Separation of Cyclosporins and Other Antibiotics by HSCCC

Wei Zheng*
Fujian Provincial Key Laboratory of Screening for Novel Microbial Products, Fujian Institute of Microbiology, Fuzhou, P.R. China, 350007

Abstract

Applications of high-speed counter-current chromatography (HSCCC) for the separations of antibiotics, cyclosporins and isoflavones, by a marine *Micromonospora* and macrolides including tacrolimus, ascomycin, and dihydroFk-506 are described. The application of silver ion HSCCC technique in the separation of tacrolimus from its components is also described.

Introduction

Antibiotics are secondary metabolites produced by various microorganisms (1). The chemical novelty and diversity of these metabolites always challenge modern separation technologies to isolate and purify them from the fermentation broth in efficient and economical ways. In the past decades, important advances have been achieved in modern separation technologies, including high-performance liquid chromatography (HPLC) and counter-current chromatography (CCC) (2). Unlike HPLC and other conventional column chromatography methods, CCC is a kind of liquid–liquid partition chromatography without any solid matrix, which eliminates irreversible adsorption of samples on solid supports (3). High-speed CCC (HSCCC), developed from CCC in the 1980's, can achieve a highly efficient separation in a short period of time. These advantages make HSCCC a useful technique in the separation of natural products, including antibiotics (4–6).

In 1998, in his review, Ito described the development of CCC including HSCCC, and its applications in the separation of more then 40 antibiotics with different kinds of chemical structures (5). Besides that, there are many other reports about the separation of antibiotics by CCC, such as herbicidins (7), nivalenol and fusaren-X (8), altromycins (9), elaiophylin and geldanamycin (10), and SCH 42282 (11); and by HSCCC, such as cephalosporin C and desacetyl cephalosporin C (12), tyrosine kinase inhibitors emodic acid, chartreusin, and three benzoic acid derivatives (13), asterriquinones (14), spiramycin components (15), ascomycin and related macrolodes (16,17), lutein (18), WAP-8294A compoents (19), coenzyme Q10 (20), and deoxynivalenol (21).

China is one of the pioneers in the development of CCC and HSCCC; the first CCC apparatus in China was made as early as in 1980 and was used successfully in the separation of traditional Chinese medicine and antibiotics (22,23). Shanghai Tauto Biotech Co., Ltd. is a professional HSCCC manufacturer and Tauto's HSCCC have been widely used in the separation processes for different purposes.

The HSCCC instrument employed in the following experiments is a TBE-300A HSCCC (Tauto Biotech, Shanghai, China) with three polytetra- fluoroethylene preparative coils (i.d. of the tubing = 2.6 mm, total volume = 300 mL) and a 20-mL sample loop. The revolution radius is 5 cm and the β-values of the multilayer coil vary from 0.5 at internal terminal to 0.8 at the external terminal. A HX 1050 constant-temperature circulating implement (Beijing Boyikang Lab Instrument, Beijing, China) was used to control the temperature. The solvent was pumped into the column with a Tauto TBP-50A pump (Tauto Biotech, Shanghai). The effluent was detected on-line at 254 nm with a Model 8823B UV detector (Beijing BINTA Instrument Technology Factory, Beijing, China). A Model N2000 chromatography data system (Zhejiang University Zhida Information Engineering Factory, Beijing, China) was used to record the chromatograms.

The HPLC used was an LC-2010A HT system (Shimadzu Corporation, Kyoto, Japan), which consisted of a quaternary low pressure gradient pump, auto-sampler, on-line degasser, block heating-type column oven, a SPD-10AVvp (PDA) detector, and a Shimadzu LC-solution workstation for data collection.

Experimental

HSCCC separation of cyclosporins

Cyclosporins are cyclic undecapeptides produced by fungi; the major analogue, cyclosporin A (CsA, Figure 1), was first developed by Sandoz and has been widely used clinically to prevent rejection after an organ transplantation (24). Now, hundreds of natural and synthetic cyclosporin analogues and derivatives have been reported, which necessitates efficient separation methods to purify them. The purifications of natural cyclosporins were originally based on column chromatography with different packing materials such as silica gel, Sephadex LH-20, and LiChroprep RP-18 (25). By silica gel column chromatography, cyclosporin A was purified and marketed in 1983. In China, cyclosporine A (CsA) was first developed in Fujian Institute of Microbiology and marketed in 1993; besides CsA, cyclosporin B (CsB), C (CsC), D (CsD), and H (CsH) were also separated and purified by silica gel column chromatography from the crude extract of *Fusarium solani* sp. No. 4–11 is a different CsA-producing strain than *Beauveria bassiana,* used in Sandoz (26–29). However, based on our experimental experiences, these column chromatographic separations

* Author to whom correspondence should be addressed.

