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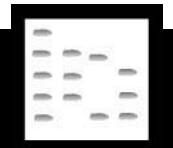
MEDICINAL HERB COMPOUNDS: HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY



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Introduction

Medicinal herbs are an important source of natural products for medicine. They include various chemical components ranging from fat-soluble to water-soluble compounds. The isolation of the biologically active components is the starting point of further research in chemistry and pharmacology as well as in the utilization of these compounds.

Traditional Chinese medicine is an extremely rich source of the experience acquired over a long period of time. In order to make greater use of traditional Chinese medicine, modern scientific methods are used to find the bioactive compounds in the traditional drugs and to use them as leading compounds for new drug design. New drugs developed in this way include anisodamine and the antimalarial agent Qinghaosu (artemisine).

For separating and purifying bioactive compounds from medicinal herbs, modern chromatographic techniques, such as gas chromatography, high performance liquid chromatography, thin-layer chromatography and electrophoresis have significantly raised the technical level and have shortened the time required for research projects.

High speed countercurrent chromatography (HSCCC) has been recognized as an effective means for separation and purification of a wide variety of bioactive components. It is a liquid–liquid partition chromatography system based on a coil planet centrifuge system without the use of any solid support. This technique has developed rapidly during the past decade. It has been demonstrated to have preparative capabilities and unique properties for fractionating a variety of natural products and medicinal herbs.

Here some applications of the separation of bioactive compounds, such as alkaloids and flavonoids, in medicinal herbs by HSCCC are described.

Separations of Alkaloids

Separations of Alkaloids Extracted from *Stephania tetrandra* S. Moore

Dried roots of *Stephania tetrandra* S. Moor (Menispermaceas) or Fenfangji in Chinese is a traditional Chinese drug used for rheumatism and arthritis. The total active alkaloid content in the natural products is 2.3%. Three major alkaloids have been identified as tetrandrine (I, 1%), fangchinoline (II, 0.5%) and cyclanoline (III, 0.2%). I and II are inseparable by conventional methods, while III is well separated from the other two. As illustrated in **Figure 1**, I and II are both bisbenzylisoquinoline alkaloids, whereas III is a water-soluble quaternary protoberberine-type alkaloid.

A sample solution was prepared as a mixture of I and II with purified III to obtain a 10 : 5 : 2 weight ratio to simulate their composition in the natural drug. 3 mg of this sample was dissolved in 0.5 mL of the upper stationary phase of the selected solvent system. The solvent system was composed of n-hexane/ethyl acetate/methanol/water at two different volume ratios of 3 : 7 : 5 : 5 in the first experiment and 1 : 1 : 1 : 1 in the second. In both cases the lower phase was used as the mobile phase at a flow rate of 60 mL h^{-1} in the normal elution mode. The apparatus used in these experiments was a Pharma-Tech Model CCC-2000 analytical countercurrent

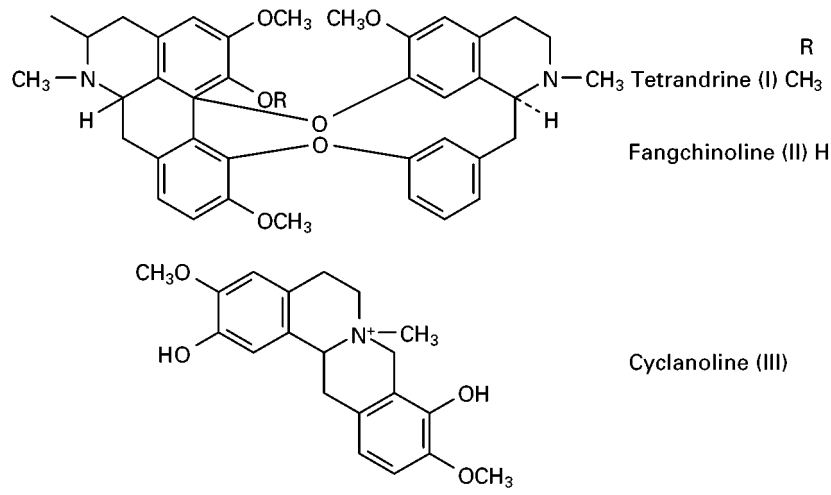

Figure 1 The chemical structures of tetrandrine(I), fangchinoline(II) and cyclanoline(III).

chromatograph made by Pharma-Tech Research Corp. It is equipped with a column holder at a 6.4 cm revolution radius. A multilayer coil prepared from a 70 m length of heavy wall 0.85 mm i.d. PTFE tubing is coaxially mounted on the holder. The total capacity of the column is 43 mL. The maximum speed of this centrifuge is 2000 rpm. The apparatus is equipped with a metering pump, a speed controller with a digital rpm display, and a pressure gauge.

