Journal of Chromatography, 538 (1991) 219–225
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 795

Studies on the preparative capability of the horizontal flow-through coil planet centrifuge and high-performance liquid chromatography in the separation of polar compounds from *Oxytropis ochrocephala* Bunge

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ABSTRACT

Horizontal flow-through coil planet centrifuge (CPC) and high-performance liquid chromatography (HPLC) techniques were used for separation of polar compounds from a crude ethanol extract of *Oxytropis ochrocephala* Bunge, a poisonous legume plant widely distributed in northwestern China. The performance of these two chromatographic methods was compared in terms of column efficiency, peak resolution, separation time, sample loading capacity, etc. The results indicated that two polar compounds in the crude extract were equally well separated by these two methods. HPLC gave comparable peak resolution in shorter separation time while its sample loading capacity was limited to the mg range. The CPC method required a long separation time, but yielded a higher purity of fractions with a much greater capacity.

INTRODUCTION

In the past, various chromatographic techniques have been developed to isolate milligram to gram quantities of pure substances from plant materials for structural studies, bioassays, pharmacological tests, etc. [1]. As a plant material often contains thousands of different chemical compounds, the choice of these chromatographic techniques needs serious consideration. Although conventional column chromatography and preparative thin-layer chromatography are both simple and inexpensive, they have a number of limitations, including low column efficiency, long separation time, irreversible adsorption loss of solutes onto the solid support, etc. Recently, high-performance liquid chromatography (HPLC) with micro-particulate column packings of a narrow size range has been widely used for preparative purposes. However, due to its low column capacity and high cost of the packing material,

0021-9673/91/$03.50

application of semi-preparative HPLC becomes impractical when a larger amount of pure compounds is desired.

For many years, preparative-scale separations of natural products have been performed with the horizontal flow-through coil planet centrifuge (CPC) which utilizes a rotating coiled column in a centrifugal force field. This counter-current chromatographic method is characterized by relatively high column efficiency, broad applicability of solvent systems and large sample-loading capacity [2,3]. Consequently, the method is useful in preparative separations of milligrams to gram range, especially for polar substances [4]. Although coil planet centrifugation is less efficient compared with the more recently developed high-speed counter-current chromatography [5–10], it provides more stable retention of the stationary phase for low-interfacial-tension solvent systems and is particularly suitable for preparative separations of natural products which often contain surface-active compounds.

The present paper describes separations of polar compounds from *Oxytropis ochrocephala* Bunge, a poisonous legume plant widely distributed in northwestern China, which causes chronic neurological disorders in livestock. The separations were performed with the horizontal CPC, semi-preparative and analytical HPLC and the results were compared in terms of resolution, separation time and sample-loading capacity. Two flavonoid glycosides were isolated for determination of their chemical structures as reported elsewhere [11].

EXPERIMENTAL

Apparatus

The horizontal CPC employed in the present study was fabricated at the Beijing Institute of New Technology Application, Beijing, China, the design of the apparatus being previously described in detail [4]. The apparatus holds a set of four coiled columns around the column holder, which is mounted on the rotary frame at a distance of 14 cm from the centrifuge axis. Each column unit consists of 1.6 mm I.D., 0.3 mm thick wall PTFE (polytetrafluoroethylene) tubing with a 30-ml capacity. Four column units are serially connected with flow tubes (0.85 mm I.D.) to make up a total capacity of 120 ml. The ratio (β value) of the distance between the coil and the holder axis to the distance between the holder axis and the centrifuge axis is about 0.26. The balance of the centrifuge system is maintained by mounting a counter-weight on the opposite side of the rotary frame.

The HPLC separations were performed using an HP-1090M Model (Hewlett-Packard, Waldbronn, F.R.G.) with three different columns: μBondapak C_{18} (300 mm × 3.9 mm I.D.) 10 μm, Herpsil ODS (100 mm × 2.1 mm I.D.) 5 μm and LiChrosorb RP-18 (200 mm × 10 mm I.D.) 10 μm.

Sample preparation

Above-ground parts of *O. ochrocephala* B. were collected in Qinhai Province of China and identified at the Institute of Plateau Biology, Academia Sinica. Milled raw plant tissue was extracted with hot ethanol under reflux over a water bath. The solvent was evaporated under vacuum, and the residue was dissolved in a 5% acetic acid aqueous solution. The supernatant was extracted with ether to remove chlorophylls and lipophilic compounds and then alkalized with ammonia to pH 9.0 for extraction of

alkaloids with dichloromethane. The upper aqueous phase was lyophilized to dryness and used as the sample.

