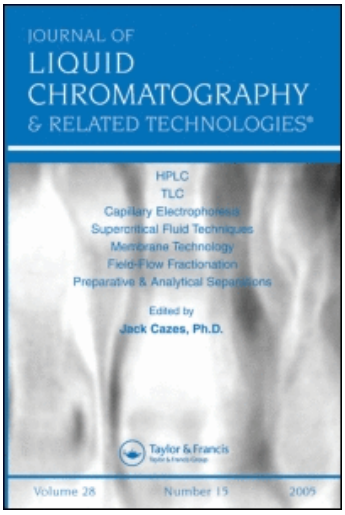


Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

High-Speed Countercurrent Chromatography Separation of Taxol and Related Diterpenoids from *Taxus Baccata*

R. Vanhaelen-Fastre[a]; B. Diallo[a]; M. Jaziri[a]; M-L. Faes[a]; J. Homes[b]; M. Vanhaelen[a]
[a] Laboratory of Pharmacognosy and Bromatology, Universitelibre de Bruxelles, Brussels, Belgium [b] Laboratory of Plant Morphology, Université Libre de Bruxelles, Brussels, Belgium

To cite this Article Vanhaelen-Fastre, R. , Diallo, B. , Jaziri, M. , Faes, M-L. , Homes, J. and Vanhaelen, M.(1992) 'High-Speed Countercurrent Chromatography Separation of Taxol and Related Diterpenoids from *Taxus Baccata*', Journal of Liquid Chromatography & Related Technologies, 15: 4, 697 — 706
To link to this Article: DOI: 10.1080/10826079208018827
URL: http://dx.doi.org/10.1080/10826079208018827

JOURNAL OF LIQUID CHROMATOGRAPHY, 15(4), 697-706 (1992)

HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY SEPARATION OF TAXOL AND RELATED DITERPENOIDS FROM *TAXUS BACCATA*

R. VANHAELEN-FASTRE[1], B. DIALLO[1], M. JAZIRI[1], M-L. FAES[1], J. HOMES[2], AND M. VANHAELEN[1]*

[1]*Laboratory of Pharmacognosy and Bromatology*
Université Libre de Bruxelles
CP 205-4, Bd du Triomphe
1050 Brussels, Belgium

[2]*Laboratory of Plant Morphology*
Université Libre de Bruxelles
CP 320, Ch. de Wavre, 1850
1160 Brussels, Belgium

ABSTRACT

Taxol and related taxane diterpenoids were isolated from pre-purified extracts of stem-barks of *Taxus bacccata* by HSCCC. The described method allowed to obtain a mixture of taxol and cephalomannine without any other interfering constituent and furthermore a partial separation of taxol (40 % of the total recovery) from cephalomannine. In addition, the HPLC and TLC separation conditions of these compounds for the semi-preparative and analytical purposes were improved. The proposed HSCCC method could be adapted to the scaling-up separation of taxol from yew material.

*Author to whom correspondence should be addressed

INTRODUCTION

Taxol is the first taxane derivative which has been demonstrated to have anticancer activity; it has been considered by the National Cancer Institute (NCI) as one of the highest priority agents at present under evaluation [1]. The results available from Phase II clinical trials show that taxol exhibits high activity against ovarian cancer; significant activity against other cancers could also be expected [1]. The reported yields of taxol from various species of yew (genus *Taxus*, family Taxaceae), a very slowgrowing evergreen, range from 0.004 to 0.016 % of dry weight [2]. The supply of taxol is therefore limited by the availability of plant material and expensive extraction methods [2]. For example, the extreme complexity related to the isolation and purification of this useful drug is such that a chemical industry has recently proposed a 50-step process for this purposes [3]. Regarding the analytical methods, high performance liquid chromatography (HPLC) [4, 5, 6] and multimodal thin-layer chromatography (MTLC) [7] methods have been described for taxol analysis. More recently, an enzyme-linked immunosorbent assay (ELISA) was developed for the semi-quantitative determination of taxol and structurally related compounds in *Taxus* plant and tissue cultures [8].

High-speed countercurrent chromatography (HSCCC) has become a widely used method for the isolation of natural products [9].

The present paper details the application of the HSCCC method to the separation of taxol and related taxane diterpenoids from a crude extract of *Taxus baccata* L. pre-purified by column chromatography (CC).

EXPERIMENTAL

Plant material

Stem-bark samples of *Taxus baccata* L. var. columnaris (Taxaceae) were collected in May 1991 from trees growing in the "Jardin Botanique Expérimental Jean Massart" de l'Université Libre de Bruxelles, B-1160 Bruxelles.

