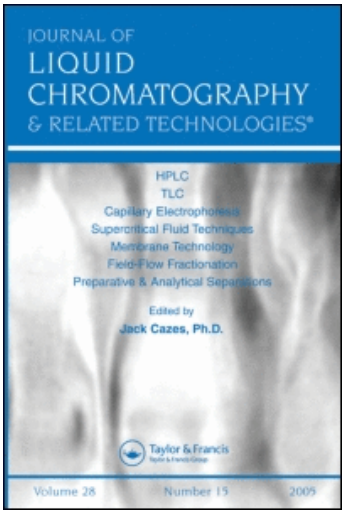


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# pH-MODULATED STEPWISE ELUTION CCC AND ITS APPLICATION TO THE PREPARATIVE SEPARATION OF HYDROXYANTHRAQUINONE COMPOUNDS FROM TRADITIONAL CHINESE MEDICINAL HERBS

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FUNDAMENTAL INVESTIGATIONS AND TRENDS IN CCC

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**Fuquan Yang,[1,*] Tianyou Zhang,[1] Guoqin Xu,[1] F. Edward Chou,[2] and Yoichiro Ito[3]**

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## ABSTRACT

Analytical and preparative high-speed countercurrent chromatography was successfully used for the separation of hydroxyanthraquinones from traditional Chinese medicine such as *Rheum officinale* Baill (Dahuang) and *Polygonum cuspidatum* Sieb. Et Zucc (Huzhang) using pH-modulated stepwise elution. Four major components including chrysophanol, emodin, physcion, and aloe-emodin were isolated each at over 98% purity.

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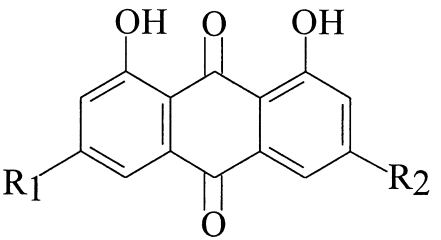
## INTRODUCTION

Hydroxyanthraquinones are rich in some important traditional Chinese medicinal herbs such as *Rheum officinale* Baill (Dahuang), *Polygomum multiflorum* Thunb, and *Polygonum cuspidatum* Sieb. Et Zucc., etc. as the major active constituents. The major active constituents in these herbs usually include chrysophanol, emodin, physcion, aloe-emodin, and rhein, and their glucosides. The chemical structures of the five main hydroxyanthraquinones are given in Figure 1. Pharmacological tests revealed that all these components are effective on dysentery bacillus, typhoid bacillus, and cholera bacillus, between which aloe-emodin is most potent, and rhein has also been found to have a strong antibacterial action on Bacteroides fragilis.[1-3] The pure chryphanol, emodin, physcion, aloe-emodin, and rhein are used for their quantitative analyses in these herbs for quality control of these herbs or their products. On the other hand, these hydroxyanthraquinones produce some problems in isolation by conventional silica gel column chromatography because they are strongly adsorbed onto the solid support.

High-speed countercurrent chromatography (HSCCC), being a support-free liquid-liquid partition chromatography, eliminates irreversible adsorption of sample onto the solid support[4] and, therefore, is considered as a suitable alternative for the separation of phenolic compounds such as flavonoids and hydroxyanthraquinones.[5-7]

The present paper describes HSCCC separation of hydroxyanthraquinones from a crude extract of *R. officinale* Baill (Dahuang) and *P. cuspidatum* Sieb. Et Zucc (Huzhang). The method uses pH-modulated stepwise elution based on their




| | $R_1$ | $R_2$ |
|---|---|---|
| Rhein | H | COOH |
| Emodin | OH | $CH_3$ |
| Aloe-emodin | H | $CH_2OH$ |
| Chrysophanol | H | $CH_3$ |
| Physcion | $OCH_3$ | $CH_3$ |

***Figure 1.*** Chemical structural illustration of hydroxyanthraquinones from *R. officinale* Baill.

characteristic acidity, which is determined by the number of carboxylic and phenolic hydroxyl groups, as well as the position (α or β) of phenolic hydroxyl group in the molecule. Analytical HSCCC is first used for selecting a two-phase solvent system and three basic mobile phases suitable for the pH-modulated stepwise elution program. The optimized HSCCC condition thus obtained led to the successful preparative HSCCC separation of hydroxyanthraquinones from crude extracts. The pH-modulated stepwise elution CCC was also compared with traditional extraction separation and purification of hydroxyanthraquinones.

