ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
14 (1996) 1215–1220

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Sample preparation for taxol and cephalomannine determination in various organs of *Taxus* sp.[1]

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Received for review 13 September 1995; revised manuscript received 5 December 1995

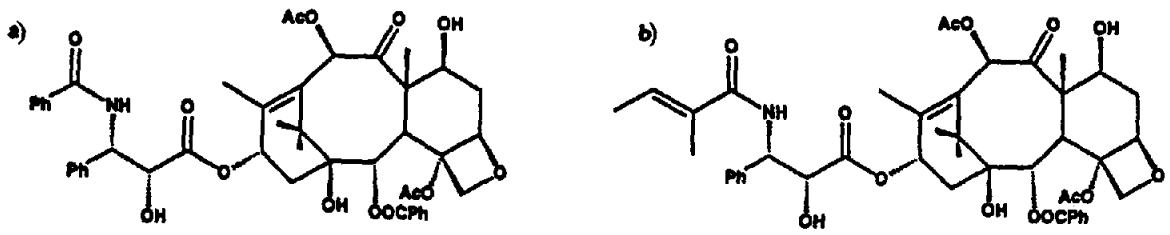
Abstract

Solid-phase extraction and preparative thin-layer chromatography were applied as sample preparation techniques for the purification of crude extracts from twigs and needles of various *Taxus* species as well as for the isolation of taxol and cephalomannine for further reversed phase high performance liquid chromatography analysis. Significant differences in the contents of taxanes examined were found. The preparative chromatographic methods used were compared and evaluated as routine and reproducible procedures for the rapid isolation and determination of taxol and cephalomannine in plant extracts.

Keywords: Cephalomannine; Quantitative analysis of taxanes; Solid-phase extraction; Taxol; *Taxus* species; Thin-layer chromatography

1. Introduction

Taxol and cephalomanine are two widely-known diterpene amides showing antimitotic and antitumour activity. The chemical structures of (a) taxol and (b) cephalamannine are shown below:



Formulae

Taxol, first isolated from the Pacific yew *Taxus brevifolia* Nutt. [1], is currently being applied and estimated in the clinical treatment of several types

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[1] Presented at the Fifth International Symposium on Drug Analysis, September 1995, Leuven, Belgium.

PII S0731-7085(96)01728-1

Table 1
Quantitative results (mean ± S.D.; $n = 5$) of the content of taxol (t) and cephalomannine (c) in *Taxus* spp. ($\mu g\ g^{-1}$ of dry wt.) obtained by application of SPE–HPLC and TLC–HPLC methods

No.	Name of specimen	SPE–HPLC		TLC–HPLC	
		t	c	t	c
1	*T. baccata* needles	25.1 ± 1.9	13.9 ± 0.6	19.4 ± 0.9	10.2 ± 0.6
2	*T. baccata* twigs	16.0 ± 1.9	4.0 ± 0.5	18.7 ± 0.9	5.5 ± 1.6
3	*T. baccata var. elegant.* needles	29.9 ± 1.6	27.1 ± 2.3	24.4 ± 2.5	20.0 ± 1.9
4	*T. baccata var. elegant.* twigs	8.6 ± 1.2	3.9 ± 0.8	6.3 ± 1.0	3.5 ± 1.3
5	*T. media var. Hicksii* needles	65.8 ± 1.8	47.0 ± 1.8	54.0 ± 1.7	40.3 ± 1.5
6	*T. media var. Hicksii* twigs	23.6 ± 0.8	22.0 ± 1.8	18.3 ± 1.9	16.2 ± 1.4
7	*T. media var. Hatfieldii* needles	12.8 ± 0.9	4.3 ± 0.6	13.0 ± 0.8	4.8 ± 0.8
8	*T. media var. Hatfieldii* twigs	20.1 ± 1.7	4.5 ± 0.5	21.1 ± 0.6	5.6 ± 1.0
9	*T. cuspidata* needles	181.0 ± 9.8	30.9 ± 2.8	128.4 ± 12.6	28.6 ± 9.8
10	*T. cuspidata* twigs	3.6 ± 0.9	1.9 ± 0.4	2.7 ± 0.5	2.4 ± 1.0