Figure 2 shows the chromatogram obtained from the first experiment. In the normal elution mode, peaks 1 and 2 were completely resolved and collected in 70 min. This was followed by a reversed elution mode without interrupting the centrifuge run to collect the third peak in an additional 30 min. As shown in the chromatogram, a very small amount of impurity present between peaks 1 and 2 was also resolved.

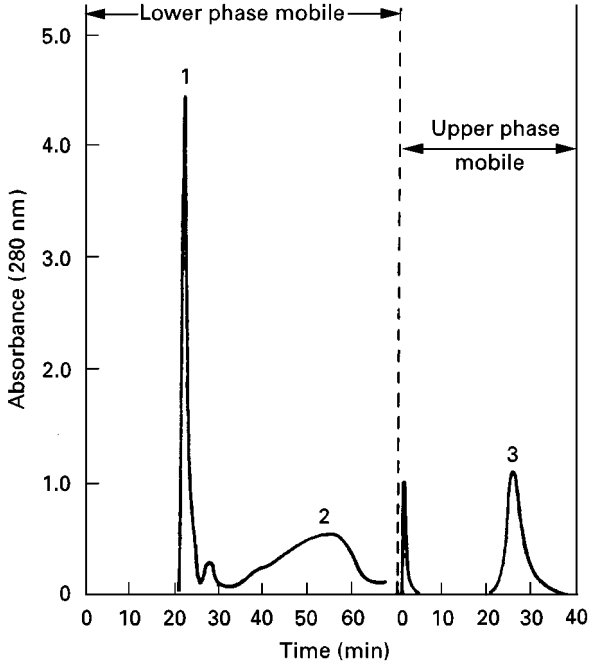

Figure 2 Chromatogram of the sample mixture of tetrandrine (1)–fangchinoline (2)–cyclanoline (3) (10 : 5 : 2). Solvent system n-hexane–ethyl acetate–methanol–water (3 : 7 : 5 : 5).

The chromatogram obtained from the second experiment is shown in **Figure 3**. It demonstrates an alternative approach where the solvent composition was adjusted to modify the partition coefficients of the compounds to shorten the separation time without the use of a reversed elution mode.

A Finnigan MAT mass spectrometer was used to analyse the peak fractions to identify the compounds in peaks 2 and 3 in Figure 3 as purified fangchinoline and cyclanoline respectively.

Semipreparative Separation of Alkaloids from *Cephalotaxus fortunei* Hook f.

The alkaloids, isoharringtonine (I), homoharringtonine (II) and harringtonine (III) isolated from *Cephalotaxus fortunei* Hook f., possess anticancer potency. Among those compounds, (I) and (III) are isomers, and (II) and (III) differ only by a $-CH_2$ group (**Figure 4**).

A crude alkaloid powder was prepared from the leaves and branches of *C. fortunei* Hook f. 20 mg of the crude powder was dissolved in 1 mL of the solvent mixture (upper phase and lower phase) as the sample solution for each separation. The two-phase solvent system was composed of chloroform/0.07 M sodium phosphate buffer solution at the volume ratio of 1 : 1 (pH 5.0).

The apparatus used in these experiments was a GS-10A HSCCC made by Beijing Institute of New Technology Application. A pair of column holders