## EXPERIMENTAL

### Apparatus



The analytical HSCCC instrument employed in the present study is a new Model GS 20 analytical high-speed countercurrent chromatograph designed and constructed at Beijing Institute of New Technology Application, Beijing, China. The apparatus holds a pair of column holders symmetrically 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 50 m x 0.85 mm I.D. PTFE (polytetrafluoroethlene) tube directly onto the holder hub, forming multiple coiled layers with a total capacity of 30 mL. The β value varied from 0.4 at the internal terminal to 0.7 at the external terminal (β=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 2000 rpm, an optimum speed of 1600 rpm was used in the present studies. Preparative HSCCC was performed using a Model GS10A2 multilayer coil planet centrifuge (Beijing Institute of New Technology Application, Beijing, China) equipped with a PTFE multilayer coil of 1.6 mm I.D. and 110 m in length with a total capacity of 230 mL and Model CCC-1000 (Pharma-Tech Scientific Corp. MD, USA) equipped with three PTFE multilayer coils of 2.6 mm I.D. and a total capacity of 860 mL.

The solvent was pumped into the column with a Model NS-1007 constant-flow pump (Beijing Institute of New Technology Application, Beijing, China). Continuous monitoring of the effluent was achieved with a Model 8823A-UV Monitor (Beijing Institute of New Technology Application) at 254 nm. A manual sample injection valve with a 1.0 mL loop (for the analytical HSCCC) and a 20-50 mL loop (for the preparative HSCCC) (Tianjin High-New Science & Technology Company, Tianjin, China) was used to introduce the sample into the column. A portable recorder (Yokogawa Model 3057, Sichuan Instrument Factory, Chongqin, China) was used to draw the chromatogram.

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 mL GU-12A degasser and a Class-VP-LC workstation (Shimadzu, Kyoto, Japan).

## Reagents

Sodium bicarbonate, sodium carbonate, sodium hydroxide, hydrochloric acid, acetic acid, and methyl-tert-butyl-ether (MTBE) used in this study are of analytical grade and were purchased from Beijing Chemical Factory (Beijing, China). Methanol used for HPLC analysis is of chromatographic grade and purchased from Tianjin Huaxi Special Reagent Factory (Tianjin, China). Chrysophanol, emodin, physcion, aloe-emodin, and rhein standard samples were purchased from National Institute for the Control of Pharmaceutical & Biological Products, Ministry of Health (Beijing, China).



## Extraction of Crude Hydroxyanthraquinones

About 1.0 kg of dried roots of *R. officinale* Baill was extracted with 1.5 L of ethanol three times at room temperature. The extracts were combined and evaporated to dryness under reduced pressure, which yielded 100 g of dry power. A 40 g amount of this dried extracts was refluxed with a solvent mixture consisting of 250 mL of ethanol and 50 mL 25% hydrochloric acid for 4 h. After cooling and removing the ethanol under reduced pressure, the total hydroxyanthraquinones were extracted with 500 mL diethyl ether for three times. The ether extract was evaporated to dryness to yield 21 g of a crude sample of hydroxyanthraquinones.

About 1.0 kg of dried roots of *P. cuspidatum* Sieb. Et Zucc was extracted with 1.5 L of ethanol three times at room temperature. After removing the ethanol under reduced pressure, the total hydroxyanthraquinones were extracted with 500 mL of diethyl ether three times. The diethyl ether extract was evaporated to dryness to yield 50 g of a crude sample of hydroxyanthraquinones.

## Separation of Hydroxyanthraquinones

Based on the difference of acidity of different hydroxyanthraquinones, a basic solution extraction separation is used for the separation of hydroxyanthraquinones from the crude diethyl ether extract of *R. officinale* Baill by using

5%$NaHCO_3$, 5% $Na_2CO_3$, 0.4% NaOH and 5% NaOH, in turn, in the first step of a traditional separation method in China. And then, recrystallization, silica gel column chromatography is used in the next several steps of separation.