Extraction of the yew stem-barks and pre-purification procedures

Powdered dried stem-barks (280 g) were percolated with a methanol-dichloromethane 1:1 (v/v) mixture (3000 ml). After solvents evaporation, the residue (55 g) was dissolved, first in methanol (350 ml), then water (350 ml) was added. The resulting hydro-methanolic solution was extracted with light petroleum (700 ml), then with dichloromethane (3 X 700 ml). The dichloromethane extractives were dried on anhydrous sodium sulfate, evaporated and the residue (5 g) was absorbed on cellulose 2100 ff (Macherey-Nagel, Düren, Germany) (10 g) and submitted to column chromatography.

Column chromatography

Column chromatography was achieved on a column (25 cm X 30 mm I.D.) filled with silica gel 60 (mean particle size 0.040-0.063 μm) (75 g) suspended in n-hexane. After introduction of the sample fixed on cellulose, the column was eluted with hexane-acetone (75:25 v/v, 1000 ml), then with hexane-acetone (70:30 v/v, 1500 ml) and the effluent was monitored by TLC. The pertinent fractions (*Rf* values closely related to the taxol Rf value) were pooled and evaporated; the residue F1 (300 mg) was further subjected to the HSCCC separation.

Thin-layer chromatography (TLC)

The precoated plates (10 X 20 cm) silica gel 60F254 Merck were developed in an unsatured tank with, either hexane-acetone (1:1 v/v) (solvent S1) either hexane-ethyl acetate (1:1 v/v) (solvent S2), or dichloromethane-methanol (95:5 v/v) (solvent S3). Water-methanol-tetrahydrofuran (5:2:3 v/v) (solvent S4, two successive developments) was used as mobile phase for the development of the precoated RP-18F254s plates (10 X 20 cm) Merck. The detection was achieved under UV at 254 nm then by spraying a 3 % sulfuric acid methanolic solution followed by heating at 115° for 5 min (visualization: UV at 366 nm and visible)

High -speed countercurrent chromatography

HSCCC was performed using an Ito multi-layer coil separator-extractor [10] (P.C. Inc. Potomac, MD, U.S.A.) equipped with a 66 m X 2.6 mm I.D. column (column capacity: 350 ml). An LDC Milton Roy (Riviera Beach, FL, U.S.A.) minipump was used to pump the solvents through the column. The rotational speed was 800 rpm. A manual sample injection valve (Lobar Column Accessories, Merck, Darmstadt, Germany) equipped with a 10 ml loop was used to introduce the sample into the column. A flow splitter was inserted between the restrictor connected at the outlet of the column and the fraction collector in order to perform the on-line monitoring by TLC of the column effluent [11]. The solvent system was prepared by equilibrating at room temperature, in a separating funnel, a light petroleum (40-65°)-ethyl acetate-methanol-water (50:70:80:65 v/v) mixture. After separation, the two phases were degassed in an ultrasonic bath. The lower phase, used as stationary phase, was pumped into the column at 6 ml/min. The sample (F1, 300 mg) was dissolved in 10 ml of a 1:1 (v/v) mixture of the two

phases of the solvent system and the solution was filtered before loading into the column. After sample injection and start of the column rotation, the upper phase, used as the mobile phase, was pumped "tail to head" into the column at 4 ml / min. The separation was performed at room temperature and 140 fractions of 12 ml each were collected using a LKB Ultrorac® 7000 (Pharmacia, Uppsala, Sweden). For the spraying of the effluent onto the silica gel plate, the settings of the Linomat C (Camag, Muttenz, Switzerland), were as follows : band width : 5 mm ; space : 5 mm; rate at which the effluent was aspirated by the syringe : 75 µl /min; peak window: 0.4 min; retention times: 1 min for the first fraction to be collected with increment of 3 min for each following fraction: run-time key was pressed when the mobile phase was coming out of the column; gas (nitrogen) pressure : 4 bars.

High-performance liquid chromatography

The HPLC system was equipped with a pump (Model 6000A from Millipore-Waters, Milford, MA, USA), a sample loop (Model U6K from Millipore-Waters) and a high-speed spectrophotometric detector (Model 1040 M from Hewlett-Packard, Avondale, U.S.A.). Detection was performed simultaneously at 3 wavelengths, 227, 254 and 270 nm and UV spectra were recorded. The semi-preparative separations of F3 dissolved in tetrahydrofuran were performed on a Hibar pre-packed column Lichrosorb® RP-18 (mean particle size 7 µm, 250 mm X 10 mm I.D.) (Cat 50994, Merck, Darmstadt, Germany), using acetonitrile-water- methanol (45:40:20 v/v) as mobile phase at a flow rate of 6 ml/min. The analytical separations were achieved either on a Hibar pre-packed column Lichrosorb RP-18 (mean particle size 7 µm, 250 mm

X 4 mm I.D.) (Cat 50394,Merck, Darmstadt, Germany), with acetonitrile-water-methanol (38 : 42 : 20 v/v) at 1 ml/min either on a Hibar pre-packed column Lichrosorb® Si 60 (mean particle size 7 μm, 250 mm X 4 mm I.D.) (Cat 50335, Merck, Darmstadt, Germany) eluted with n-hexane-1,2-dichlorethane- 1-propanol (50:50:5 v/v) at 1 ml/min .

