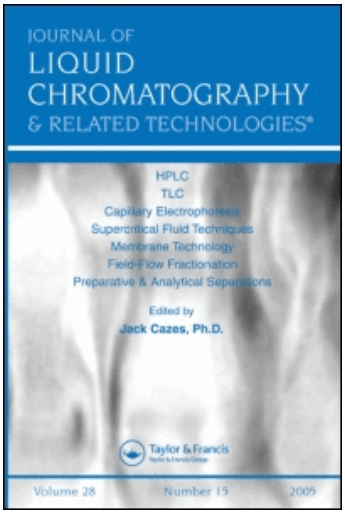

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High Performance Liquid Chromatographic Determination of Taxol and Related Taxanes from *Taxus* Callus Cultures

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF TAXOL AND RELATED TAXANES FROM *TAXUS* CALLUS CULTURES

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ABSTRACT

This study presents a protocol for analyzing taxol and five related taxanes from tissue culture samples of *Taxus* spp. by high performance liquid chromatography (HPLC) using a reverse phase C_{18} column. Sep-Pak C_{18} cartridges were used for semi-purification of the crude extracts of the samples prior to analyses. Taxol, cephalomannine, 10-deacetyltaxol, 10-deacetylcephalomannine, baccatin III and 10-deacetylbaccatin III were well separated by the mobile phase of methanol : acetonitrile : water (25:35:45) at a flow rate of 1.0mL/min and detected at 227nm.

INTRODUCTION

Taxol[I], a cytotoxic diterpenoid natural product initially isolated from the stem bark of *Taxus brevifolia* Nutt. by Wani et al.,[1] is of great interest for its unique structure and excellent activity against ovarian cancer, breast cancer

and other types of cancer.[1,2] Presently, the commercial source of taxol is the bark of *Taxus brevifolia*, which grows slowly and yields relatively low amounts of taxol. Although others species of *Taxus* also contain taxol,[1] and semi-synthesis from natural taxoids,[3] as well as total synthesis of taxol have been successful,[4] cell culture of *Taxus* spp., which provides rich stable supply of the antineoplastic agent taxol and related taxanes, may be viewed as a potential alternative to plant extraction currently in short supply.

The closely related taxanes of taxol[**I**], cephalomannine[**II**], 10-deacetyl-taxol[**III**] and 10-deacetylcephalomannine[**IV**] have shown less anticancer activity than taxol; baccatin III[**V**] and 10-deacetylbaccatin III[**VI**] can be converted to taxol through a semi-synthetic route. The analysis of these compounds[**I~VI**] from cultures of *Taxus* spp. can be used to select particular cell lines with high and stable yield of taxol/taxanes.

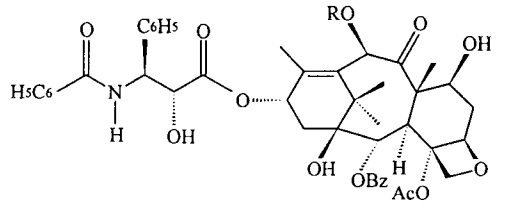
It is rather difficult to completely separate taxol[**I**] from cephalomannine[**II**] and 10-deacetyltaxol[**III**] from 10-deacetylcephalo-mannine[**IV**] by a common HPLC method, so several reported methods[1,2,5] utilized gradient elution technique or/and selected phenyl, cyano, and other special columns to modify the separation of taxol and the closely related taxanes. Although, it was reported, that a common HPLC method using a C_{18} column eluted in the isocratic mode for the determination of four taxanes[**I~IV**],[6] the determination of baccatin III[**V**] and 10-deacetylbaccatin III[**VI**] was not mentioned.

On the basis of reported methods,[1,2,6] we developed a HPLC method using a C_{18} column, eluted in isocratic mode, for analyzing taxol[**I**], cephalomannine[**II**], 10-deacetyltaxol[**III**], 10-deacetylcephalomannine[**IV**], baccatin III[**V**], and 10-deacetyl-baccatin III[**VI**] (Figure 1) from cultures of *Taxus* spp.

MATERIALS

A Spherisorb C_{18}, 5μm (250mm × 4.6mm) column was used for analysis. Sep-Pak C_{18} cartridges (Waters/Millipore Co., Milford, MA, USA) were used for pretreatment of the crude extracts of culture samples before HPLC analyses.

