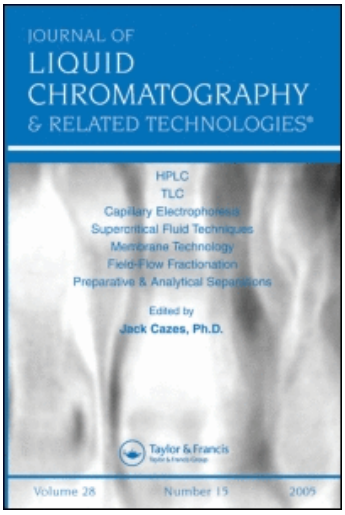

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METHODOLOGY FOR THE IDENTIFICATION AND PURIFICATION OF TAXOL AND CEPHALOMANNINE FROM *TAXUS* CALLUS CULTURES

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ABSTRACT

In this study we have developed a new protocol for the extraction and purification of taxol and cephalomannine from plant tissue culture samples. This protocol allows large numbers of samples to be processed with a minimum use of solvents and can be completed in a relatively short period of time, compared to existing published protocols. The procedure involves a methanol extraction followed by the use of C_{18} Sep-pak cartridges, before analysis by isocratic high performance liquid chromatography (HPLC) using a reverse-phase analytical phenyl column. Our purification protocol consisted of a 65% methanol : chloroform partitioning step followed by C_{18} bonded silica column chromatography and finally, C_{18} preparatory HPLC.

INTRODUCTION

Taxol (NSC-125973) is a cytotoxic diterpene initially identified from the stem bark of *Taxus brevifolia* Nutt. by Wall and Wani in 1967 (1). Its structure along with its potent antileukemic and tumor inhibitory properties were elucidated in

1971 by Wani and co-workers (2). Taxol is the only plant secondary metabolite known to promote the assembly of microtubules and inhibit the tubulin disassembly process (3,4), and therefore appears to be the prototype of a new class of cancer chemotherapeutic agents (5).

Clinical trials indicate that taxol is effective in the treatment of patients with refractory ovarian cancer, breast cancer, non-small cell lung cancer and malignant melanoma (6,7). However, the limited supply of taxol has precluded extensive clinical studies.

Several other species of yew (genus *Taxus*) have also been reported to contain taxol, including: *T. baccata, T. cuspidata, T. canadensis*, and *T.* x *media* (2,8,9). Chemists all over the world have been involved in an attempt to chemically synthesize taxol, and have had no success to date (10,11).

The production of antineoplastic compounds in callus cultures from a number of antineoplastic agent-producing plants have been demonstrated (12,13). Plant tissue culture can provide large amounts and a stable supply of compounds exhibiting antineoplastic activity, and has been of major interest since the 1970's.

Screening for taxol-producing cell lines requires the availability of both extraction and analytical protocols that would facilitate the identification of taxol and other related diterpenoids in plant tissue cultures. Techniques used to analyze whole plants and plant parts (8) are not practical for screening large numbers of plant tissue culture samples, and therefore, need to be modified or new protocols have to be developed.

In most cases, the protocols developed for the analysis of whole plants and plant parts cannot be extrapolated to the cell cultures (13). Cell cultures in general, accumulate only a fraction of the levels of the secondary products found in field-grown plants. In addition, the metabolites may be stored in different tissues and/or compartments than found in the whole plant, or bound in such a way that they may

not be retrieved by conventional extraction methods. Therefore, the objective of this study was to develop extraction and purification protocols for taxol and cephalomannine from plant tissue cultures.

MATERIALS AND METHODS

Extraction and Identification from Plant Tissue Cultures

Callus cultures were established from different *Taxus* taxa as described by Wickremesinhe and Arteca (14). Six- to eight-month old, slow-growing callus samples were harvested and freeze-dried. Callus (50 mg) was extracted in methanol (1.5 ml) by homogenizing in an Omni-mix homogenizer (Omni International, Waterbury, Connecticut) for 2 minutes followed by sonication for 5 minutes. The extract was transferred to a 2-ml Eppendorf tube and centrifuged to pellet cell debris. The supernatant was recovered and filtered through a 0.2 micron nylon filter for high performance liquid chromatography (HPLC) analysis. However, for critical analysis, samples were purified as follows. The extract was diluted to 20% methanol (final volume less than 3.0 ml), loaded on a C_{18} Sep-pak cartridge (Waters, Milford, Massachusetts) and washed with 25, 50 and 65% methanol. A final elution step with 85% methanol recovered more than 90% of the total taxol.