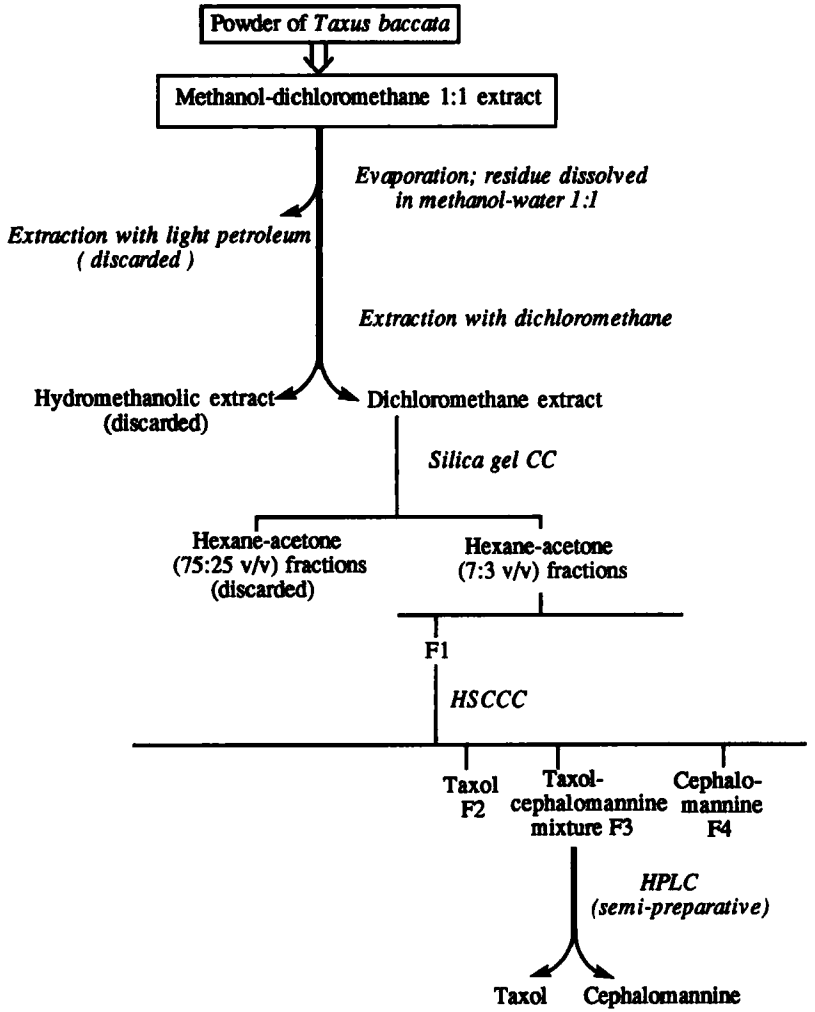
RESULTS AND DISCUSSION

Previously described methods for the isolation of taxol from crude extracts of *Taxus brevifolia* mainly involved preparative HPLC of pre-purified extracts [6]. However, these procedures are time consuming and preliminary results indicate that the on-column precipitation of taxane derivatives from the crude extracts resulted in backpressure problems on normal phase as well as on reversed phases [4]. These precipitation problems limit the use of the HPLC method for large scale application. Therefore, chromatographic procedures which require no solid supports would represent a useful option; in this purpose, the application of the HSCCC method was investigated.