Reproduction (photocopying) of editorial content of this journal is prohibited without publisher's permission.

are usually time-consuming processes, and it is difficult to obtain pure minor components from cyclosporin's crude extracts. Therefore, more efficient and economical ways were sought after, and HSCCC has provn to be a good technology, in addition to column chromatography, in the separation and purification of natural and semi-synthetic cyclosporins.

Unlike column chromatography, HSCCC is a technique based on the liquid–liquid partition principle. Therefore, the selection of a suitable two-phase solvent system is the decisive factor for the successful separation of target products in HSCCC. Usually, the criteria described here should be considered for a good separation: (*i*) setting time of the solvent systems should be 30 s or less to keep a desirable retention of the stationary phase ($\geq 50\%$); (*ii*) each phase should have, approximately, an equal volume so as to avoid excessive waste of solvent; (*iii*) the partition coefficient (K) of the target compounds should be close to 1, and the separation factors between two components ($\alpha = K_2/K_1$, $K_2 > K_1$) should be larger than 1.5 (4,6). Following the described criteria and using HPLC for the measurements of partition coefficient and separation factors as well as analysis of products, HSCCC was run for the separation of cyclosporins.

In the separation of a mixture (200 mg) of cyclosporin A, B, C, and D, a solvent system of petroleum ether–acetone–water (3:3:6, v/v/v) was chosen with 78% of retention of the stationary phase and K values of 1.4, 0.6, 0.2, and 6.5, respectively. After 8 h running at 600 rpm speed by selecting the lower phase as the mobile phase, these four cyclosporins were separated successfully in 450 min with high purities and good recoveries (30).

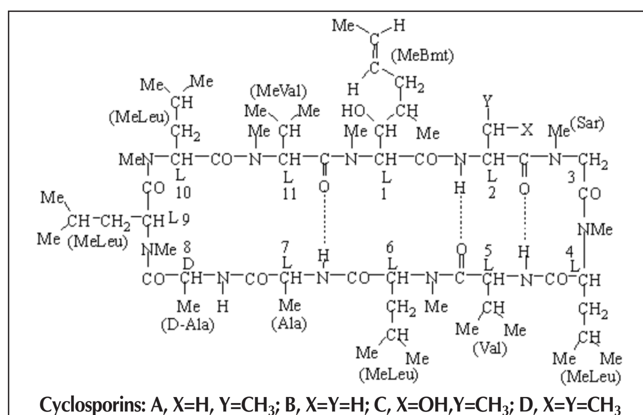

Figure 1. Chemical structures of cyclosporins.

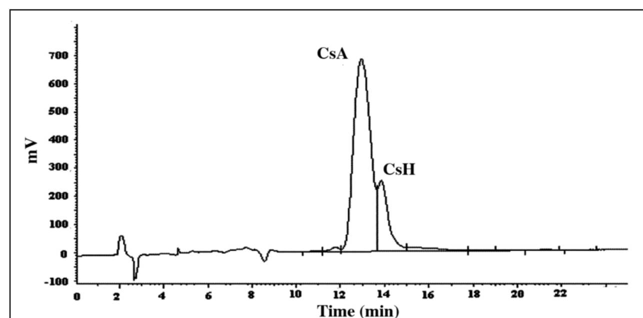

Figure 2. HPLC analysis of mixture of CsA and CsH. Conditions: column, Nucleosil C18 column (250 mm × 4.6 mm i.d : 5 µm); elutant, acetonitrile–ethyl ether–water–formic acid (450:130:415:1, v/v/v/v); flow rate, 1.0 mL/min; temperature, 60°C; detecting wavelength, 210 nm.

CsH is another CsA analogue; the only difference between CsH and CsA is the configuration of amino acid residue at position 11. The amino acid residue at position 11 in CsH is *N*-methyl-D-valine instead of *N*-methyl-L-valine as in CsA (31). The chemical structural similarity between CsH and CsA makes the separation difficult by column chromatography. In our unpublished experiment, HSCCC separation of CsA and CsH (Figure 3) from their mixture (Figure 2) was completed successfully by modifying the process described earlier, which involved three major aspects: (*i*) reducing the sample load from 200 mg to 100 mg; (*ii*) increasing the rotation speed of the column from 650 rpm to 850 rpm; (*iii*) reducing the flow-rate of the mobile phase from 2.0 mL/min to 1.0 mL/min.