Analytical HPLC was performed on a Dynamax 60 Å 8 µm phenyl column (4.6 mm x 250 mm) with a phenyl guard module (Rainin Instrument Co. Inc., Woburn, Massachusetts). A mobile phase consisting of methanol : 50 mM acetate buffer (pH = 4.4) : acetonitrile (20:41:39), at a flow rate of 1 ml per minute was used. Taxol was detected by monitoring absorbance at 227 nm.

Duplicate injections were made from every sample and the average of the two peak areas was used to quantify taxol and cephalomannine. Authentic samples of taxol and cephalomannine (from Dr. Ken Snader, National Cancer Institute, Bethesda, MD) were used for the identification of peaks. A taxol and cephalomannine linearity curve was established by injecting amounts of authentic taxol ranging from 0.005 to 10 μg per injection.

Preparatory-Scale Extraction and Purification of Taxol from Plant Tissue Cultures

Callus cultures of *Taxus media* cv. Hicksii harvested during routine subculture procedures were bulked, frozen, and subsequently freeze-dried (15). Samples were extracted in three ways, the first was according to the methylene chloride : water extraction protocol of Witherup et al. (8), and the second utilized the multi-step solvent extraction procedure of Cardellina (16). Both protocols were designed and used for the analysis of *Taxus* stem and needle samples, by their respective authors. The third extraction procedure was developed in our laboratory and is described as follows: callus was extracted in methanol (at a ratio of 10 g dry cells per 250 ml methanol), homogenized, extracted on a gyratory shaker at 200 rpm for 12 hours, and finally, sonicated for 10 minutes. The homogenates were filtered through Whatman # 1 filter paper, the filtrate was adjusted to 65% methanol and partitioned with chloroform (3:1 v/v).

The methylene chloride and chloroform extracts, obtained from the three protocols, were dried *in vacuo* and resuspended in 100% methanol. The samples were then diluted to 40% methanol (in water) and loaded onto a luer-lock glass column (15 mm x 200 mm) packed with preparative C_{18} bonded amorphous silica 125 Å, 55 to 105 mm (Waters, Milford, Massachusetts) and washed with 2 column volumes of 60% and 85% methanol. The latter eluted the taxol-rich fraction.

This semi-purified taxol-rich fraction was injected onto a μBondapak C_{18} (7.8 mm x 300 mm) preparatory column (Waters, Milford, Massachusetts) using a water : methanol : acetonitrile mobile phase (45:20:35) at a flow rate of 3 ml/min.

RESULTS AND DISCUSSION

The extraction procedures mentioned used by Witherup et al. (8) and Cardellina (16) were not practical for routine screening of a large number of plant tissue culture samples. Therefore, it was necessary to develop an efficient extraction protocol for plant tissue culture samples.

Very few highly hydrophobic components such as oils, waxes etc. were found in our plant tissue cultures, especially in those cultured in darkness. Therefore, the use of crude methanol extracts from freeze-dried callus cultures were satisfactory for the analysis and identification of taxol and cephalomannine. However, for critical analysis, samples were further purified as described in the materials and methods section. This protocol efficiently removed most of the lesser hydrophobic components from the methanol extract, and therefore, sufficed as a semi-purification step.

The HPLC protocol reported by Witherup et al. (8) caused damage to the column due to clogging, which resulted in very high back pressures, beyond the operational limits of the column. The substitution of water with a 50 mM ammonium acetate buffer (pH 4.4) in the mobile phase prolonged column life remarkably. This procedure was capable of detecting taxol at amounts as low as 5 nanograms. Separation of taxol and cephalomannine from crude plant tissue culture samples was achieved, with the two peaks eluting after 16.5 and 19.3 minutes, respectively.

TABLE 1

Taxol and cephalomannine contents in callus derived from different *Taxus* taxa. The values represent the highest taxol and cephalomannine contents (% dry matter basis) observed from at least five samples of callus derived from each taxa. The highest values are represented because some samples did not contain detectable amounts of either compound, and therefore averaging these values would tend to make the results biased.