## Preparation of Two-Phase Solvent System and Sample Solutions

The two-phase solvent system utilized in the present study was prepared by mixing MTBE and distilled water at suitable volume ratio and thoroughly equilibrating in a separatory funnel at room temperature. After the two phases were separated, the organic phase was used as stationary phase, and the aqueous phase as the mobile phase. For performing stepwise elution, a portion of the aqueous phase was basified by adding 4.0% $NaHCO_3$ (wt/wt; pH 8.4), 0.7% $Na_2CO_3$ (wt/wt; pH 11.10), and 0.4% NaOH (wt/wt; pH 12.20), and these three mobile phases at different pHs were successively eluted through the column in an increasing order of pH for the separation.

The sample solutions were prepared by dissolving the crude extract in the upper organic phase at a suitable concentration for analytical and preparative purposes.

## Separation Procedure

The analytical HSCCC was performed with a Model GS 20 HSCCC instruments as follows: The multilayer coiled column was first entirely filled with the upper organic stationary phase. The lower aqueous mobile phase was then pumped into the head end of the inlet column at a flow-rate of 1.0 mL/min, while the apparatus was run at a rotational speed of 1600 rpm. After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (10 mg in 1 mL of upper organic phase) was injected through the sample port. Then, the stepwise elution was started by successively eluting the column with three different mobile phases containing 4.0% $NaHCO_3$ (pH 8.4), 0.7% $Na_2CO_3$ (pH 11.10), and 0.4% NaOH (pH 12.20), respectively, in an increasing order of pH. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm. 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.

The preparative HSCCC was performed with a Model GS10A2 HSCCC centrifuge equipped with a multilayer coil column of 1.6 mm I.D. and 230 mL in total volume, and a 20 mL loop. The mobile phase was eluted at a flow-rate of 2.0 mL/min at a revolution speed of 800 rpm. The peak fractions were collected with test tubes according to the chromatogram.

The preparative HSCCC was also performed with a Model CCC-1000 HSCCC centrifuge equipped with three multilayer coil columns of 2.6 mm I.D. and 860 mL in total volume and a 50 mL loop. The mobile phase was eluted at a flow-rate of 3.5 mL/min at a revolution speed of 1000 rpm. The peak fractions were collected with test tubes according to the chromatogram.

## HPLC Analyses and Identification of CCC Peak Fractions

The HPLC analyses were performed with a Shim-pack VP-ODS column (4.6mm I.D. × 150mm) at a column temperature of 40°C. The mobile phase, composed of methanol-1% HAc (A:B), was gradiently eluted at a flow-rate of 1.0 mL/min and the effluent monitored by PDA detector. Identification of HSCCC



