased from Beijing Chemical Factory (Beijing, China). Methanol used for HPLC analysis was of chromatographic grade and purchased from Tianjin Huaxi Special Reagent Factory (Tianjin, China). Tripdiolide (90%) and crude sample from *Tripterygium wilfordii* Hook F. were gifts from an acquaintance of one of us (Y.W.).

Preparation of Two-Phase Solvent System and Sample Solutions

Two biphasic solvent systems composed of n-hexane-dichloromethane-methanol-water (3:22:17:8, v/v/v/v) and chloroform-methanol-water (4:3:2 v/v/v) were each thoroughly equilibrated in a separatory funnel at room temperature, and the two phases separated shortly before use. The sample solutions were prepared by dissolving the crude extract in the lower phase at a suitable concentration according to the preparative purpose.

Separation Procedure

HSCCC was performed as follows: the multiplayer coiled column was first entirely filled with the upper phase. The lower phase was then pumped into the head end of the inlet column at a flow-rate of 2.0 mL/min, while the apparatus was run at a revolution speed of 800 rpm. After hydrodynamic equilibrium was established, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (1000.7 mg in 20 mL lower phase at a 50 g/L concentration) was injected through the sample port. The effluent from the tail end of the column was continuously monitored with a UV detector at 214 nm. Each peak fraction was collected according to the chromatogram. The retention of the stationary phase relative to the total column capacity was computed from the volume of the stationary phase collected from the column after the separation was completed.

MDCCC separation was performed as follows: The switching valve shown in Figure 1 was initially set in position A, and HSCCC systems 1 and 2 were simultaneously filled with the upper aqueous polar stationary phases using pumps 1 and 2, respectively (Figure 1). The n-hexane-dichloromethane-methanol-water (3:22:17:8, v/v/v/v) biphasic liquid system was used in System 1 and the chloroform-methanol-water (4:3:2 v/v/v) mixture was used in System 2. Both apparatuses were rotated at 800 rpm, while the lower phases were eluted through HSCCC systems 1 and 2 using their respective pumps at a flow-rate of 2.0 mL/min. In System 1, the mobile phase was the organic hexane-dichloromethane lower phase. In System 2, it was the chloroform-rich lower mobile phase. After hydrodynamic equilibrium was reached in each column, the sample solution (1000 mg in 20 mL of lower phase) was injected into HSCCC 1 through the injection valve, while pump 2 was stopped to save solvents. The effluent from the outlet of HSCCC 1 was continuously monitored with the UV detector 1 at 214 nm, and collected according to the chromatogram. When the target peak appeared, the effluent from HSCCC 1 was cut and introduced into the HSCCC 2 column by turning the switching valve to position B. After the target peak was completely introduced from HSCCC 1 to HSCCC 2 columns, the switching valve was returned to position A, while restarting pump 2 to resume the elution of the target peak with detector 2 and recorder 2.

HPLC Analyses and Identification of HSCCC Peak Fractions

The crude sample of *Tripterygium wilfordii* Hook, tripdiolide, and HSCCC peak fractions were each analyzed by HPLC. The analyses were performed with a Supelcosil ODS column (250 × 4.6 mm I.D.) at column temperature of 35°C. The mobile phase, composed of methanol: water (30:70, v/v), was isocratically eluted at a flow-rate of 1.0 mL/min and the effluent monitored at 214 nm by a PAD detector. Identification of the target compound

(tripdiolide) was based on retention time referenced with a pure standard of tripdiolide, together with MS, ^{1}H-NMR; and ^{13}C-NMR spectra.

RESULTS AND DISCUSSION

The HPLC analysis of the crude extract from *Tripterygium wilfordii* Hook F. indicated that it contained several compounds as shown in Figure 2. The tripdiolide content in the crude extract is about 1.1% w/w based on the external standard curve determined by HPLC. The CCC chromatogram of tripdiolide from *Tripterygium wilfordii* Hook F. is shown in Figure 3. Peak 3 contains tripdiolide.

Using analytical HSCCC, a pair of two-phase solvent systems composed of n-hexane-dichloromethane-methanol-water (3:22:17:8, v/v/v/v), and chloroform-methanol (4:3:2, v/v/v) were selected for the MDCCC method for purification of tripdiolide.