Combination with other techniques in the separation of cyclosporins

Although HSCCC was successful for the separation of the major cyclosporin analogues such as CsA, CsB, CsC, CsD, and CsH, it can not always be used alone to separate all the cyclosporins, especially the minor cyclosporin components. So the combination of HSCCC and other separation techniques, such as column chromatography, is usually a good choice. A good example of combination was reported for the separation of destruxin A-, B-, D-, E-, and E-diol (32). In the mother liquid of CsA crystallization, there are some minor components that can not be separated and purified by HSCCC alone; so a technique using a Mitsubishi DIAION HP20 macroporous adsorptive resin column for chromatography combined with HSCCC was applied. By resin column chromatography, the minor components were enriched and decolored; the enriched fractions were then purified by HSCCC to obtain the pure compounds. In one experiment, two components were enriched after resin column chromatography and purified by HSCCC with two modified solvent systems: *n*-hexane–ethyl acetate–methanol–water (5:6:6:5, v/v/v/v) for component 1 and petroleum ether–methanol–acetone–water (3:1:3:2, v/v/v/v) for component 2, respectively (33). Comparative analyses of mass spectra and HPLC of these two products and standard samples indicated that component 2 was identical with cyclosporin E ([Val^{11}]CsA), which has the amino acid residue of valine in position 11 instead of methyl valine in CsA. Component 1 was identical with [Leu^{4}]CsA, which

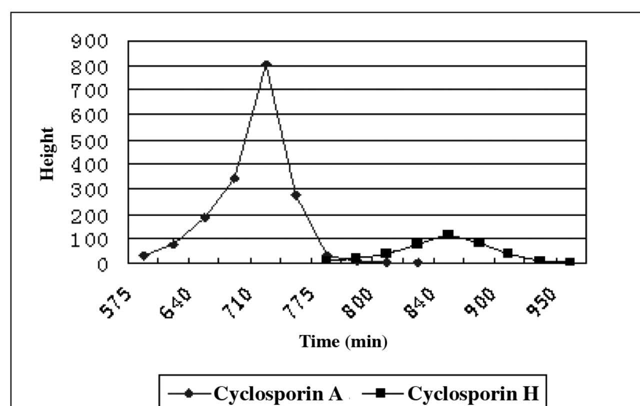

Figure 3. HSCCC separation process of CsA and CsH. Conditions: solvent system, petroleum ether-acetone–water (3:3:2, v/v/v); sample loading, 100 mg; rotation speed, 850 rpm; flow-rate of mobile phase, 1.0 mL/min.

was first discovered in the fermentation broth of *Tolypocladium terricola*, another cyclosporins-producing strain belonging to fungi (34). The fact that *Fusarium solani* sp. No. 4–11 can produce the same cyclosporins, such as CsA, CsB, and CsE, as that of *Beauveria bassiana* (as well as $[Leu_4]$CsA as in *Tolypocladium terricola*) prove again that different cyclosporins-producing fungi can produce same components as well as different components (35).

In another unpublished experiment, two minor components were detected in the mother liquid of CsA crystallization; the molecular weight of component A is 1187.6 and that of component B is 1184.1. The mother liquid, treated by flocculating, was loaded, and HP20 macroporous adsorptive resin column chromatography was run with acetone–water as the elutant. Components A and B were separated, and the contents of A and B in their enriched fractions were increased from 26.0% and 8.3% (Figure 4) to 60.9% and 13.8% (Figure 5), respectively. Then, the enriched fraction of component A was purified by HSCCC with the same conditions as those in the separation of a mixture of cyclosporin A, B, C, and D. Component 1 was obtained with 99.0% purity and proved to be cyclosporine L ($[Bmt_1]$CsA) after identification by modern spectrometric methods. Unfortunately, the HSCCC trials for the purification of component B failed, although many modifications have been tried. As an alternative choice, preparative HPLC was run successfully for purification, and component B was obtained with 93.0% purity; the experimental date showed that it could be attributed to dehydrated CsA or dehydrated CsH. This experiment also indicated that HSCCC is not the only choice for the purifications of all natural products, such as cyclosporins, even though most of the components can be purified by this technique.