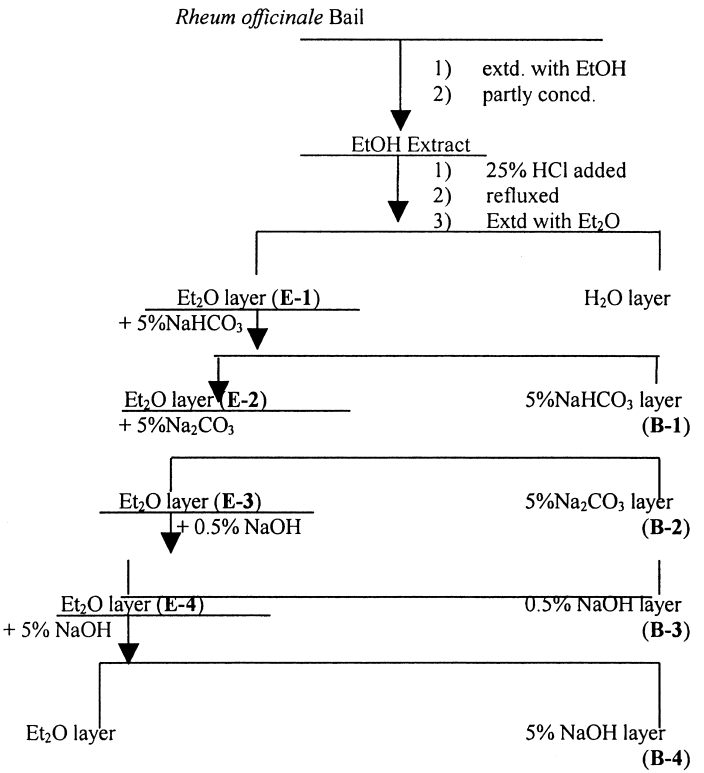



***Figure 2.*** Illustration of the aqueous base extraction separation of hydroxyanthraquinones from a crude extract of *R. officinale* Baill.

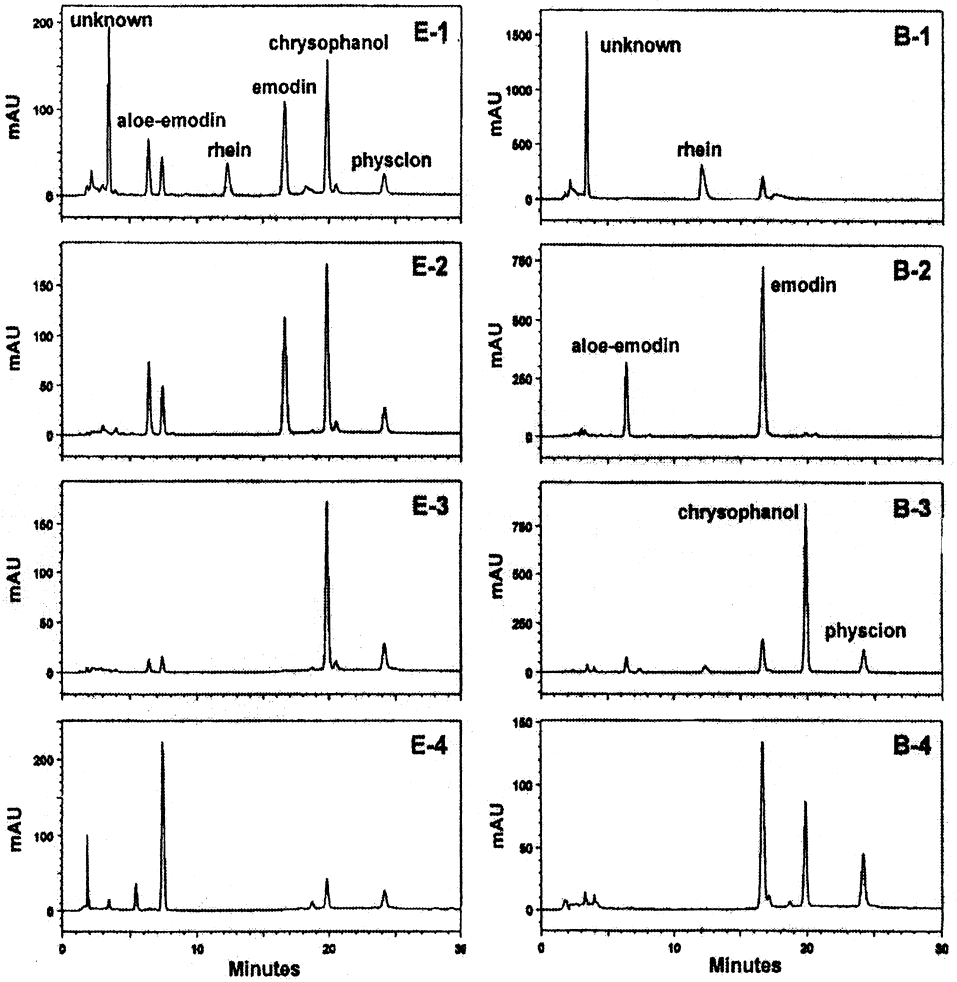



***Figure 3.*** The result of HPLC analyses of each basic extract and ether layer of hydroxyanthraquinones from *R. officinale* Baill shown in Figure 2. Column: Shim-pack VP-ODS column (150mm × 4.6mmI.D.); column temperature: 40°C; mobile phase and gradient program: methanol-1%HAc(A:B, v/v): 0.01 min (70%A)-10 min (70%A)-15 min (80%A)-30 min (80%A); flow-rate: 1.0 mL/min; UV wavelength: 430 nm.