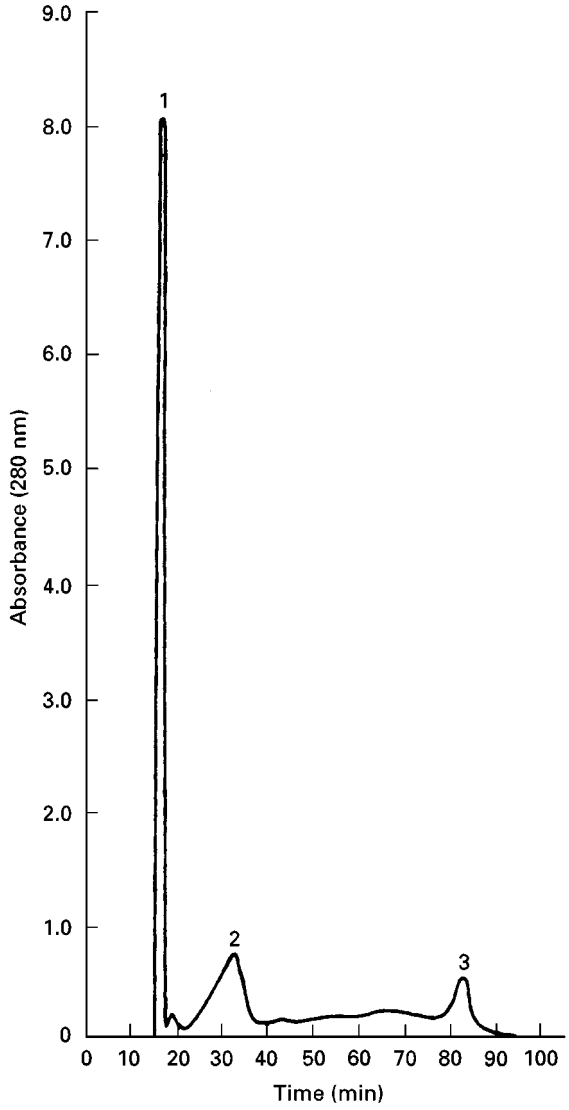

Figure 3 Chromatogram of the sample mixture of tetrandrine (1)–fangchinoline (2)–cyclanoline (3) (10 : 5 : 2). Solvent system n-hexane–ethyl acetate–methanol–water (1 : 1 : 1 : 1).

are held symmetrically on the rotary frame at a distance of 8 cm from the central axis of the centrifuge. The multilayer coil separation column was prepared by winding a 130 m length of 1.5 mm i.d. PTFE tubing directly on to the holder hub. The total capacity of the 14 layer column was 230 mL. The speed of this machine was regulated with a speed controller in a range between 0 and 1000 rpm, while 800 rpm was used as the optimum speed for the conventional two-phase solvent system.

In each separation, the column was first entirely filled with the stationary aqueous phase followed by injection of the sample solution. The apparatus was rotated at 800 rpm while the nonaqueous mobile phase was pumped into the column at a flow rate of 2 mL min^{-1}. Effluent from the outlet of the column was continuously monitored with a UV monitor at 254 nm and collected with a fraction collector at 2 min intervals. After a group of nonpolar compounds was eluted, the centrifuge was stopped while pumping was resumed to collect a polar compound still retained on the column. **Figure 5** shows the chromatogram obtained. Each fraction was analysed by TLC and the fractions corresponding to peaks 2, 4 and 6 were identified as isoharringtonine, homoharringtonine and harringtonine.

In order to increase the yield of the component homoharringtonine, 300 mg of crude extract was applied to produce 70 mg of the pure product. The method is useful for semipreparative separation of alkaloids and other natural products with similar polarity.

Separation of Flavonoids

Separation of Flavonoids in Crude Extract from Sea Buckthorn

The results of separations of a crude ethanol extract from dried fruits of sea buckthorn (*Hippophae rhamnoides*) are described. Five flavonoid constituents were successfully separated by P.C. Inc. A two-phase solvent system composed of chloroform–methanol–water at a 4 : 3 : 2 volume ratio was used. The sample solution was prepared by dissolving the crude ethanol extract in the solvent mixture of upper and lower phases at a concentration of 2.2 g %.

Separation was performed as follows: The coiled column was first entirely filled with the upper phase as the stationary phase followed by injection of sample solution containing 100 mg crude mixture through the sample port. The apparatus was rotated at the optimum speed of 800 rpm while the lower phase was pumped into the head end of the column as the mobile phase at 200 mL h^{-1} flow rate. Effluent from the outlet end of the column was continuously monitored at 278 nm and fractionated into test tubes. An aliquot of each fraction was diluted with methanol and the absorbance was determined at 260 nm with a spectrophotometer. **Figure 6** shows the chromatogram obtained. Five flavonoid components were completely resolved from each other as symmetrical peaks and eluted in 2.5 h. Partition efficiencies computed from the equation $N = (4R/W)^2$ (where N is the efficiency expressed in terms of theoretical plates (TP), R is retention time of the peak maximum, W is the peak width expressed in the same unit as R) range from 800 TP (2nd peak) to 530 TP (5th peak). By calculating the partition coefficients of each peak and comparing the values