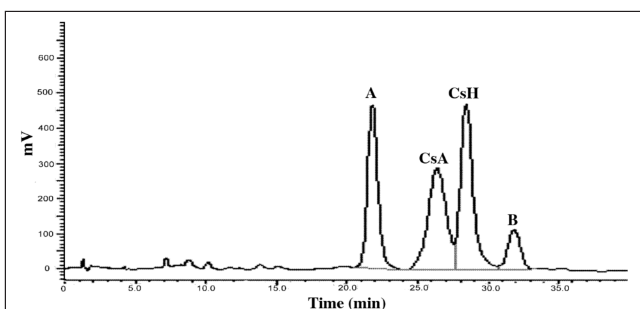

Figure 4. HPLC analysis of cyclosporins before resin column chromatography. Retention times, component A, 22.0 min; CsA, 26.5 min; CsH, 28.7 min; component B, 32.0 min. conditions: column, Nucleosil C18 (250 mm × 4.6 mm i.d., 5 µm); elutant, methanol–water (80:20, v/v); flow rate, 1.0 mL/min; temperature, 60°C; detecting wavelength, 210 nm.

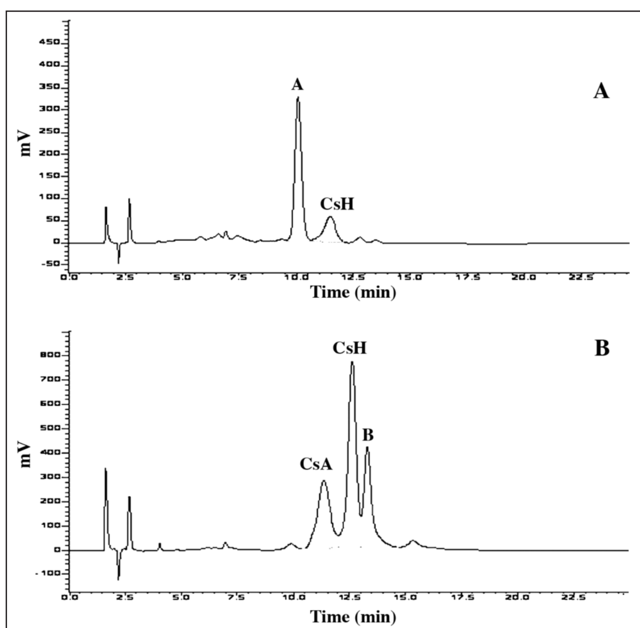

Figure 5. HPLC analysis of cyclosporins after resin column chromatography. Conditions: column, Nucleosil C18 (250 mm × 4.6 mm i.d., 5 µm); elutant, acetonitrile–ethyl ether–water–formic acid (450:130:415:1, v/v/v/v); flow rate, 1.0 mL/min; temperature, 60°C; detecting wavelength, 210 nm.

HSCCC separation of isoflavones by a *Micromonospora* of marine source

In the course of screening for novel immunosuppressive agents from marine *Micromonospora*, two bioactive compounds were targeted in the culture broth of a marine *Micromonospora* sp, FIM 02-635. After extraction and silica gel column chromatography, crude light yellow powder containing these two compounds was obtained (Figure 6). Because of the close retention times of target compounds 1 (FW635I-1) and 2 (FW635I-2) in HPLC, preparative HPLC separation trials for these two compounds failed. Finally, HSCCC was run successfully to get pure FW635I-1 and FW635I-2, respectively (Figure 7), by selecting a solvent system of chloroform–methanol–water (4:3:2, v/v/v) and a lower phase as mobile phase, with rotation speed of 800 rpm. Physico-chemical experiments proved that FW635I-1 and FW635I-2 were identical to daidzein and genistein, respectively (Figure 8); these two isoflavones usually originate from plants, especially from the soy plant (36).

HSCCC separation of tacrolimus

FK-506 (tacrolimus) is an important and widely used immunosuppressive agent; the FK-506-producing strains usually also produce other minor components such as ascomycin and dihydroFK-506 (Figure 9) (37). Because of the chemical structural similarity of these three macrolides, separation of FK-506 from the other two components is a challenging process. Besides column chromatography (38), the application of HSCCC in the separation and purification of FK-506 from its components has also been reported, but the results were not encouraging (16,17). In our unpublished experiments, the dif-

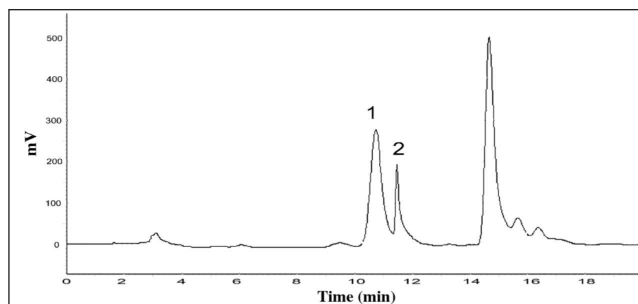

Figure 6. HPLC analysis of crude powder containing FW635I-1 (1) and FW635I-2 (2). Retention times: FW635I-1(1), 10.8 min; FW635I-2(2), 11.4 min. Conditions: column, Nucleosil ODS-C18 (250 mm × 4.6 mm i.d., 5 µm); temperature, 50°C; elutant, water–methanol (6:4, v/v); flow rate, 1.0 mL/min; detecting wavelength, 250 nm.

ferent R groups in FK-506 and its components attracted our attention. The R groups in ascomycin and dihydroFK-506 belong to the alkanes, while the R group in FK-506 belongs to alkene. Therefore, it is possible to separate them according to this slight structural difference.