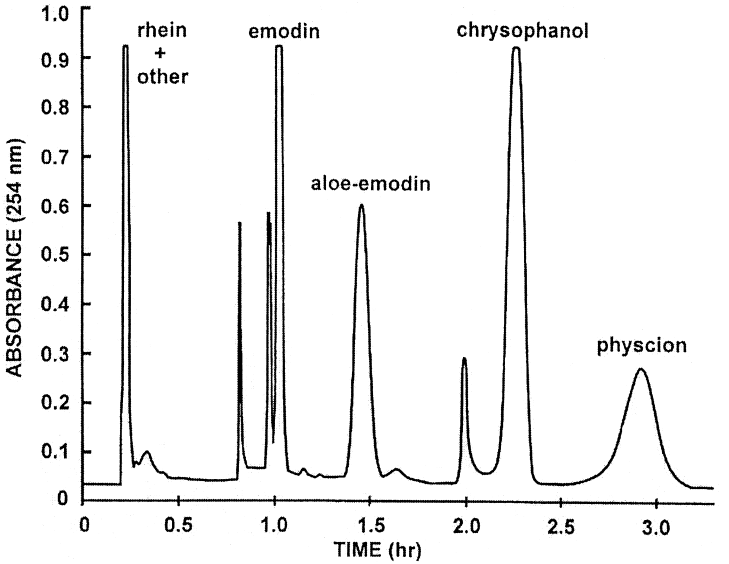



***Figure 4.*** Chromatogram of the crude sample of hydroxyanthraquinones from *R. officinale* Baill by analytical HSCCC. Solvent system: MTBE-aqueous basic system; stationary phase: upper organic phase; mobile phase: 30 mL of 4.0% $NaHCO_3$ and 60 mL of 0.7% $Na_2CO_3$ and 90 mL of 0.4% NaOH; flow-rate: 1.0 mL/min; revolutionary speed: 1600 rpm; sample size: 10 mg dissolved in 1 mL stationary phase. Retention of the stationary phase was 51%.

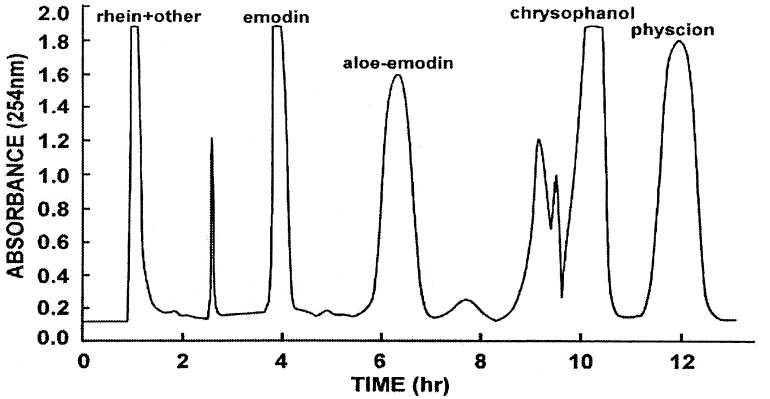



***Figure 5.*** Chromatogram of the crude sample of hydroxyanthraquinones from *R. officinale* Baill by Model CCC-1000 preparative HSCCC with a MTBE-aqueous basic system. Stationary phase: upper organic phase; mobile phase: 420 mL of 4.0% $NaHCO_3$ and 1050 mL of 0.7% $Na_2CO_3$ and 1050 mL of 0.4% NaOH; flow-rate: 3.5 mL/min; revolutionary speed: 1000 rpm; sample size: 600 mg dissolved in 50 mL stationary phase; retention of the stationary phase was 50%.