Two pre-purification steps before HSCCC were found essential, especially to reduce the total amount of sample to be injected into the column. They include a partition between two phases (methanol-water-dichloromethane) and a column chromatography (Table 1). The solvent system selected for our actual HSCCC investigation was adapted from a countercurrent distribution method previously used as one of the pre-purification steps for the isolation of taxol and related diterpenes from *Taxus wallichiana* [12]. In this respect, the proposed composition, hexane-ethyl acetate-methanol-water (60:40:65:35), was modified to allow the separation and the elution

TABLE 1

Separation scheme of taxol and cephalomannine from *Taxus baccata* stem-barks



of taxol by increasing the ethyl acetate and water concentrations and by substitution of hexane by light petroleum to afford an efficient separation within a convenient analysis time (from 180 to 330 min). The partition coefficients (K) of taxol was determined by a simple test-tube experiment using HPLC for the quantitative determination [13]. They were calculated by dividing the corresponding peak area of taxol retained in the upper phase by that in the lower phase; with light petroleum-ethyl acetate-methanol-water (50:70:80:65 v/v), K = 2. The direct coupling of HSCCC to TLC with solvent S1 allowed an easy monitoring of the column effluent; in addition, normal phase HPLC (taxol and cephalomannine retention times were respectively 7.5 and 8.5 min) and TLC with solvent S2 and S3 were used to confirm the first conclusions. The HSCCC allowed to discard all interfering constituents of the extract from the fractions containing taxol and cephalomannine; in addition, 40 % of the total amount of recovered taxol was present (97 % pure form, 3% cephalomannine as contaminant) in fraction F2.

The fractions F3 were pooled with the help of a new TLC separation method using reversed phase silica gel and solvent S4.

Fractions F4 (4 mg) corresponded to cephalomannine in a 90 % pure form.

The reversed phase HPLC conditions for the semi-preparative separation of taxol and cephalomannine in fractions F3 were further optimized in order to reduce analysis time; with the proposed solvent system and column, their retention times were respectively 9 and 8.2 min.

At the end of the overall process, 43 mg of taxol and 29 mg of cephalomannine were recovered. Expressed in % plant dry weight, the taxol yield reached 0.015 %; therefore, a high recovery of this constituent from the plant was expected especially as the extraction

procedure was applied to a *Taxus baccata* material which in comparison with *T. brevifolia* is considered as poor source of taxol [5].

In comparison with the procedure of Cardellina [6], the most interesting feature of the proposed method was that, after the HSCCC separation step, the only fraction (F3) which remain to be further purified by HPLC contained taxol and cephalomannine without any interfering constituent. This last purification step could be achieved using our proposed HPLC conditions; however, for large scale separations of taxol, the normal phase separation [6] could be more convenient as the solubility of taxol and cephalomannine is poor in the solvent systems used in our reverse phase conditions.

In conclusion, the proposed method which includes a HSCCC step in the overall process of taxol purification is efficient and could also be very interesting for the scaling-up extraction of taxol from plant material. Studies are now in progress in order to adapt this method to the isolation of taxane diterpenoids from extracts of needles and other parts of *Taxus* sp.

ACKNOWLEDGMENTS

B. Diallo thanks the "Administration Générale de la Coopération au Développement (AGCD, Belgium)" for financial support.

REFERENCES

1. D.G.I. Kingston, G. Samaranayake, and C. A.. Ivey, J. Nat. Prod. 53: 1-12 (1990).

2. J.N. Denis, A.E. Greene, D. Guenard, F. Gueritte-Voegelin, L. Mangatal and P. Potier, J. Am. Chem. Soc. 110: 5917-5919 (1988).

3. Washington Insight, volume IV ,number 3, (1991).

4. K. M. Witherup, S.A. Look, M.W. Stasko, T.G. MC Cloud, H. J. Issaq and G. M. Muschik, J. Liq. Chromatogr. 12: 2117-2132 (1989)

5. N. Vidensek, P. Lim, A. Campbell and C. Carlson, J. Nat. Prod 53: 1609-1610 (1990).

6. J. H. Cardellina II, J. Liq. Chromatogr. 14: 659-665 (1991).

7. M. W. Stasko, K. M. Witherup, T. J. Ghiorzi, T. G. MC Cloud, S. Look, G.M. Muschik and H. J. Issaq, J. Liq. Chromatogr. 12: 2133-2143 (1989).

8. M. Jaziri, B. Diallo, M. Vanhaelen, R. Vanhaelen-Fastré, A. Zhiri, A. Bécu and J. Homès, J. Pharm. Belg. 46: 93-99 (1991).

9. K. Hostettman, M. Hostettmann and A. Marston, Preparative Chromatography Techniques Springer-Verlag, New York, 80-126 (1986).

10. Y. Ito, Anal. Chem., 17: 65 (1986).

11. B. Diallo, R. Vanhaelen-Fastré and M. Vanhaelen, J. Chromatogr. (in press).

12. R. W. Miller, R. G. Powell and C. R. Smith, Jr. J. Org. Chem. 46: 1469-1474 (1981).

13. H. Oka, Y. Ikai, N. Kawamura, M. Yamada, K. I. Harada, Y. Yamazaki and M. Suzuki, J. Chromatogr. 462: 315-322 (1989).