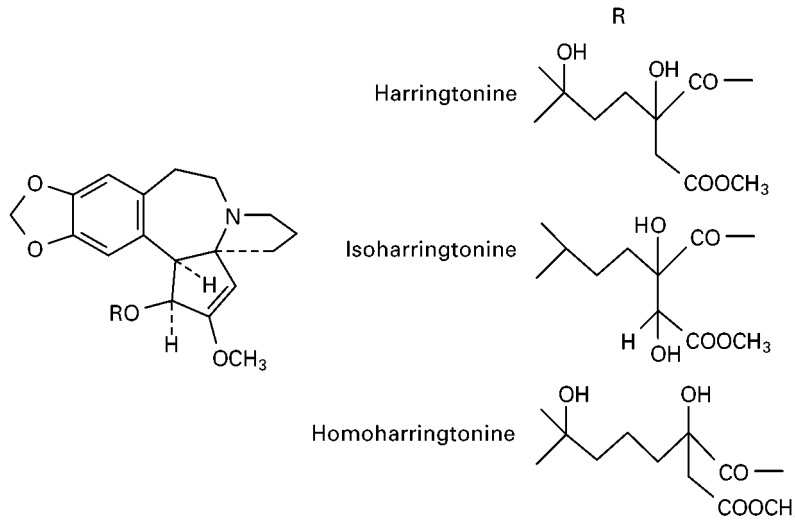

Figure 4 Chemical structures of harringtonine, isoharringtonine and homoharringtonine.

obtained with those of the pure compounds, quercetin and isorhamnetin peaks were identified as labelled in the chromatogram.

The same crude sample of ethanol extract from dried fruits of sea buckthorn can be separated with an analytical HSCCC for a series of rapid separations. The apparatus was a Pharma-Tech Model CCC-2000 made by Pharma-Tech Research Corp. The multilayer coiled column was prepared from a single piece of 0.85 mm i.d. heavy wall PTFE tubing. The total capacity of the column is 43 mL including 3 mL in the flow tubes. The revolution speed of the centrifuge can be continuously adjusted up to 2000 rpm. A LDC/Milton Roy Pump, a speed controllor with digital rpm display and a pressure gauge are also included.

In these separations, 3 mg of sample of the flavonoid mixture were dissolved in 0.5 mL of solvent mixture and loaded for each experiment. The centrifuge was rotated at the optimum speed of 1800 rpm. The effluent from the outlet of the column was continuously monitored with an LKB Uvicord S at 278 nm and then collected as 1 mL fractions with an LKB fraction collector. Each fraction was diluted with 2 mL of methanol and the absorbance was determined at 260 nm with a Zeiss spectrophotometer Model PM6.

Figure 7 shows the result of separation obtained at a flow rate of 60 mL h^{-1}. The high efficiency of the separation is evidenced by a minor peak present between the first and the second major peaks, which

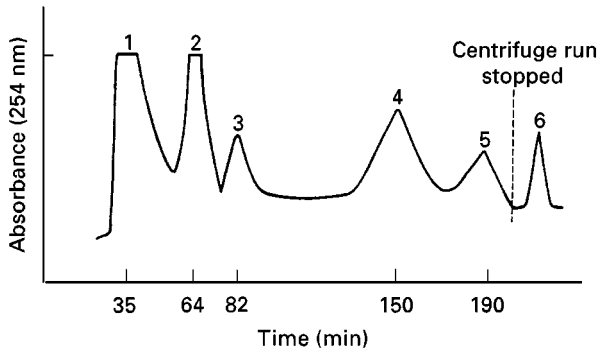

Figure 5 High speed CCC separation of harringtonine, isoharringtonine and homoharringtonine from a crude extract of *Cephalotaxus fortunei* Hook f. Peak 2, isoharringtonine; 4, homoharringtonine and 6, harringtonine.

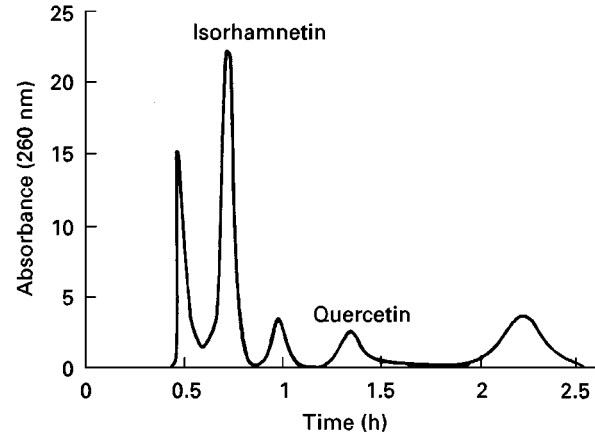

Figure 6 Countercurrent chromatogram of flavonoids in crude extract from dried fruits of sea buckthorn by a multilayer coil planet centrifuge. Conditions for CCC: sample size, 100 mg; solvent system, chloroform–methanol–water (4 : 3 : 2); mobile phase, lower phase; flow rate, 200 mL h^{-1}; speed, 800 rpm; fraction volume, 6 mL.