Taxus taxa	Taxol %	Cephalomannine %
brevifolia	0.00059	0.00043
baccata cv. Repandens	0.00021	0.00021
x *media* cv. Densiformis	0.00078	0.00056
cuspidata	0.00109	0.00064
x *media* cv. Hicksii	0.00089	0.00030

The amounts of taxol and cephalomannine detected in the slow-growing callus cultures derived from the five explant sources (14) are given in **Table 1**. These values represent the highest amounts observed among at least five callus samples analyzed from each taxa. We decided to report the highest values because approximately half of all the samples analyzed contained non-detectable amounts of either compound, and therefore, representing this data as an average would tend to bias the results. Analysis of callus line CR-1 over a culture period of 14 weeks exhibited taxol levels ranging from <0.0001 to 0.0131%, expressed on a dry weight basis (15).

Approximately a milligram of purified taxol was needed to perform nuclear magnetic resonance spectroscopy (NMR) to confirm the presence of taxol in our culture samples. This was a challenge because published protocols on scale-up purification includes techniques that involve several silica and Florisil

TABLE 2

Total dry matter and taxol recovery from the extraction protocols of Witherup et al. (1990), Cardellina (1991) and the one-step 65% methanol : chloroform partitioning protocol. The data is presented as a percent of the initial dry cell weight.

Extraction protocol	Dry matter recovery (%)	Taxol recovery (%)
Witherup et al. (1990)	8.2	81
Cardellina (1991)	2.4	73
65% methanol : chloroform	2.5	87

chromatographies, several partitioning steps, multimodal thin layer chromatography, and high-speed countercurrent chromatography (16 - 19).

We obtained 87% recovery of taxol using our 65% methanol : chloroform extraction protocol, which is superior to both the methylene chloride : water extraction protocol of Witherup et al. and the multi-step partitioning protocol of Cardellina (**Table 2**). It could be argued that the overall taxol yields obtained through this protocol are comparable to the yields obtained from the protocol of Witherup et al.; however, the 65% methanol : chloroform partitioning gave a cleaner fractionation resulting in a dry matter yield of 2.5%, compared to 8.2% for the methylene chloride : water partitioning.

Although the protocol reported by Cardellina gave similar dry matter yields, it resulted in poor taxol recovery (**Table 2**). This could be due to its three-step partitioning protocol consisting of hexane, carbon tetrachloride, and finally chloroform. The hexane and carbon tetrachloride partitioning steps recovered only