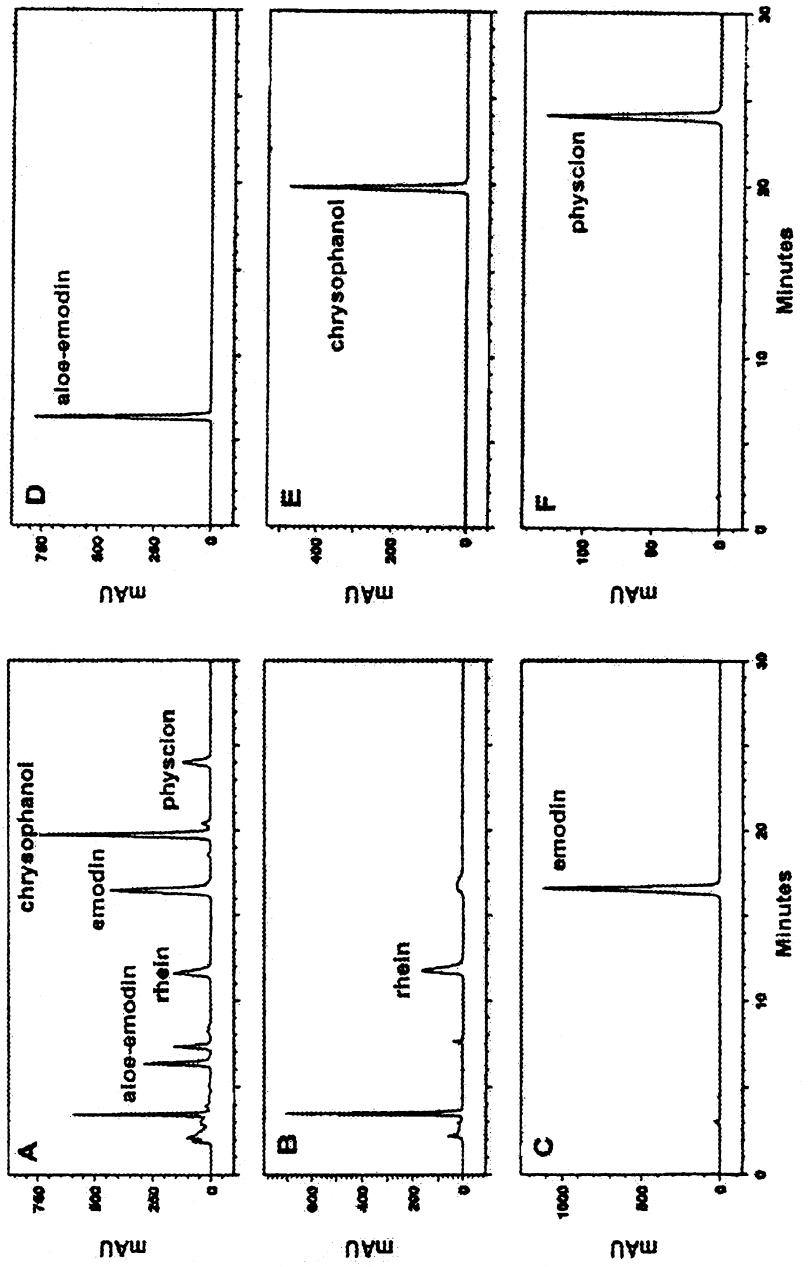



*Figure 6.* The result of HPLC analyses of the crude sample and CCC fractions of hydroxyanthraquinones from *R. officinale* Baill. (A) crude sample; (B) peaks 1; (C) peak 2; (D) peak 3; (E) peak 4; (F) peak 5 of the HSCCC separation shown in Figure 5. Column: Shim-pack VP-ODS column (150mm × 4.6mm I.D.); column temperature: 40°C; mobile phase and gradient program: methanol-1%HAc (A:B, v/v): 0.01 min (70%A)-10 min (70%A)-15 min (80%A)-30 min (80%A); flow-rate: 1.0 mL/min; UV wavelength: 430 nm.