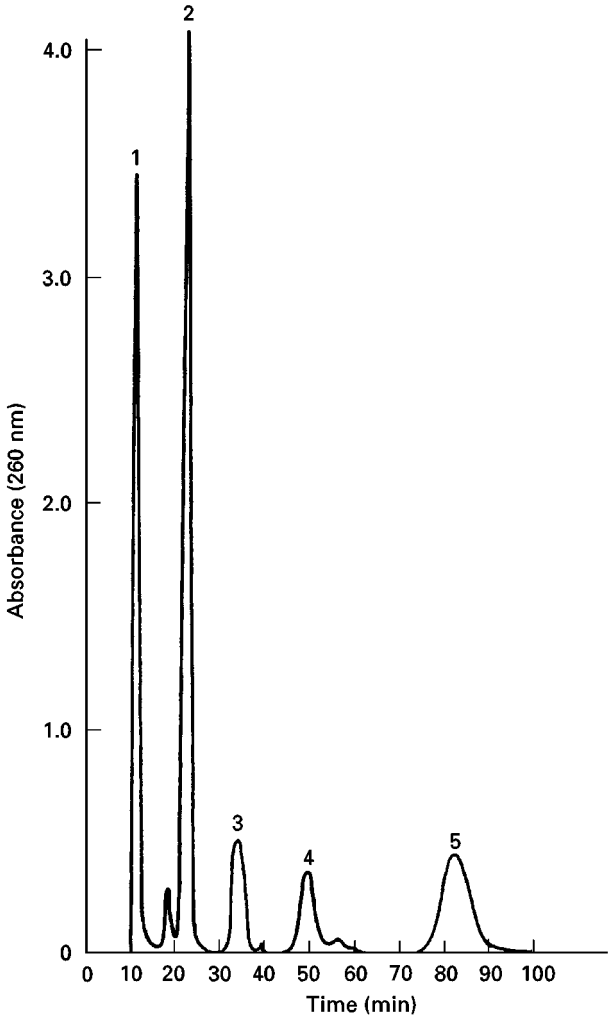

Figure 7 Chromatogram obtained from 3 mg extract by analytical countercurrent chromatography, flow rate of 60 mL min^{-1}.

was not detected in the semipreparative separation shown in Figure 6. Flow rates as high as 300 mL h^{-1} can be used to give a separation time of only 15 min, which is quite comparable with that of analytical HPLC.

The analytical HSCCC can be used to interface with MS to provide a new analytical methodology HSCCC-MS, combining the versatility and high resolution of HSCCC with the identification capability and low detection limit of mass spectrometry.

Preparative Separation of Kaempferol, Isorhamnetin and Quercetin from Extracts of *Ginkgo biloba* L.

Extracts of the leaves of *Ginkgo biloba* L. are used to increase peripheral and cerebral blood flow. *Ginkgo* extracts contain active compounds such as flavonoids and terpene lactones (ginkgolides and bilobalide). They show effects on vascular and cerebral metabolic processes and inhibit platelet-activating factor. The standard compounds used to control the quality of *Ginkgo* extracts and its preparations, such as isorhamnetin, kaempferol and quercetin, the three major flavone aglycones in the leaves of *Ginkgo biloba* L., are expensive and difficult to obtain. Some commercial quercetin standards, usually used in quantitative analysis of total flavonoids, are not pure and contain isorhamnetin, kaempferol and impurities. The preparative separation and purification of flavonoids from plant materials by classical methods are tedious and usually require multiple chromatographic steps. HSCCC as a form of liquid–liquid partition chromatography without using a solid support matrix is very suitable for the separation of flavonoids.

HSCCC was performed using a Model GS-10A multilayer coil planet centrifuge made by the Beijing Institute of New Technology Application. A NS1007 constant-flow pump, a model 8823A UV monitor operating at 254 nm and a manual sample injection valve with a 35 mL loop, were used in the experiment. A Rainin Model SD-200 HPLC was used for analysis.

In these studies, a two-phase solvent system composed of chloroform–methanol–water at a volume ratio of 4 : 3 : 2 was used. The crude *Ginkgo* flavone aglycones were prepared by several steps, from the extract of *Ginkgo* leaves. A second sample was a commercial quercetin standard. The sample solutions were prepared by dissolving these two samples in equal volumes of upper and lower phases.

In each separation, the column was first entirely filled with the upper aqueous phase as stationary phase, and then the apparatus was rotated at 800 rpm, while the lower chloroform phase was pumped into the column at a flow rate of 2 mL min^{-1}. After the mobile phase front emerged and the two phases had established hydrodynamic equilibrium in the coil column, the sample solution containing 200 mg of crude *Ginkgo* flavone aglycones or commercial quercetin standard was introduced through the injection value. HPLC analysis of the crude *Ginkgo* flavone aglycones is shown in **Figure 8A**. According to MS analysis, quercetin, kaempferol, isorhamnetin and some impurities were present. HPLC analysis of the commercial quercetin standard is shown in Figure 8B indicating that a larger amount of unknown impurity was present.