of ovarian, breast and skin cancer because of its unique ability to bind to and stabilize microtubules and, in this way, to interfere with their depolymerization [2]. Although the synthesis of taxol [3], as well as biosynthesis [4] on the basis of cell cultures, has already been reported, plant material, i.e. the bark of *T. brevifolia*, is still the richest source of taxol and related compounds. Hence, numerous trials [5,6] searching for other taxane sources, especially within *Taxus* sp., are currently being carried out [5]. As these compounds are present in very small quantities in plant material and show low stability in stored extracts [7] it is necessary to develop rapid methods for their isolation and purification for final quantitative high performance liquid chromatography (HPLC) analysis. There are only a few reports dealing with the problem mentioned above using solid-phase extraction (SPE) and thin layer chromatography (TLC) preparative techniques. Vidensek et al. [5] and Auriola et al. [8] utilized octadecylsilane (C-18) cartridges and methanol for the elution of taxol. Similarly, TLC has been very seldom used as a preparative technique for taxanes [5]. Therefore, in this research SPE and TLC procedures for the purification and isolation of taxol and cephalomannine from twigs and needles of various *Taxus* spp. as well as an HPLC method for their determination were elaborated. Numerous HPLC systems applying reversed-phase (RP) chromatography for the separation of taxanes have been reported. Isocratic elution of taxol and related compounds on pentafluorophenyl packing material by Richheimer et al. [7] and on octadecylsilane by Vidensek et al. [5] and Fang et al. [9] was performed.

Witherup et al. [10] used cyano- and phenyl-bonded silica HPLC columns and a mobile phase in the gradient mode for the determination of taxanes. In this paper a quick and sensitive isocratic RP-18-HPLC method for the quantification of taxol and cephalomannine was developed.

2. Experimental

2.1. Plant material

The investigation was performed on the needles and twigs of *Taxus baccata* L., *Taxus baccata var. elegantissima*, *Taxus cuspidata* Sieb. et Zucc., *Taxus media* Rehder: *var. Hicksii* and *var. Hatfieldii* collected from the Botanical Garden of the Polish Academy of Sciences in Powsin and the Botanical Garden of the University of Warsaw in July 1995.

2.2. Regents

Taxol was purchased from Sigma (St. Louis, MO) and cephalomannine was supplied by the Drug Synthesis and Chemistry Branch of the National Cancer Institute (Bethesda, MD). The taxane standards were dissolved in acetonitrile to obtain a concentration of 0.04 mg ml^{-1}. Aliquots (10 μl) were injected into the HPLC system. The solvents used (methanol, acetonitrile, heptane and dichloromethane) were of chromatographic grade (Baker Chemikalien, Gros-Gerau, Germany); acetone and ethyl acetate were of reagent grade (POCH, Gliwice, Poland). In all experiments doubly-distilled water was used.

2.3. Apparatus

The HPLC system consisted of a Hewlett-Packard (Palo Alto, CA) Model 1050 liquid chromatograph equipped with a 20 μl sample injector (Rheodyne, Cotati, CA) and a variable-wavelength UV–Vis detector. A stainless-steel column (200 × 4.6 mm i.d.) packed with ODS Hypersil (Shandon, UK), $d_p = 5$ μm, was applied.

2.4. HPLC conditions

The separation of taxol and cephalomannine was accomplished by isocratic elution. A mixture of acetonitrile–water (50:50 v/v) was used as a mobile phase, at a flow rate of 1 ml min^{-1} and at ambient temperature. The compounds examined were detected at 200 nm.

2.5. Assay procedure

Fresh plant material (5 g of each specimen) was cut into tiny pieces (about 2 mm), immediately placed in 100 ml of methanol and heated under reflux on a water bath at 90°C for 2 h. After cooling, the liquids were decanted and the residues submitted twice to the same extraction procedure. Combined methanolic extracts, evaporated to dryness under reduced pressure, were dissolved in acetonitrile and, after filtration, placed in 10 ml volumetric flasks. 2.5 ml portions of these extracts, accurately measured, were purified prior to HPLC analysis by means of TLC and SPE.

2.6. SPE method

Diluted (70%) acetonitrilic extracts were passed through octadecyl SPE microcolumns (500 mg, J.T. Baker Inc., Phillipsburg, NJ) previously conditioned with 100% acetonitrile (10 ml), water (10 ml) and 70% acetonitrile (10 ml). After the application of crude acetonitrilic samples and the collection of eluates each sorbent was washed with 1 ml of 70% acetonitrile to remove matrix. In this way non-polar ballast compounds (e.g. chlorophyll, waxes) were retained on the microcolumns and the collected eluates containing taxol and cephalomannine were directly analysed by HPLC.