It is well known that silver ion can form a π complex with double bonds of olefins. Based on this phenomenon, silver ion chromatography was developed and has been applied in thin-layer, column, high-performance liquid, and supercritical fluid chromatography (39). There are also patents about the separation of FK-506 from ascomycin and dihydroFK-506 by silver ion column chromatography. In the experimental examples of these patents, FK-506 can form a tight complex with silver ion by its olefinic R group; ascomycin and dihydroFK-506 can not form such a complex because of their non-olefinic R groups. Because of this, FK-506 was separated successfully from ascomycin and dihydroFK-506 (40–42).

In one of our experiments with silver ion HSCCC, separating FK-506 in the crude extract containing ascomycin and dihydroFK-506 as impurities (Figure 10), a solvent system of petroleum ether–acetone–water (3:3:1, v/v/v) was selected, 0.15 mol/L $AgNO^3$ was added to the lower phase (stationary phase), 80 mg of sample was loaded, and HSCCC was run with rotation speed of 850 rpm and 3.0 mL/min flow-rate of mobile phase (upper phase) at 25°C. After 150 min running with 82% of retention of the stationary phase, ascomycin and dihydroFK-506 flowed out with the mobile phase, and FK-506 remained in the stationary phase because of the π complex with silver ion. The stationary phase was pumped out and combined with methanol to clean the column; after evaporation in vacuum, FK-506 in the concentrated liquid was extracted with ethyl acetate, and silver ion remained in the aqueous phase; after evaporation of ethyl acetate and crystallization, FK-506 with a purity higher than 99.0% was obtained (Figure 10). This experiment proves that silver ion chromatography can also be applied in HSCCC.

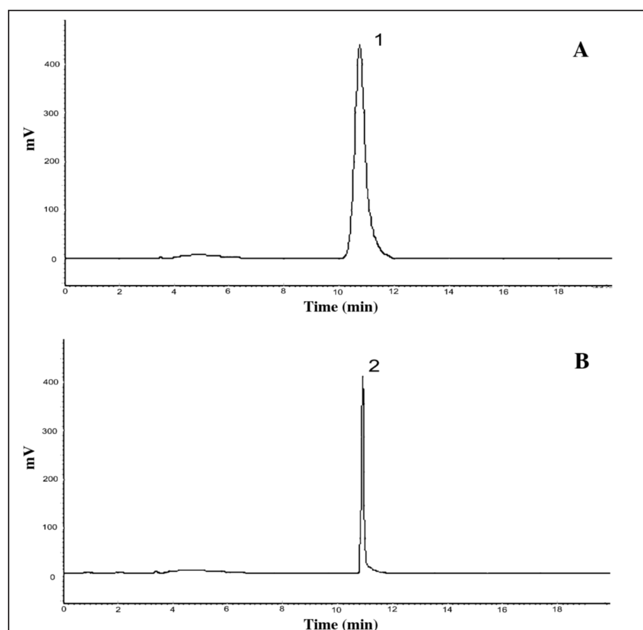

Figure 7. HPLC analysis of FW635I-1 (1) and FW635I-1 (2) after HSCCC separation. HSCCC separation conditions: retention of stationary phase, 82.0%; solvent system, chloroform–methanol–water (4:3:2 v/v/v); stationary phase, upper phase; mobile phase, lower phase; flow-rate, 1 mL/min; rotation speed, 800 rpm; temperature, 25°C; detecting wavelength, 254 nm; sample loading, 80 mg/10 mL.

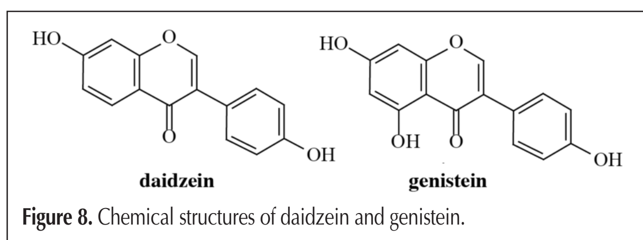

Figure 8. Chemical structures of daidzein and genistein.