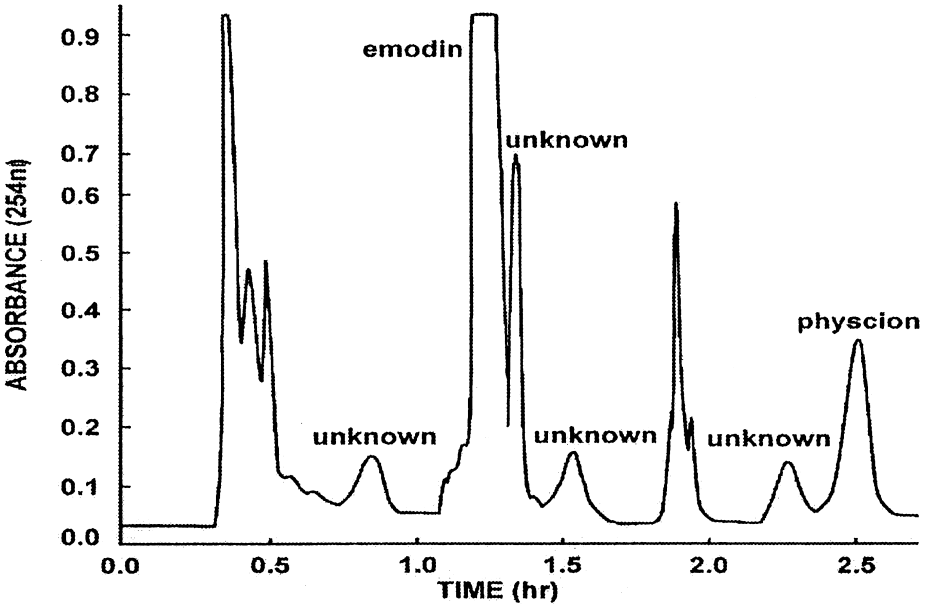



***Figure 7.*** Chromatogram of the crude sample of hydroxyanthraquinones from *P. cuspidatum* Sieb. Et Zucc by analytical HSCCC. Solvent system: MTBE-aqueous basic system; stationary phase: upper organic phase; mobile phase: 35 mL of 4.0% $NaHCO_3$ and 55 mL of 0.7% $Na_2CO_3$ and 70 mL of 0.4% NaOH; flow-rate: 1.0 mL/min; revolutionary speed: 1600 rpm; sample size: 10 mg dissolved in 1 mL stationary phase; Retention of the stationary phase was 51%.



peak fractions were carried out by comparing retention time and UV spectra with those of standard samples.

## RESULTS AND DISCUSSION

In order to compare with newly set pH-modulated stepwise elution CCC, a basic solution extraction, based on the difference of acidity of different hydroxyanthraquinones, was used in the first step of a traditional method for the separation of hydroxyanthraquinones from the crude diethyl ether extract of *R. officinale* Baill by using 5% $NaHCO_3$, 5% $Na_2CO_3$, 0.4% NaOH, and 5% NaOH solution, respectively (Figure 2). Figure 3 shows the results of the HPLC analyses of each basic extract and ether layer. The results indicated that each basic extract contains two or three compounds. Further purification steps were neces-