2.7. TLC preparative method

Parallel samples of crude acetonitrilic extracts (2.5 ml) were condensed in an evaporator under vacuum to a volume of 0.5 ml and spotted linearly on TLC plates. Preparative chromatography was performed on 10 × 20 cm^2 glass plates covered with a 0.5 mm layer of Kieselgel 60 HF_{254} (E. Merck, Darmstadt, Germany) by means of a coat-

ing apparatus (Quickfit, UK) and activated before use at 110°C for 1 h. Chromatograms were developed with a mobile phase of *n*-heptane–dichloromethane–ethyl acetate (50:40:5 v/v/v) over a distance of 9 cm in horizontal DS chambers (Chromdes, Lublin, Poland). After development, plates were observed under UV light ($\lambda = 254$ nm). The located common band of taxol and cephalomannine ($R_F \approx 0.55$) was scraped off and the compounds were eluted from silica with a methanol–acetone mixture (1 + 1 v/v; 3 × 25 ml) by sonication (10 min), followed by centrifugation (10 min) with a MPW-2 centrifuge (Warsaw, Poland). Supernatants were combined, evaporated to dryness, dissolved in 5 ml of acetonitrile and finally analysed by HPLC.

2.8. HPLC analysis

A Hewlett-Packard (Palo Alto, CA) Model 1050 liquid chromatograph was used for the quantitative determination of taxanes on the stainless-steel column: 200 mm × 4.6 mm i.d., packed with 5 μm ODS Hypersil (Shandon, UK). As a mobile phase 50% acetonitrile was used. Chromatograms were detected at 200 nm. 10 μl volumes of the eluates after SPE and TLC were injected and analysed by HPLC. The content of each taxane in the samples examined was calculated from the ratio of the sample peak area or height to the standard peak area (height).

2.9. Recovery tests

The determination of recovery was performed for the SPE and TLC methods. 2 ml of the standard solution of taxol and cepthalomannine ($c = 0.04$ mg ml^{-1}) was added to selected acetonitrilic extracts of *Taxus* (see Section 2.5) before their submission to both preparative methods described.

All eluates obtained were analysed by HPLC. The comparison of peak heights and areas of normal and fortified samples as well as the standard solutions of taxol and cephalomannine ($c =$ 0.04 mg ml^{-1}) and the same solutions (2 ml) subjected to SPE and TLC methods enabled the calculation of recoveries (Table 2). The recovery procedure was repeated five times for both standards and samples in each of the preparative methods presented.

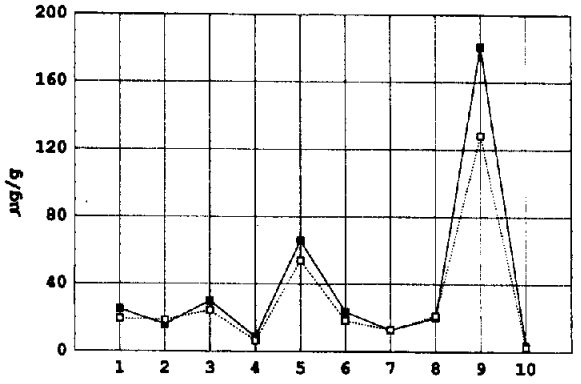

Fig. 1. Differences in the content of taxol within *Taxus* spp. obtained by applying combined SPE–HPLC (■) and TLC–HPLC (□) methods. The numbers on the abscissa correspond to the Taxus samples presented in Table 1.

3. Results and discussion

In this report the determination of taxol and cephalomannine by the application of two combined methods (SPE–HPLC and TLC–HPLC) was performed. Significant differences in the concentrations of these compounds within *Taxus* spp.

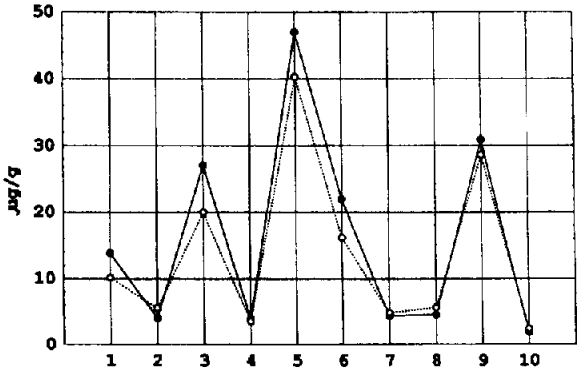

Fig. 2. Differences in the content of cephalomannine within *Taxus* spp. obtained by applying combined SPE–HPLC (●) and TLC–HPLC (○) methods. The numbers on the abscissa correspond to the *Taxus* samples presented in Table 1.