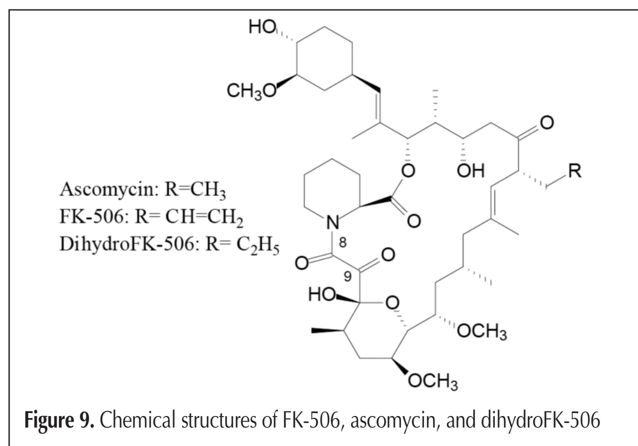

Figure 9. Chemical structures of FK-506, ascomycin, and dihydroFK-506

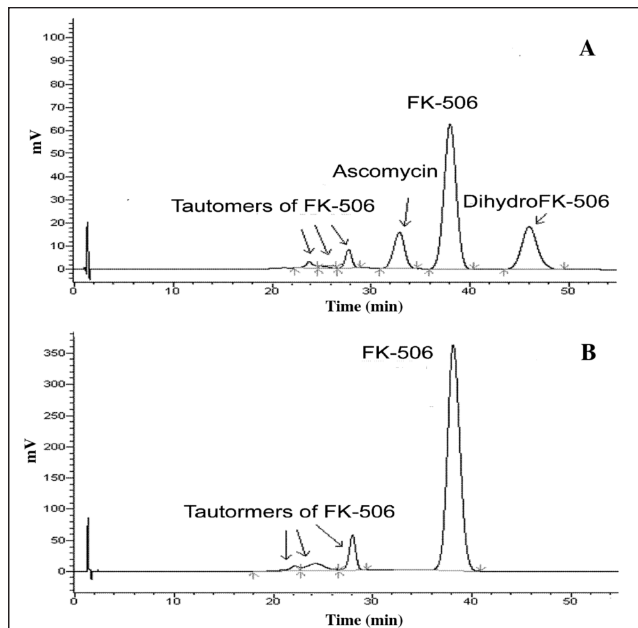

Figure 10. HPLC analysis of FK-506 crude sample (A) and pure sample (B) after silver ion HSCCC. Retention times: tautomer 1, 23.0 min; tautomer 2, 25.1 min; tautomer 3, 27.9 min; ascomycin, 33.1 min; FK506, 38.2 min; dihydroFK-506, 46.0 min. HPLC conditions: column, Kromasil CN (250 mm × 4.6 mm i.d. 5 µm); elutant, acetonitrile–water (30:70, v/v); flow rate, 1.5 mL/min; temperature, 40°C; detecting wavelength, 210 nm. HSCCC separation conditions: retention of stationary phase, 82.0%; solvent system, petroleum ether–acetone–water (3:3:1, v/v/v); stationary phase, lower phase; mobile phase, upper phase; flow-rate, 3.0 mL/min; rotation speed, 850 rpm; temperature, 25°C; detecting wavelength, 254 nm; sample loading, 80 mg/10 mL.

Acknowledgments

The author is indebted to Yaoming Wen for technical assistance.