Acetonitrile (HPLC grade, Zhejiang Huangyan Experimental Chemical Factory, Zhejiang, China), methanol (G. R. grade, Beijing Chemical Factory, Beijing, China) and water (ultrapure) were filtered through G_5 filters prior to



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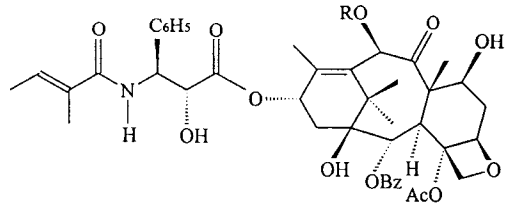
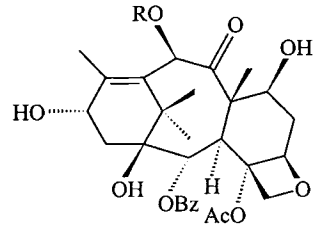
III R=H



II R=Ac
IV R=H



V R=Ac
VI R=H

Figure 1. Structures of taxol and related taxanes.

use. Methanol and methylene chloride (both A. R. grade, Beijing Chemical Factory, Beijing, China) were used for the extraction of samples. Callus cultures were established from *Taxus yunnanensis, T. cuspidata*, and etc. Callus samples were harvested after 21~25 days of growth and dried at 55°C. Authentic samples of standards **I~VI** were generously provided by Dr. Weishuo Fang of our institute.

Instrumentation consisted of a LC-6A pump (Shimadzu), a Rheodyne 7125 Manual injector (Cotat, California, USA) and SPD-6A ultraviolet detector (Shimadzu) with a C-R3A integrator (Shimadzu).

METHODS

Dried callus cultures were pulverized and passed through 40-mesh sieves and extracted ultrasonically with a mixture of methanol and methylene chloride (10:1) for 30 min. The crude extract was evaporated to dryness and the residue was dissolved in methanol. A portion of the sample solution was loaded on a Sep-Pak C_{18} cartridge and eluated with water, 30%, and 85% methanol, respectively. The final eluate was evaporated to dryness and redissolved in a minimum amount of methanol.

HPLC analysis of 10μL fractions of the preparation was made with a mobile phase of methanol : acetonitrile : water (25:35:45) at a flow rate of 1.0mL/min with UV detection at 227nm.

All presumptive taxanes[**I~VI**] in calli of different species of *Taxus* cell lines were identified by comparison of the retention times of the chromatographic peaks with those of a standard mixture containing **I**, **II**, **III**, **IV**, **V**, and **VI** chromatographed in the same conditions. The co-injection of the sample and the standard mixture was done to confirm the compound existing in the sample. Each sample was injected duplicately and the average of the peak areas was used to quantify **I~VI** against external standard curves of the standards.

To inspect the recovery, the spiked samples containing definite amounts of the six standards[**I~VI**] were analyzed by the same procedure. By comparing different peak areas of the six compounds[**I~VI**] in samples and spiked samples, the recovery rates were obtained.

RESULTS AND DISCUSSION

The extraction solvent used by Wickremesinhe et al.[1] was methanol, which could effectively reduce the extraction of the highly hydrophobic components, such as oils, waxes, etc. in the plant tissue cultures. In our cultures, not only non-polar components, but also highly polar impurities were found. Therefore, methanol : methylene chloride (10:1) was used as the extraction solvent which could give satisfactory results for the analysis.

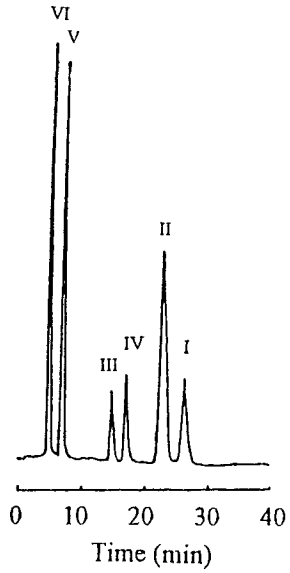


Figure 2. Separation of taxane standards. Peaks: I= taxol; II=cephalomannine; III= 10-deacetyltaxol; IV= 10-deacetylcephalomannine; V= baccatin III; VI= 10-deacetyl-baccatin III.

Sep-Pak C_{18} cartridges played an important role in the semi-purification step as Wickremesinhe et al.[1] reported. Neither non-polar components nor highly polar impurities were seen in the semi-purified fraction collected from the cartridge compared with the crude extract. Nevertheless, the following fraction eluted by pure methanol did not show any peaks of **I~VI**.