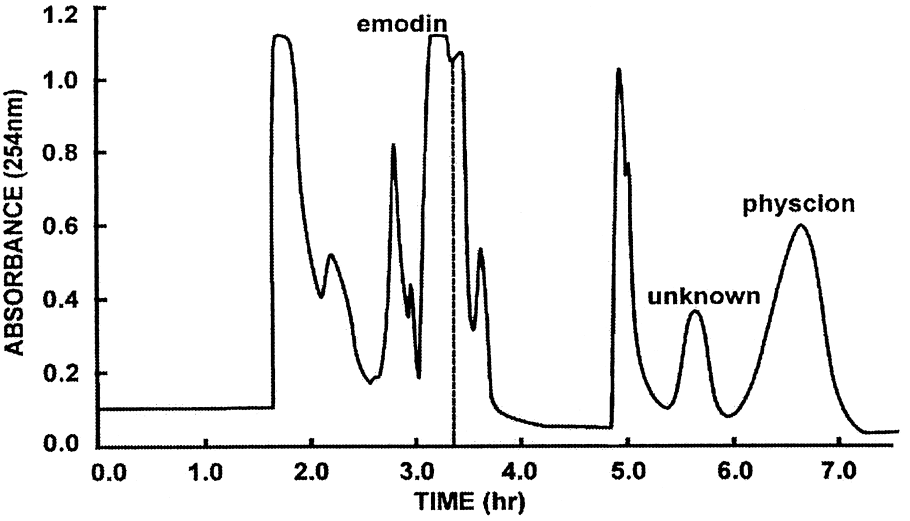



***Figure 8.*** Chromatogram of the crude sample of hydroxyanthraquinones from *P. cuspidatum* Sieb. Et Zucc by Model GS10A2 preparative HSCCC with a MTBE-aqueous basic system. Stationary phase: upper organic phase; mobile phase: 240 mL of 4.0% $NaHCO_3$ and 180 mL of 0.7% $Na_2CO_3$ and 420 mL of 0.4% NaOH; flow-rate: 2.0 mL/min; revolutionary speed: 800 rpm; sample size: 200 mg dissolved in 20 mL stationary phase; retention of the stationary phase was 40%.



sary and yielded rhein, emodin, and aloe-emodin, each, at over 90% purity and a mixture of chrysophanol and physcion.

Based on their characteristic acidity of hydroxyanthraquinones, pH-modulated stepwise elution was performed for HSCCC separation of hydroxyanthraquinones. Using analytical HSCCC, a suitable two-phase solvent system was obtained from MTBE by adding dilute bases to the aqueous mobile phase. Figure 4 shows the separation of hydroxyanthraquinones from the crude extract of *R. officinale* Baill by analytical HSCCC. In this separation, although rhein was eluted together with impurities in the first peak, the rest of the components were all resolved well and eluted within 3 hours.

Figure 5 shows the result obtained from 600 mg of the crude hydroxyanthraquinone extract of *R. officinale* Baill by preparative HSCCC (Model CCC-1000), using pH-modulated stepwise elution. Emodin, aloe-emodin, chrysophanol, and physcion were all well resolved. After separation, each purified compound was extracted from the peak fraction with ethyl acetate after acidification by concentrated hydrochloric acid. HPLC analyses given in Figure 6 indicated that the purity of all four major compounds thus obtained was over 99%.

Figure 7 and Figure 8 show the results separately obtained from 10 mg and 200 mg of the crude ether extract of *P. cuspidatum* Sieb. Et Zucc by analytical and preparative (Model GS10A2) HSCCC using pH-modulated stepwise elution. Emodin and physcion were purified well, and each at over 98% purity.

The results of our studies clearly demonstrated that the HSCCC procedure using the pH-modulated stepwise elution program with a suitable two-phase solvent system is cost-effective and very efficient for the separation of hydroxyanthraquinones from the crude extracts of traditional Chinese medicinal herbs. Because of the high boiling point of MTBE, the MTBE solvent system is safer and more stable than the diethyl ether solvent system reported before,[8] and easier to use at much higher room temperatures.

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## REFERENCES

1. Silva, M.G.V. Ann. Pharm. Fr. **1988**, *46* (2), 83.
2. Marini, D. Domenico Rass Chim **1982**, *34* (3), 125.
3. Liu, Q.Z.; Wang, J.L. Chin. Trad. Herbal Drugs **1987**, *18* (1), 41.
4. Ito, Y. CRC Crit. Rev. Anal. Chem. **1986**, *17*, 65.
5. Zhang, T.-Y.; Hua, X.; Xiao, R.; Knog, S. J. Liq. Chromatogr. **1988**, *11* (1), 233.
6. Zhang, T.-Y.; Xiao, R.; Xiao, Z.-Y.; Pannell, L.K.; Ito, Y. J. Chromatogr. **1988**, *445*, 199.
7. Yang, F.-Q.; Zhang, T.-Y.; Mo, B.-X.; Yang, L.-J.; Gao, Y.Q.; Ito, Y. J. Liq. Chromatogr. & Rel. Technol**. 1988**, *21* (1&2), 209.
8. Yang, F.-Q.; Zhang, T.-Y.; Tian, G.; Cao, H.; Liu, Q.; Ito, Y. J. Chromatogr. A **1999**, *858*, 103.

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