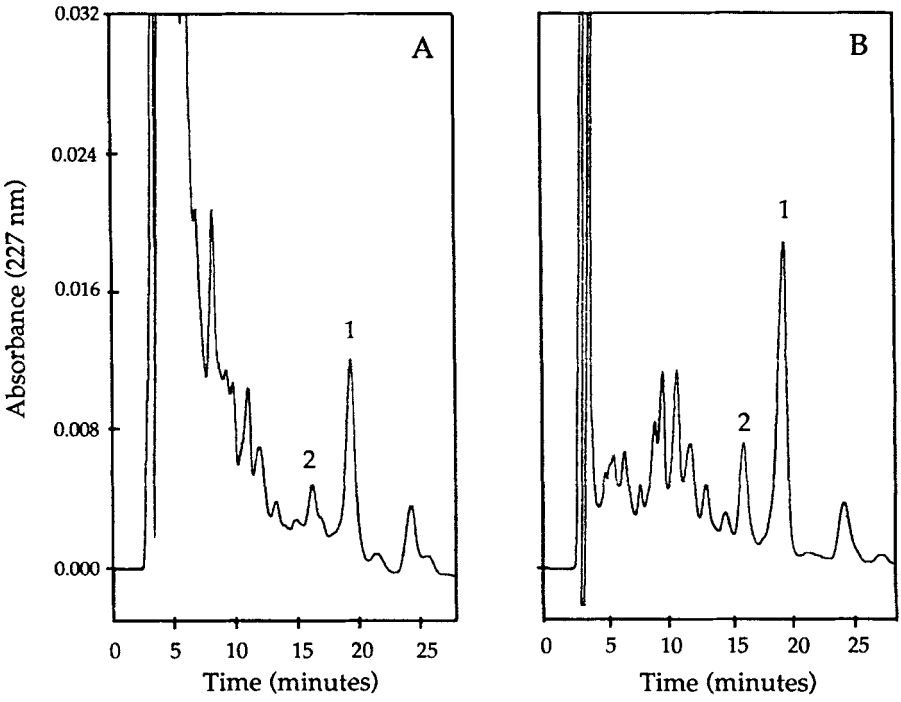

FIGURE 1. Crude methanol extract (A), and taxol-rich semi-purified fraction obtained from the 25 ml C_{18} bonded amorphous silica packed glass column (B), from callus cultures of *Taxus media* cv. Hicksii. HPLC was performed on a Dynamax 60 Å 8 µm phenyl column. A mobile phase consisting of methanol : 50 mM acetate buffer (pH = 4.4) : acetonitrile (20:41:39) at a flow rate of 1 ml/min was used. Peak number 1 = taxol, and 2 = cephalomannine.

0.6 and 5.5% of the dry matter and 0.04 and 6.8% taxol, respectively. The very low dry matter yields in the hexane fraction reflects the absence of extremely hydrophobic compounds, such as waxes and other cell wall components. Cardellina (16) claims that taxol was located in the carbon tetrachloride phase; however, in this study, taxol was contained in the chloroform phase.

Since the Sep-pak C_{18} cartridges performed well as an intermediate semi-purification step, the use of the same solid phase, packed in a 25 ml luer-lock glass

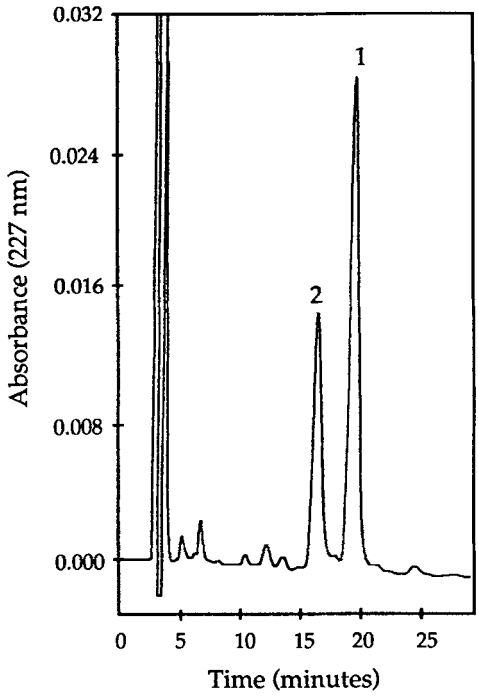

FIGURE 2. Taxol and cephalomannine purified from *Taxus media* cv. Hicksii callus cultures. HPLC was performed on the Dynamax 60 Å 8 μm phenyl column. A mobile phase consisting of methanol : 50 mM acetate buffer (pH = 4.4) : acetonitrile (20:41:39) at a flow rate of 1 ml/min was used. Peak number 1 = taxol, 2 = cephalomannine.

column gave similar results. The semi-purified taxol-rich fraction collected from the C_{18} column did not contain any of the hydrophilic peaks (**Figure 1b**) seen in the crude extract (**Figure 1a**). The column efficiency with respect to the overall recovery of taxol was greater than 90%, similar to the recovery rates obtained from the Sep-pak C_{18} cartridges.

The peaks corresponding to taxol and cephalomannine collected from the C_{18} column were concentrated *in vacuo* and finally lyophilized to yield a white powder. Approximately two milligrams of taxol was purified from 33.4 grams of

freeze-dried callus. This sample consisted of callus collected over a period of time and bulked together. A sample of this white powder yielded a chromatogram shown in **Figure 2**, with peaks eluting at the same retention time as authentic taxol and cephalomannine. The efficiency of this purification system was not critically evaluated for the recovery of cephalomannine. However, it was evident that the protocol was equally efficient for the purification of both taxol and cephalomannine (data not shown).

The absorbance spectrum of purified taxol exhibited an absorption peak at 227 nm, which corresponds to authentic taxol. Taxol activity was confirmed by the microtubule-stabilizing bioassay using tubulin isolated from calf brains (15). The structure was confirmed by NMR spectroscopy using one-dimensional ^{1}H and two-dimensional ^{1}H - ^{13}C inverse detected HMQC spectrum analysis (15).