The mobile phase consisting of methanol, acetonitrile, and water was used on a phenyl, cyano, and any other special column except a C_{18} column to analyze taxanes[1,2] and on a μBondapak C_{18} preparative column to prepare taxol.[1] A mobile phase of water : acetonitrile : tetrahydrofuran (55:35:10) was used on a C_{18} column[6] but this took too much time to equilibrate the column before analysis, and tetrahydrofuran (THF) must be reevaporated in order to avoid baseline drift. Comparing these two kinds of mobile phases, we found that the mobile phase consisting of methanol, acetonitrile, and water was better and methanol : acetonitrile : water (20:35:45) which used to prepare taxol on a C_{18} preparative column[1] could be adopted for our analysis on a C_{18} analytic column. Equilibration time is relatively short and baseline is stable under this kind of mobile phase.

Table 1

The Linearity of Determination of Taxol and Related Taxanes

Compound	Range of Linearity (ng)	Equation of Linear Regression	Correlation Coefficient
Taxol	11.5 - 92	Y=328.1X - 565.8	0.9968
Cephalomannine	16.9 - 135	Y=98.51X + 1178	0.9994
10-Deacetyltaxol	9.6 - 76.8	Y=370.1X - 5.857	0.09996
10-Deacetylcephalo-mannine	14.5 - 116	Y=218.7X + 379.8	0.9998
Baccatin III	11.6 - 92.8	Y=165.0X - 86.30	0.9996
10-Deacetylbaccatin III	11.8 - 94.4	Y=135.6X - 80.61	0.9983

Table 2

Recovery Rate of Taxol and Related Compounds in Callus Cultures of Taxus spp.

Sample	I(%)	II(%)	III(%)	IV(%)	V(%)	VII(%)
TY-37	103.7	98.3	97.4	96.0	N.D.	N.D.
TY(2)2500-5	N.D.	N.D.	N.D.	N.D.	101.9	96.8
TN-20	N.D.	102.2	N.D.	N.D.	98.7	N.D.

I: taxol
II: cephalomannine
III: 10-deacetyltaxol
IV: 10-deacetylcephalomannine
V: baccatin III
VI: 10-decetyllbaccatin III
N.D.: not detected

Tests of different proportions of this kind of mobile phase showed that the proportion of methanol has an apparent effect on the separation and the retention times, but slight effect on the shape of the chromatographic peak. **I**~*VI* were well separated by the mobile phase of methanol : acetonitrile : water (25:35:45) at a flow rate of 1.0mL/min and detected at 227nm (Figure 2).

Table 3

The Contents of Taxol and Related Taxanes in Callus Cultures from Taxus spp.

Number of Cell Line	Kind of Medium	I(%)	II(%)	III(%)	IV(%)	V(%)	VI(%)
TY-37	D	0.052	0.13	0.018	0.029	ND	ND
TY-55	D	0.021	0.011	0.016	ND	ND	ND
TY(2)-500-5	D	0.0043	0.0044	0.0043	ND	ND	ND
TY(2)-500-6	D	0.0055	0.035	trace	ND	ND	0.042
TY(2)-500-8	D	0.011	0.010	0.0084	ND	ND	0.019
TN-20	C	ND	0.013	ND	ND	0.11	ND
TY(2)-1000-5	C	ND	ND	ND	ND	0.011	0.0081
TY(3)-7	B	ND	ND	ND	ND	0.0086	0.0057
TY(2)-2500-2	B	ND	ND	ND	ND	0.041	0.094
TY(2)-2500-4	B	ND	ND	ND	ND	0.012	0.0044
TY(2)-2500-5	B	ND	ND	ND	ND	0.076	0.031

I: taxol
II: cephalomannine
III: 10-deacetyltaxol
IV: 10-deacetylcephalommanine
V: baccatin III
VI: 10-deacetylbaccatin III
ND: Not detected

The isocratic analytical HPLC method showed good linearity for all six compounds **I~VI**. The mass of each taxoid was determined by comparison to its external standard curve over the range of approximately 10ng to 100ng (Table 1). Recovery rates of taxol and related taxanes in callus cultures of *Taxus* spp. were from 96.0% to 103.7% (Table 2).

The contents of **I~VI** in callus cultures from various species of *Taxus* were determined. A compound could be assumed to be present in the sample, if the peak of that compound remained single when co-injected with an authentic standard. The results showed that the contents of **I~VI** varied in different cell lines established in various kinds of medium (Table 3), where medium A was 6, 7-V basic medium contained 0.1mg/L KT, 1.0mg/L IAA and 1.0mg/L 2, 4-D, medium B was medium A which contained 20mg/L phenylalanine(Phe), medium C was medium A which contained 20mg/L Phe and 2.5g/L soybean

powder (SP), and medium D was medium C which contained 50mg/L vanadyl sulphate. The cell line TY-37 has the highest contents of **I** and **II**, and TY(2)2500-5 the highest content of **V**. Details of the work on *Taxus* callus culture will be reported elsewhere.

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