After repurification of the isorhamnetin peak by HSCCC, the purities of the three flavone aglycones were more than 99% according to the results of HPLC analysis. The identities of the three flavone aglycones were confirmed by MS analysis.

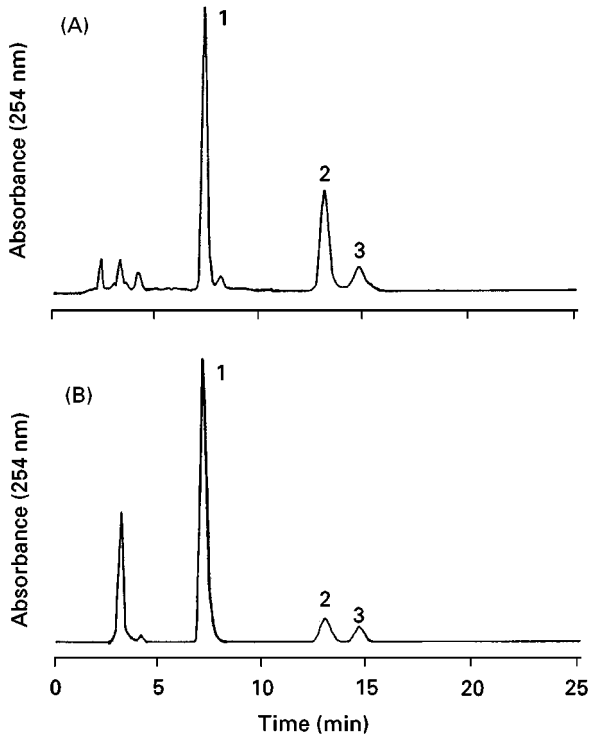

Figure 8 Chromatograms of the HPLC analyses of the crude *Ginkgo* flavone aglycones from the Ginkgo extracts (A) and the commercial quercetin standard (B). Mobile phase: methanol-0.04% H_3PO_4 (50 : 50, v/v); flow rate: 1.0 mL min^{-1}; detection: 254 nm. Peak 1: quercetin: Peak 2: kaempferol; Peak 3: isorhamnetin.

Semipreparative Separation of Taxol Analogues

Taxol is a promising new anticancer drug. Currently, taxol intended for human consumption is mainly obtained from the bark of *Taxus*. Although taxol is the compound most commonly used in clinical treatment, numerous analogues have also been identified. Some of them are of similar biological activities to taxol, and some can be used as natural precursors to semisynthesize taxol by simply modifying the C13 side chains. Meanwhile, the study of these compounds may lead to the discovery of new alternatives with improved pharmaceutical properties or economic benefits. The separation and purification of taxol analogues by classical methods such as preparative TLC, open column chromatography and HPLC in general are complicated and have low recoveries. HSCCC can be used for the semipreparative separation of some taxol analogues such as cephlomanine and 7-epi-10-deacetyltaxol.

HSCCC was performed with a model GS-10A multilayer coil planet centrifuge made by the Beijing Institute of New Technology Application. The chromatography system was the same as that described previously.

A crude extract containing 10% taxol from *Taxus yunnannesis* was pre-separated through a C_{18} column eluted by an acetonitrile–water gradient. After recovering the majority of the taxol and removing some other polar components, the fraction mainly containing cephalomannine, 7-epi-10-deacetyltaxol, residual taxol and an unknown compound was collected, concentrated and dried. The sample solution was prepared by dissolving the sample in the solvent mixture of upper and lower phases.

The two-phase solvent system, a quaternary system of n-hexane–ethyl acetate–ethanol–water was selected. In those studies a two-step separation was chosen. Firstly, the solvent system in 1 : 1 : 1 : 1 proportion was employed to separate the sample into two groups, A and B. Secondly, the system in 3 : 3 : 2 : 3 and 4 : 4 : 3 : 4 proportions was employed to separate the two compounds in A and B. In each separation, the coiled column was first entirely filled with the upper phase as the stationary phase. After the apparatus was rotated at 800 rpm, the lower phase was pumped into the column at a flow rate of 2 mL min^{-1}, while the sample solution was introduced via the injection valve. The effluent from the outlet of the column was continuously monitored at 254 nm. Twelve consecutive injections of 10–16 mg of sample mixture were performed without changing the stationary phase in the coiled column.