Separation procedures

The CPC separation was performed with a two-phase solvent system composed of chloroform–ethyl acetate–methanol–water (2:4:1:4, v/v). The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature, and the two phases were separated shortly before being applied to the column. The separation was initiated by filling the entire column space with the upper aqueous stationary phase. This was followed by injection of the sample solution containing 60 mg of the crude extract in the upper phase. Then the lower non-aqueous mobile phase was eluted through the column at a flow-rate of 1 ml/min while the apparatus was run at 280 rpm. Effluent from the outlet of the column was continuously monitored with a UV monitor at 254 nm, and the peak fractions were manually collected for later analysis. After 270 ml of the mobile phase were eluted, the centrifuge run was stopped, and the column contents were slowly purged with nitrogen gas to collect the peak fractions retained in the column. The column was then washed with methanol and dried with nitrogen.

The HPLC separations were performed in a gradient-elution mode using methanol and water. The detailed gradient patterns are indicated under the captions of Figs. 2 and 4. The analytical columns were eluted at a flow-rate of 1 ml/min and the semi-preparative column at 4 ml/min. The effluent was monitored at 254 nm in all cases.

RESULTS AND DISCUSSION

CPC separation

According to the previous studies [12], the oxytropis species contains flavonoids which can be isolated by a solvent system composed of chloroform–methanol–water. The present study was focused on isolation of flavonoid glycosides with higher polarity, which required modifying the polarity of the above ternary solvent system by addition of ethyl acetate.

Fig. 1 shows a typical chromatogram of the crude extract of *O. ochrocephala* B. obtained with the CPC method. The separation was performed with a quaternary two-phase solvent system composed of chloroform–ethyl acetate–methanol–water (2:4:1:4, v/v) using the lower non-aqueous phase as the mobile phase. Multiple peaks were eluted in increasing order of polarity and six fractions corresponding to the main peaks, labeled 1–6 in the chromatogram, were manually collected and subjected to HPLC analysis.

HPLC separations

Because polar compounds are most conveniently separated by reversed-phase HPLC, all HPLC separations in the present study were performed with reversed-phase columns. Fig. 2 illustrates an analytical chromatogram of the crude *O. ochrocephala B.* extract obtained by a gradient elution of methanol in water using a μBondapack C_{18} column. Because of the reversed-phase HPLC, peaks were eluted in decreasing order of polarity in contrast to the CPC separation above. Among many compounds present in the crude extract, only two components corresponding to peaks 3 and 4 are obtained in relatively high concentrations.

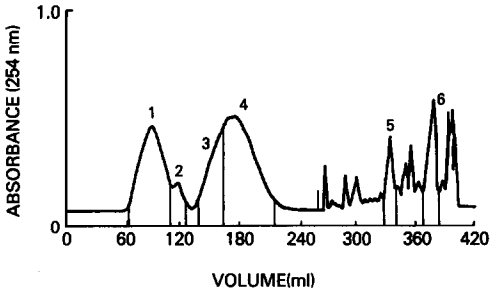

Fig. 1. CPC separation of ethanol extract of *O. ochrocephala* B. Experimental conditions: apparatus, horizontal coil planet centrifuge with 14 cm revolution radius, four coiled columns of 1.6 mm I.D. and 0.3 mm wall thickness and 120 ml capacity; sample size, 60 mg; solvent system, chloroform–ethyl acetate–methanol–water (2:4:1:4, v/v); mobile phase, lower non-aqueous phase; flow-rate, 1 ml/min; revolution, 280 rpm, detection at 254 nm.

Fig. 3 shows a semi-preparative separation of the same sample similarly obtained in a preparative mode using a 10-mm I.D. column operated under overloaded conditions with a 3-mg sample size. Four peak fractions indicated in Fig. 3 were manually collected, concentrated by evaporation in vacuum and subjected to HPLC analysis.

Comparison of separation efficiencies of CPC and HPLC

As described above, peak fractions obtained from the preparative separations (Figs. 1 and 3) were analyzed with HPLC under the same conditions applied to the separation shown in Fig. 2. The qualitative analyses revealed that the compounds found in CPC fractions 1, 3 and 5 (Fig. 1) correspond to those found in HPLC fractions 4, 3 and 1 (Figs. 2 and 3), respectively. It can be seen directly from Figs. 1, 2 and 3 that HPLC has a higher column efficiency, shorter separation time, but lower sample