CONCLUSIONS

The presence of taxol and cephalomannine in plant tissue cultures samples was identified by isocratic reverse-phase analytical HPLC methods using a simple extraction technique. The protocol developed in this study was capable of handling a large number of plant tissue culture samples with minimal use of solvents, and could be completed over a relatively short period of time. The use of C_{18} Sep-pak cartridges as an intermediate purification step was efficient in separating taxol and cephalomannine from less hydrophobic compounds before HPLC analysis. Pure taxol and cephalomannine was obtained using the following extraction and purification protocol. Callus samples were extracted in methanol, partitioned between 65% methanol : chloroform, loaded onto a luer-lock glass column (15 mm x 200 mm) packed with preparative C_{18} bonded amorphous silica. Taxol and

cephalomannine was eluted by 2x column volumes of 85% methanol. Finally, pure taxol and cephalomannine was obtained by C_{18} preparatory HPLC. Using this protocol, we purified approximately 2 milligrams of taxol and 1 mg of cephalomannine from a plant tissue culture sample.

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REFERENCES

1. Wall, M.E., and Wani. M.C., 153rd National Meeting of the American Chemical Society (paper no. M-006), Miami Beach, Florida, 1967.

2. Wani, M.C., Taylor, H.L., Wall, M.E., Coggon, P., and McPhail, A.T., J. Am. Chem. Soc. **93**, 2325-2327 (1971)

3. Schiff, P.B., Fant, J., Auster L.A., and Horowitz, S.B., J. Supramolec. Struct., **8**, suppl. 2, 328, (1978)

4. Horowitz, S.B., Lothsteia, L., Manfredi, J.J., Mellado, W., Parness, J., Roy, S.N., Schiff, P.B., Sorbara L., and Zeheb, R., Ann. New York Acad. Sci., **466**, 733-743, (1986)

5. Suffness, M., and Cordell, G.A., "Antitumor alkaloids" in The Alkaloids, vol. XXV, A. Brossi, ed., Academic Press, Inc., Orlando 1985, pp. 6-20.

6. Slichenmyer, W.J., and Von Hoff, D.D., Anti-Cancer Drugs, **2**, 519-530, (1991)

7. Arbuck, S.G. Second National Cancer Institute Workshop on Taxol and *Taxus*, (abstract) September 23-24, 1992, Alexandria, Virginia (1992)

8. Witherup, K.M., Look, S.A., Stasko, M.W., Ghiorzi, T.J., and Muschik, G.M., J. Nat. Prod., **53**, 1249-1255, (1990)

9. Wickremesinhe E.R.M., Ph.D. Dissertation, The Pennsylvania State University, University Park, Pennsylvania, (1992)

10. Kingston, D.G.I., The chemistry of taxol. Pharmac. Ther., **52**, 1-34, (1991)

11. Wender, P.A., Second National Cancer Institute Workshop on Taxol and *Taxus*, (abstract), September 23-24, 1992, Alexandria, Virginia, (1992)

12. Misawa, M., Hayashi M.M., and Takayama, S., "Accumulation of antineoplastic agents by plant tissue cultures" in Primary and Secondary Metabolism of Plant Cell Cultures, K. H. Neumann, W. Barz and E. Reinhard, eds., Springer-Verlag, New York 1985, pp. 235-246.

13. Stafford, A., "Natural products and metabolites from plants and plant tissue cultures", in Plant Cell and Tissue Culture, A. Stafford and G. Warren, eds., Open University Press, Buckingham 1991, pp. 125-162.

14. Wickremesinhe, E.R.M. and Arteca, R.N., Submitted to Plant Cell Reports, (1993)

15. Wickremesinhe, E.R.M. and Arteca, R.N., Submitted to Plant Cell Tissue and Organ Culture, (1993)

16. Cardellina, J.H. II., J. Liq. Chromatogr., **14**, 659-665 (1992)

17. Magri, N.F., Ph.D. Dissertation, Virginia Polytechnic Institute and State University, Blacksburg, VA, (1985)

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

19. Vanhaelen-Fastre, R., Jaziri, M., Faes, M.-L., Homes, J., and Vanhaelen, M., J. Liq. Chromatogr., **15**, 697-706, (1992)

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