The four compounds were first separated into two groups as shown in **Figure 9**. Peak A contains mainly cephalomannine and taxol, while peak B contains mainly 7-epi-10-deacetyltaxol and the unknown compound. After 12 consecutive injections, 84.4 mg of A and 42.1 mg of B were obtained. Cephalomannine and taxol in peak A were well separated in the second step with the 3 : 3 : 2 : 3 solvent system

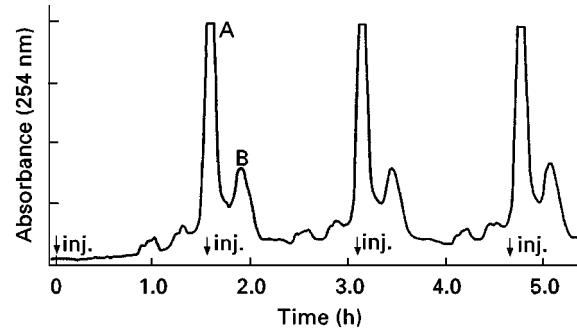

Figure 9 First step separation of taxol and its analogues by HSCCC with three consecutive injections. Solvent system: n-hexane–ethyl acetate–ethanol–water (1 : 1 : 1 : 1, v/v/v/v). A, cephalomannine + taxol; B, unknown + 7-epi-10-deacetyltaxol.

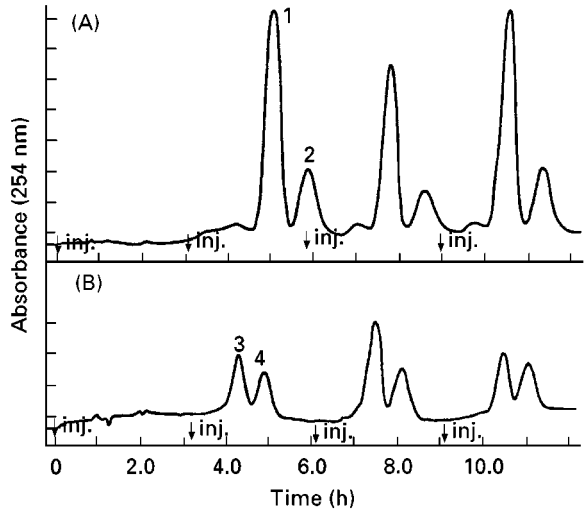

Figure 10 Second step separations of peak fractions (A) and (B) in Figure 9 by HSCCC with three consecutive injections. Solvent system: n-hexane–ethyl acetate–ethanol–water. (A) 3 : 3 : 2 : 3, (B) 4 : 4 : 3 : 4. 1, Cephalomannine; 2, taxol: 3, unknown; 4, 7-epi-10-deacetyltaxol.

as shown in **Figure 10**. 49.3 mg of cephalomannine and 14.6 mg of taxol were obtained after six consecutive injections. The purities were about 99% and 87% respectively according to the results of HPLC analysis. Similarly with peak B, 7-epi-10-deacetyltaxol and the unknown compound were separated with the 4 : 4 : 3 : 4 solvent system as shown in Figure 10B: 12 mg of 7-epi-10-deacetyltaxol and 10 mg of the unknown compound were obtained after three consecutive injections. The purities were about 85% according to the results of HPLC analysis.

Conclusions

Many applications of HSCCC such as the separation of alkaloids, flavonoids, hydroxyanthraquinone derivatives, etc., have been performed successfully. The main advantages of HSCCC are as follows:

1. Since no solid supports are used, all compounds in the sample solution are recovered. The crude sample can be injected directly into the column, which simplifies sample preparation.
2. Numerous two-phase solvent systems with a broad spectrum of polarity can be used with either the aqueous or organic phase as the mobile phase. Some examples are shown in **Table 1**.
3. The quantity of purified compound may range from milligrams to grams. Consecutive injection can be applied for large scale separation.
4. HSCCC will become widely used in the research and production of natural drugs, since the instrument is inexpensive, convenient to operate, solvent saving and has preparative capacity.