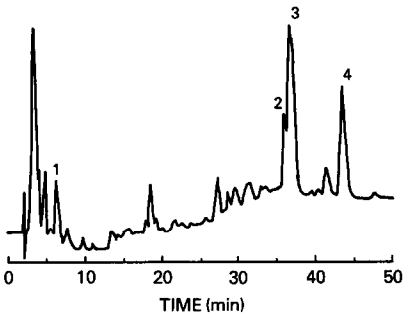

Fig. 2. HPLC analysis of ethanol extract of *O. ochrocephala* B. Experimental conditions: column, μBondapak C_{18} (300 × 3.9 mm I.D.), 10 μm; Mobile phase, water–methanol: initial composition, 100:0; changed between 0 and 10 min to 90:10; between 10 and 15 min to 80:20; between 15 and 30 min to 50:50; between 30 and 50 min to 30:70. Flow-rates, 1 ml/min; detection, HP-1040M photodiode array detector, 254 nm.

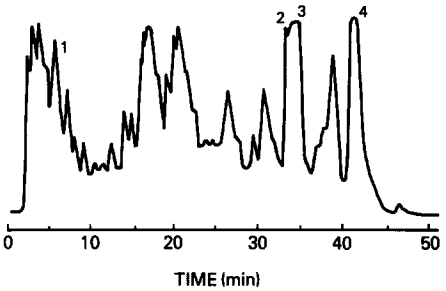

Fig. 3. Semi-preparative HPLC separation of ethanol extract of *O. ochrocephala* B. Experimental conditions: column, LiChrosorb RP-18 (200 × 10 mm I.D.), 10 μm; flow-rate, 4 ml/min; other conditions as in Fig. 2.

loading capacity. As indicated in Figs. 2 and 3, once the analytical HPLC conditions are optimized, the separations can be performed on a much larger scale with the semi-preparative column. However, the sample loading capacity of the semi-preparative column is still limited to a milligram range where further increase of the sample size would cause substantial loss in peak resolution.

In contrast, CPC has a lower column efficiency, requires a longer separation time, but provides a much greater sample loading capacity. Although the column efficiency of CPC is lower than that in the semi-preparative HPLC column, the method is still suitable for the separation of polar compounds such as those corresponding to peaks 1 and 3 in Fig. 1. Disadvantage of the longer separation time of CPC can be compensated by its larger sample loading capacity. For example, the CPC separation time in Fig. 1 is five times that of HPLC in Fig. 3, while the sample size in Fig. 1 is 20 times that applied in Fig. 3. The elution profile of the desired peaks in Fig. 1 suggests that the sample size may be further increased without a detrimental loss in peak resolution.

In order to compare the efficiency in preparative separations between CPC and semi-preparative HPLC, the purities of the peak fractions obtained from these two methods were quantitatively determined by analytical HPLC. The results are summarized in Fig. 4A–F where chromatograms on the left (I) were obtained from CPC fractions 1–6, and those on the right (II) were from semi-preparative HPLC fractions 4–1. In order to facilitate the comparison, these chromatograms are arranged from A to F in such a way that each matched pair represents analysis of the same main compound separated by the two methods. It can be seen that purities of the compound separated by CPC and HPLC are quite similar in Fig. 4A, but CPC yields substantially higher purity in Fig. 4C. Fig. 4E further indicates that the more polar compound in CPC fraction 5 shows a much higher purity than the same compound in semi-preparative HPLC fraction 1. The most polar compounds in CPC fraction 6 (Fig. 4F) contains a large amount of impurities, but it would neither be well separated by semi-preparative HPLC, as suggested from the elution profile in the chromatogram (Fig. 3).

Overall results of the above comparative studies on the preparative performance of CPC and HPLC indicate that CPC requires a longer separation time, but it has important advantages of yielding higher purity of fractions and providing a much