Figure 4 shows the chromatogram of tripdiolide from *Tripterygium wilfordii* Hook obtained by MDCCC. Figure 4 A shows the chromatogram obtained from HSCCC 1 and recorder 1. Peak 3 containing tripdiolide was

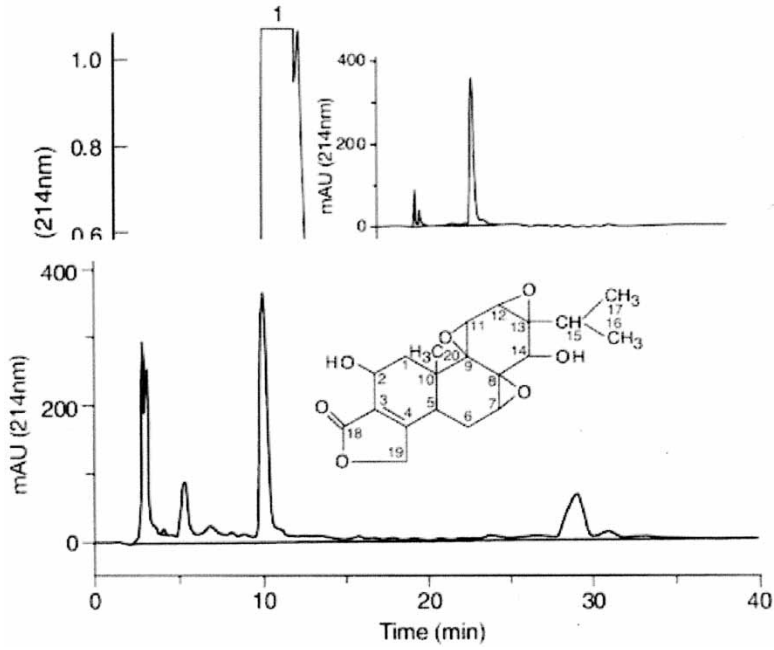

Figure 2. 1
HPLC analyses of the crude extract from *Tripterygium wilfordii* Hook with the chemical structure of tripdiolide. HPLC conditions: Supelcosil ODS column (250 × 4.6 mm I.D.), column temperature: 35°C. Mobile phase: methanol: water (30:70, v:v), flow-rate: 1.0 mL/min, monitored at 214 nm by a PAD detector.

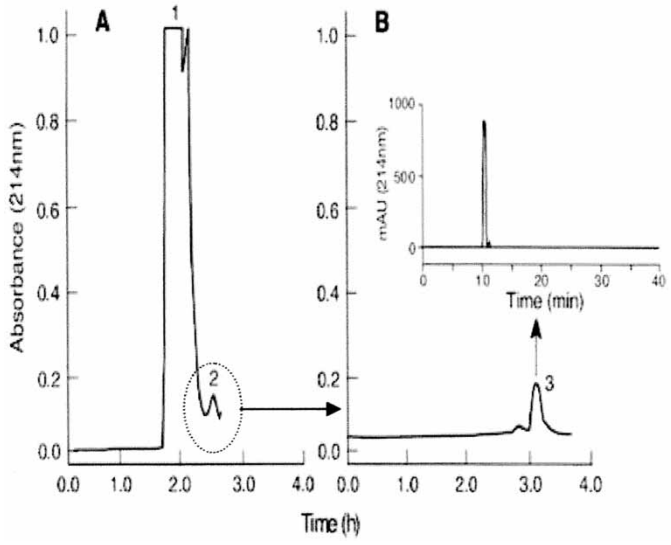

Figure 3. Chromatogram of tripdiolide from *Tripterygium wilfordii* Hook by preparative HSCCC. Solvent system: n-hexane-dichloromethane-methanol-water (3:22:17:8, v/v/v/v); stationary phase: upper polar aqueous phase; mobile phase: lower organic apolar phase; flow-rate: 2.0 mL/min; revolution speed: 800 rpm; detection: 214 nm; sample: 1000.7 mg dissolved in 20 mL of lower phase. Peak 3 contains tripdiolide as shown by the HPLC inset. HPLC conditions listed in Figure 2 caption.

cut and introduced into the HSCCC 2 column. The chromatogram in Figure 4 B was obtained by the cut fraction of HSCCC 1 (corresponding to the shaded part of the peak 3 in Figure 3) introduced into and eluted from the HSCCC 2 column. This separation yielded 7.8 mg of tripdiolide at over 98% purity based on HPLC analysis. The injected 1 g of crude extract contained about 11 mg of tripdiolide. The CCC yield is then about 71%.