References

1. J. Berdy. Bioactive microbial metabolites. *J. Antibiot.* **58:** 1–26 (2005).
2. A. Marston and K. Hostettmann. Modern separation methods. *Nat. Prod. Rep.* **8:** 391–413 (1991).
3. Y. Ito. Recent advances in counter-current chromatography. *J. Chromatogr. A* **538:** 3–25 (1991).
4. A.P. Foucault and L. Chevolot. Counter-current chromatography: instrumentation, solvent selection and some recent applications to natural product purification. *J. Chromatogr. A* **808:** 3–22 (1998).
5. H. Oka, K-I. Harada, Y. Ito, and Y. Ito. Separation of antibiotics by counter-current chromatography. *J. Chromatogr. A* **812:** 35–52 (1998).
6. Y. Ito. Golden rules and pitfalls in selecting optimum conditions for high-speed counter-current chromatography. *J. Chromatogr. A* **1065:** 145–68 (2005).
7. T. Haneish, A. Terahara, H. Kayamori, J. Yabe, and M. Arai. Herbicidins A and B, two new antibiotics with herbicidal activity II. Fermentation, isolation and physico-chemical characterization. *J. Antibiot.* **29:** 870–75 (1976).
8. Y. Onju, Y. Aoki, Y. Yamazoe, Y. Doki, and T. Moriyama. Isolation of nivalenol and fusarenon-X from pressed barley culture by centrifugal partition chromatography. *J. Liq. Chromatogr. Rel. Technol.* **11:** 2537–46 (1988).
9. G.M. Brill, J.B. McAlpine, D.N. Whittern, and A.M. Buko. Altromycin, novel pluramycin-like antibiotics. II. Isolation and elucidation of structure. *J. Antibiot.* **43:** 229–37 (1990).
10. K.A. Alvi, J. Peterson, and B. Hofmann. Rapid identification of elaiophylin and geldanamycin in *Streptomyces* fermentation broths using CPC coupled with a photodiode array detector and LC-MS methodologies. *J. Ind. Microbiol.* **15:** 80–84 (1995).
11. V.R. Hegde, M.G. Patel, A.C. Horan, A.H. King, F. Gentile, M.S. Puar, and D. Loebenberg. A novel macrolactam-trisaccharide antibiotic. Taxonomy, fermentation, isolation, physico-chemical properties, structure elucidation and biological activity. *J. Antibiot.* **51:** 464–70 (1998).
12. P.-C. Lin and I.-M. Chu. Separation of cephalosporin C and desacetyl cephalosporin C by high speed counter-current chromatography in aqueous two-phase systems. *Biotechnol. Tech.* **9:** 549–52 (1995).
13. K. A. Alvi, B. Nair, C. Gallo, and D. Baker. Screening of microbial extracts for tyrosine kinase inhibitors. *J. Antibiot.* **50:** 264–66 (1997).
14. K. A. Alvi, H. Pu, M. Luche, A. Rice, H. App, G. McMahon, H. Dare, and B. Margolis. Asterriquinones produced by *Aspergillm candidus* inhibit binding of the Grb-2 adapter to phosphorylated EGF receptor tyrosine kinase. *J. Antibiot.* **52:** 215–23 (1999).
15. H. Oka, K. Harada, M. Suzuki, and Y. Ito. Separation of spiramycin components using high-speed counter-current chromatography. *J. Chromatogr. A* **903:** 93–98 (2000).
16. W. Wang-Fan, E. Küsters, O. Lohse, C-P. Mak, and Y. Wang. Application of centrifugal counter-current chromatography to the separation of macrolide antibiotic analogues: I. Selection of solvent systems based on solubility and partition coefficient investigations. *J. Chromatogr. A* **864:** 69–76 (1999).
17. W. Wang-Fan, E. Küsters, C.P. Mak, and Y. Wang. Application of coil centrifugal counter-current chromatography to the separation of macrolide antibiotic analogues: III. Effects of flow-rate, mass load and rotation speed on the peak resolution. *J. Chromatogr. A* **925:** 139–49 (2001).
18. H.-B. Li, F. Chen, T.-Y. Zhang, F.-Q. Yang, and G.-Q. Xu. Preparative isolation and purification of lutein from the microalga *Chlorella vulgaris* by high-speed counter-current chromatography. *J. Chromatogr. A* **905:** 151–55 (2001).
19. K.-I. Harada, M. Suzuki, A. Kato, K. Fujii, H. Oka, and Y. Ito. Separation of WAP-8294A components, a novel anti-methicillin-resistant *Staphylococcus aureus* antibiotic, using high-speed counter-current chromatography. *J. Chromatogr. A* **932:** 75–81 (2001).
20. X.-L. Cao, Y.-T. Xu, G.-M. Zhang, S.-M. Xie, Y.-M. Dong, and Y. Ito. Purification of coenzyme Q10 from fermentation extract: High-speed counter-current chromatography versus silica gel column chromatography. *J. Chromatogr. A* **1127:** 92–96 (2006).