Table 1 Solvent systems for separation of some substances by high speed countercurrent chromatography

Substances separated	*Solvent system*
Alkaloids	nC_6H_{14}–EtOAc–MeOH–H_2O (1 : 1 : 1 : 1)
	nC_6H_{14}–EtOH–H_2O (6 : 5 : 5)
	$CHCl_3$–MeOH–H_2O (4 : 3 : 2)
	$CHCl_3$–MeOH–0.5%HBr–H_2O (5 : 5 : 3)
	Bu^nOH–0.1 M NaCl (1 : 1)
Flavonoids	$CHCl_3$–MeOH–H_2O (7 : 13 : 8), (4 : 3 : 2)
Flavonoid glycosides	$CHCl_3$–EtOAc–MeOH–H_2O (2 : 4 : 1 : 4)
	EtOAc–Bu^nOH–H_2O (2 : 1 : 2)
Indole	nC_6H_{14}–EtOAc–MeOH–H_2O (3 : 7 : 5 : 5), (1 : 1 : 1 : 1)
Xanthone glycosides	$CHCl_3$–MeOH–H_2O (4 : 4 : 3)
Lignans	nC_6H_{14}–CH_3CN–EtOAc–H_2O (8 : 7 : 5 : 1)
Lignan glycosides	$CHCl_3$–MeOH–H_2O (5 : 5 : 3)
	nC_6H_{14}–CH_2Cl_2–MeOH–H_2O (2 : 4 : 5 : 2)
Phenolic glycosides	$CHCl_3$–MeOH–H_2O (7 : 13 : 8)
	C_6H_{12}–Me_2CO–EtOH–H_2O (7 : 6 : 1 : 3)
Polyphenols	$CHCl_3$–MeOH–H_2O (7 : 13 : 8)
Tannins	Bu^nOH–0.1 M NaCl (1 : 1)
Coumarins	nC_6H_{14}–EtOAc–MeOH–H_2O (3 : 7 : 5 : 5)
Saponins	$CHCl_3$–MeOH–Bu^iOH–H_2O (7 : 6 : 3 : 4)
	$CHCl_3$–MeOH–Pr^iOH–H_2O (5 : 6 : 1 : 4)
	$CHCl_3$–MeOH–H_2O (7 : 13 : 8)
Hydroxyanthraquinone derivatives	nC_6H_{14}–EtOAc–MeOH–H_2O (9 : 1 : 5 : 5)
25-Hydroxycholecalciferol	nC_6H_{14}–EtOAc–MeOH–H_2O (5 : 1 : 5 : 1)

See also: **II/Chromatography:** Countercurrent Chromatography and High-Speed Countercurrent Chromatography: Instrumentation. **Chromatography: Liquid:** Countercurrent Liquid Chromatography. **III/Alkaloids:** Gas Chromatography; High-Speed Countercurrent Chromatography; Liquid Chromatography; Thin-Layer (Planar) Chromatography.

Further Reading

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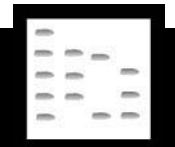
MEDIUM-PRESSURE LIQUID CHROMATOGRAPHY



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Introduction

Medium-pressure liquid chromatography (MPLC) is one of the various preparative column chromatography techniques. Separation under pressure renders the use of smaller particle size supports possible and increases the diversity of usable stationary phases. MPLC was introduced in the 1970s as an efficient technique for preparative separation of organic compounds. MPLC overcame one major drawback of low pressure liquid chromatography (LPLC), i.e. the limited sample loading. This separation method is now routinely used beside or in combination with the other common preparative tools: open-column chromatography, flash chromatography, LPLC or preparative high performance liquid chromatography (HPLC). The distinction between low pressure, medium pressure and high pressure LC is based on the pressure ranges applied in these techniques and the overlap is often considerable. MPLC allows purification of large compound quantities and, unlike open-column chromatography and flash chromatography, faster and improved separations are obtained. Packing of material with lower particle size under pressure enhances separation quality and moreover the solid phase can be reused. **Table 1** provides a comparative description of these different methods. Simplicity and availability of the instrumentation, together with recycling of packing materials and low maintenance costs, contribute to the attractiveness of this technique. More details about experimental conditions are given below.

Instrumentation

A schematic representation of a simple MPLC setup is shown in **Figure 1**. The instrumentation is made up of

Table 1 Comparative description of various preparative column chromatographic techniques

Technique	*Stationary phase particle size (μm)*	*Pressure (bar)*	*Flow rate (mL min^{-1})*	*Sample amount (g)*	*Solvents*	*General*
Open-column chromatography	63–200	Atmospheric	1–5	0.01–100	General solvent used	Frequent packing; RP not possible
Flash chromatography	40–63	1–2	2–10	0.01–100	General solvent used	Frequent packing
Low pressure LC	40–63	1–5	1–4	1–5	More solvent required	Prefilled Lobar columns
Medium pressure LC	15–40	5–20	3–16	0.05–100	More solvent required	Infrequent packing
Preparative HPLC	5–30	> 20	2–20	0.01–1	High purity solvent required	Higher resolution