The structural identification of tripdiolide was carried out by MS, ^{1}H-NMR, and ^{13}C-NMR spectra as follows: the EI-MS: m/z 376, 361, 358, 343, 340, 297, 269, 250, 149, 43. It showed the molecular ion at m/z 376, which is in agreement with the molecular formula $C_{20}H_{24}O_7$ of tripdiolide.

^{1}H-NMR (300 MHz, DMSO) δ ppm: 1.33 (1C-2H, m), 4.33 (2C-2H, br), 2.50 (5C-1H, m), 2.22 (6C-1H, m), 1.93 (6C-1H, t), 3.36 (7C-1H, d), 3.89 (11C-1H, d), 3.54 (12C-1H, d), 3.32 (14C-1H, d), 2.15 (15C-1H, m), 0.79 (16C-3H, d), 0.91 (17C-3H, d), 4.85 (19C-2H, br), 0.96 (20C-3H, s), 5.10 (2C-OH, d), 4.63 (14C-OH, d).

^{13}C-NMR (300 MHz, DMSO) δ ppm: 39.07 (C-1), 58.32 (C-2), 125.52 (C-3), 164.50 (C-4), 40.73 (C-5), 22.63 (C-6), 60.22 (C-7), 61.32 (C-8), 64.84 (C-9), 35.48 (C-10), 56.24 (C-11), 54.53 (C-12), 64.95 (C-13), 71.63 (C-14), 27.80 (C-15), 17.02 (C-16), 17.95 (C-17), 172.73 (C-18), 70.03.18 (C-19), 15.96 (C-20).

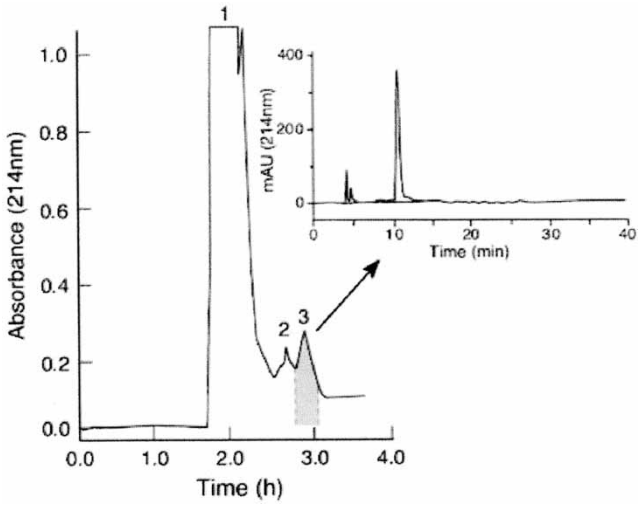

Figure 4. Chromatogram of tripdiolide from *Tripterygium wilfordii* Hook by multi-dimensional CCC. A. Chromatogram obtained with the HSCCC 1. Solvent system: n-hexane-dichloromethane-methanol-water (3:22:17:8, v/v/v/v); stationary phase: upper polar aqueous phase; mobile phase: lower chlorinated organic phase; flow-rate: 2.0 mL/min; revolution speed: 800 rpm; detection: 214 nm. Sample: 1 g extract dissolved in 20 mL of lower phase. B. Chromatogram of the cut fraction (corresponding to the shaded part of the peak 3 in Figure 3) obtained by HSCCC 2. Solvent system: chloroform-methanol-water (4:3:2, v/v/v); stationary phase: upper polar aqueous phase; mobile phase: lower chlorinated organic phase; flow-rate: 2.0 mL/min; revolution speed: 800 rpm; detection: 214 nm. Peak 3 is tripdiolide. HPLC conditions listed in Figure 2 caption.

The results of this study demonstrated that MDCCC is a useful method for the preparative separation of tripdiolide from a crude extract of *Tripterygium wilfordii* Hook.

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