21. J.-W. He, R. Yang, T. Zhou, R. Tsao, J.C. Young, H.-H. Zhu, X.-Z. Li, and G.J. Boland. Purification of deoxynivalenol from *Fusarium graminearum* rice culture and mouldy corn by high-speed counter-current chromatography. *J. Chromatogr. A* **1151:** 187–92 (2007).
22. Y. Wei and X.-L. Cao. Peered separation science technology-countercurrent chromatography technique. *World Sci. Technol. Moder. Trad. Chin. Med.* **3:** 17–22 (2001).
23. R.-Y. Bi, Y.-M. Kong, F.-J. Yu, and Y.-Z. Sheng. Analysis and identification of Kangdisu. *Kangshensu* **6:** 370–82 (1982).
24. R. Kunz and H.-H. Neumayer. Maintenance therapy with triple versus double immunosuppressive regimen in renal transplantation: A meta-analysis. *Transplantation* **63:** 386–92 (1997).
25. A. von Wartburg and R. Traber. Cyclosporins, fungal metabolites with immunosuppressive activities. *Prog. Med. Chem.* **25:** 1–33 (1988).
26. X.-D. Tang, L.-N. Hong, C.-C. Miao, and Y. Wang. Studies on cyclosporin produced by *Fusarium solani* sp.No. 4-11 and its biological characterization. *Kangshensu.* **1:** 1–4 (1984).
27. J.-R. Fang, L.-Y. Ren, X.-D. Tang, Q. Lin, and Y.-H. Du. Isolation and identification of cyclosporine D. *Kangshensu* **4:** 279–82 (1987).
28. J.-R. Fang, Y-F. Shi, and B-S. Yu. Isolation and structural assignment of cyclosporine B and C produced by *Fusarium solani* sp. No. 4-11. *Chin. J. Antibiot.* **3:** 197–201 (1989).
29. W. Zheng, J.M. Zhuo, Q.X. Wen, and J. Zhang. Cyclosporin H produced by *Fusarium solani*: purification and structural identification. *Chin. J. Antibiot.* **6:** 417–19 (2001).
30. D-S. Fang, Y. Xie, Y. Chen, X.M. Chen, and W. Zheng. Separation of cyclosporins by high speed counter current chromatography. *Chin. J. Antibiot.* **1:** 48–51 (2005).
31. R. Traber, H. R. Loosli, H. Hofmann, M. Kuhn, and A. von Wartburg. Isolierung und Strukturermitt-lung der neuen Cyclosporine E, F, G, H und I. Helv. *Chim. Acta* **65:** 1655–77 (1982).
32. C. Seger, K. Eberhart, S. Sturm, H. Strasser, and H. Stuppner. Apolar chromatography on Sephadex LH-20 combined with high-speed counter-current chromatography: high yield strategy for structurally closely related analytes—Destruxin derivatives from *Metarhizium anisopliae* as a case study. *J. Chromatogr. A* **1117:** 67–73 (2006).
33. X.M. Chen, J.Y. Fang, Z.W. Chen, Y-M. Wen, and W. Zheng. Separation and purification of cyclosporin minor components by macroporous adsorption resin and high speed countercurrent chromatography. *Chin. J. Antibiot.* **5:** 276–79 (2008).
34. A. Jegorov, V. Maha, P. Sedmera, V. Havlíek, J. Stuchlík, P. Seidel, and P. Imek. Cyclosporins from *Tolypocladium terricola*. *Phytochemistry.* **38:** 403–407 (1995).
35. R. Traber and M.M. Dreyfuss. Occurrence of cyclosporins and cyclosporin-like peptolides in fungi. *J. Ind. Microbiol.* **17:** 397–401 (1996).
36. H. Jiang, Y.R. Cheng, and W. Zheng. Daidzein and genistein produced by a marine *Micromonospora carbonacea* FIM 02-635. *Chin. J. Mar. Drugs* **26:** 8–12 (2007).
37. P.E. Wallemacq and R. Reding. FK506 (tacrolimus), a novel immunosuppressant in organ transplantation: clinical, biomedical, and analytical aspects. *Clin. Chem.* **39:** 2219–28 (1993).
38. T. Kino, H. Hatanak, M. Hashimoto, M. Nishiyama, T. Goto, M. Okuhara, M. Kohsaka, H. Aoki, and H. Imanaka. FK-506, A novel immunosuppressant isolated from a *Streptomyces* I. Fermentation, isolation, and physico-chemical and biological characteristics. *J. Antibiot.* **40:** 1249–55 (1987).
39. G. Dobson, W.W. Christie, and B. Nikolova-Damyanova. Silver ion chromatography of lipids and fatty acids. *J. Chromatogr. B: Biomed. Appl.* **671:** 197–222 (1995).
40. C. Ladislav, B. Martin, J. Alexandr, B. Pavel, K. Vilmos, C. Andrea, S. Angela, and M. Gyorgyne. U. S. Patent 20080000834 (2008).
41. C. Walter, P. Paolo, R. Jacopo, and M. Luca. U.S. Patent 20080160586 (2008).
42. M. Luca, R. Jacopo, L. Giovanni, C. Walter, and P. Paolo. U.S. Patent 20080161555 (2008).

Manuscript received October 30, 2008;
revision received December 20, 2008.