Table 2
Recoveries (mean% ± S.D.; $n = 5$) of taxol and cephalomannine for standard solutions (a) and spiked samples (b) submitted to TLC and SPE procedures

Standard	SPE		TLC	
	a	b	a	b
Taxol	98.8 ± 1.3	92.9 ± 3.3	83.6 ± 4.4	80.6 ± 6.0
Cephalomannine	93.3 ± 0.8	90.8 ± 2.9	83.1 ± 5.1	84.3 ± 6.5

were demonstrated (Table 1). The needles of *T. cuspidata* turned out to be the richest source of taxol and cephalomannine. Both methods used showed good reproducibility (low SD values presented in Table 1) and the convergence of the quantitative results of the content of taxanes in *Taxus* samples (Figs. 1 and 2).

The comparison of these methods also showed that the SPE procedure enabled higher recoveries of taxol and cephalomannine (Table 2) but a slightly poorer clean-up of plant extracts from polar compounds than TLC (Fig. 3). Moreover, it was observed in earlier investigations (not published), for the purpose of the protection of taxanes from structural changes in alcoholic extracts, acetonitrile can be recommended as a reagent that ensures a better stability of these compounds than other solvents (e.g. methanol) and a proper medium for SPE.

The TLC chromatographic system elaborated for the separation of taxanes enabled the satisfactory resolution of compounds examined from ballast fractions such as chlorophyll and waxes as well as suitable purity of eluates for further HPLC analysis. Under the chromatographic conditions applied taxol and cephalomannine were observed as a common narrow band ($R_F \approx 0.55$) easy to remove from silica plates and well separated from the bands of neighbouring compounds (Fig. 4).

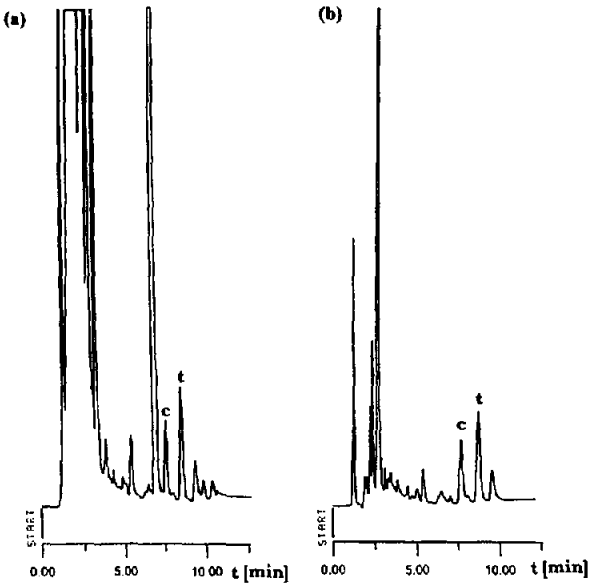

Fig. 3. Experimental chromatograms of extracts from *T. media var. Hicksii* needles after their submission to SPE–HPLC (a) and TLC–HPLC (b) methods. HPLC analysis carried out on ODS Hypersil (200 mm × 4.6 mm i.d., $d_p = 5$ μm) column under isocratic conditions; mobile phase: acetonitrile–water (50:50 v/v); flow rate 1 ml min^{-1}; detection at 200 nm; c, cephalomannine; t, taxol.

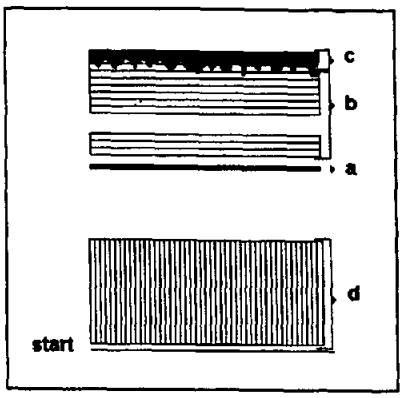

Fig. 4. Preparative TLC separation of crude acetonitrilic extract from *T. cuspidata* needles: (a) common band of taxol and cephalomannine ($R_F \approx 0.55$); (b) chlorophyll; (c) waxes; (d) polar compounds.

The quantitative analysis of taxol and cephalomannine was carried out using an HPLC method also developed in the authors' laboratory. With the reversed-phase (RP-18) system applied and 50% acetonitrile as a mobile phase, good separation, characterized by narrow, symmetrical peaks, was obtained in a short time of analysis (< 10 min). The detection of compounds determined, established in the short ultraviolet region ($\lambda = 200$ nm), ensured the high sensitivity of the method used. A detection limit of 10 ng (for both taxol and cephalomannine) at a signal-to-noise ratio of 3:1 for the peak heights was determined.

Summing up the results of this work, combined SPE–HPLC and TLC–HPLC methods can be recommended for the screening analysis of the content of taxol and cephalomannine in various *Taxus* organs as easy, rapid and reproducible chromatographic procedures. However, regarding the precision of quantitative analysis, SPE seems to be a superior technique to preparative TLC.

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