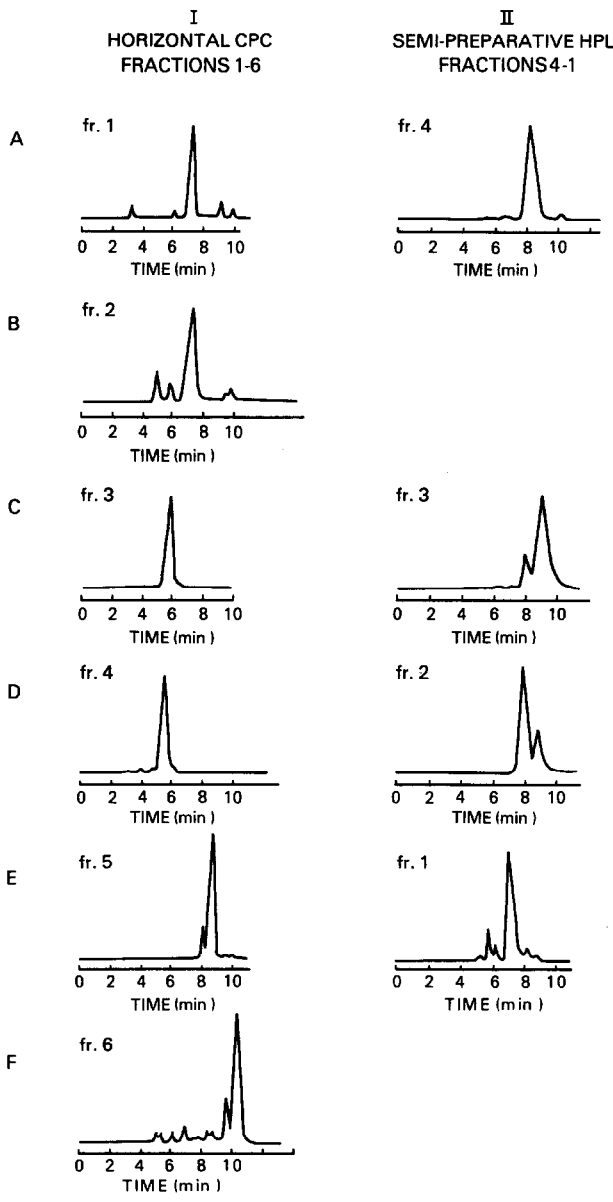

Fig. 4. HPLC analyses of peak fractions obtained from CPC (I) and semi-preparative HPLC (II). Fractions (fr.) 1–6 in I (left column) are corresponding to peak fractions 1–6 in Fig. 1 and fractions 4–1 in II (right column) to peak fractions 4–1 in Fig. 3. Matched pairs of chromatograms in A, C, D and E indicate analysis of the same components separated by the two different methods. Experimental conditions: I. Column: Herpsil ODS (100 × 2.1 mm I.D.), 5 μm; mobile phase: water–methanol: (A–D) initial composition 54:46; changed between 0 and 10 min to 32:68; (E,F) 95:5; otherwise same as described in Fig. 2. II. Mobile phase: water–methanol: (E) initial composition 100:0; changed between 0 and 10 min to 90:10; (C,D) 60:40; (A) initial composition 60:40; changed between 0 and 20 min to 40:60; other conditions are same as in Fig. 3.

greater sample loading capacity. These findings suggest that the isolation of natural products may be efficiently performed in two steps: CPC is used first to clean up the crude extract and HPLC is used next to effect the final purification of the desired compounds. Thus, the combined use of these two methods can be advantageous.

ACKNOWLEDGEMENTS

We are deeply indebted to Professor Lu Wan-Zhen and Dr. Long Yicheng for HPLC analysis of CPC fractions (Fig. 4-I) and to Hewlett-Packard for providing us an HP-1090M HPLC system. We also thank Dr. Henry M. Fales for his valuable suggestions in preparing the manuscript.

REFERENCES

1 K. Hostettmann, M. Hostettmann and A. Marston, *Preparative Chromatography Techniques —Applications in Natural Product Isolation*, Springer, Berlin, Heidelberg, 1986, p. 56.
2 N. B. Mandava and Y. Ito (Editors), *Countercurrent Chromatography —Theory and Practice*, Marcel Dekker, New York, 1988.
3 Y. Ito, *Trends Biochem. Sci.*, 7 (1982) 47.
4 T.-Y. Zhang, *J. Chromatogr.*, 315 (1984) 287.
5 Y. Ito, *CRC Crit. Rev. Anal. Chem.*, 17 (1986) 65.
6 T.-Y. Zhang, R. Xiao, Z.-Y. Xiao and Y. Ito, *J. Chromatogr.*, 445 (1988) 199.
7 T.-Y. Zhang, D.-G. Cai and Y. Ito, *J. Chromatogr.*, 435 (1988) 159.
8 T.-Y. Zhang, L. K. Pannell, Q.-L. Pu, D.-G. Cai and Y. Ito, *J. Chromatogr.*, 442 (1988) 455.
9 T.-Y. Zhang, X. Hua, R. Xiao and S. Kong, *J. Liq. Chromatogr.*, 11 (1988) 233.
10 T.-Y. Zhang, L. K. Pannell, D.-G. Cai and Y. Ito, *J. Liq. Chromatogr.*, 11 (1988) 1661.
11 P. Li, R. Zhang, W. Yu and B. Zhou, *Tsu Wu Tsu Zhu*, in press.
12 D.-L. Cheng, R.-Q. Sun, X.-R. Li, P.-X. Zou and Z.-Q. Zhu, *Acta Bot. Sin.*, 28 (1986) 404.