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Extraction, purification by solid-phase extraction and high-performance liquid chromatographic analysis of taxanes from ornamental *Taxus* needles

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Abstract

Needles from ornamental yews (*Taxus* spp.) are known to contain significant amounts of the anticancer agent Taxol (paclitaxel), as well as other analogous taxane compounds. Methanol extracts the taxanes from the needles efficiently, but the crude methanolic extract contains large amounts of co-extractives in addition to the taxanes of interest. The crude extract may be fractionally partitioned using C_{18} solid-phase extraction (SPE), permitting the collection of a fraction in which the taxanes elute quantitatively. This fraction can then be analyzed directly by means of high-performance liquid chromatography. We have successfully scaled-up the quantity of crude extract partitioned by means of the SPE technique, using larger SPE cartridges prepared in our laboratory as well as using Empore extraction disks. SPE using the Empore disks permits us to collect large quantities of the taxane fraction for studies on its cytostatic effect on non-mammalian eukaryotic cells.

1. Introduction

Studies on the chemistry of Taxol (paclitaxel, Bristol-Myers-Squibb, New York, NY, USA) and analogous taxane compounds span four decades, beginning in 1963 with the observation that extracts from the Pacific yew (*Taxus brevifolia*) were cytotoxic [1], up to the present attempts to elucidate paclitaxel's mode of action [2]. The structures of some diterpenoid taxanes are shown in Fig. 1. The magnitude of current interest in taxanes is attested to by symposia dedicated to paclitaxel chemistry [3], as well as by the increased magnitude of the taxane literature. By far the main focus of this literature is the interaction of taxanes with mammalian cells. In contrast, the number of reports on the effects of taxanes on non-mammalian, eukaryotic systems is few. For example, limited studies of paclitaxel effects on frog eggs [4], on sea urchin eggs [5], on hemoflagellates [6], on higher plants [7] and on fungi [8–11] have been reported.

All of the reports in the literature to date and alluded to above have utilized pure, authentic taxanes, e.g. paclitaxel, cephalomannine and baccatin III, from Pacific yew bark. Data from our earlier investigations [12] indicated that the needles from ornamental yew (*Taxus* spp.) shrubs contain paclitaxel and other taxanes in quantities equal to or higher than the amounts

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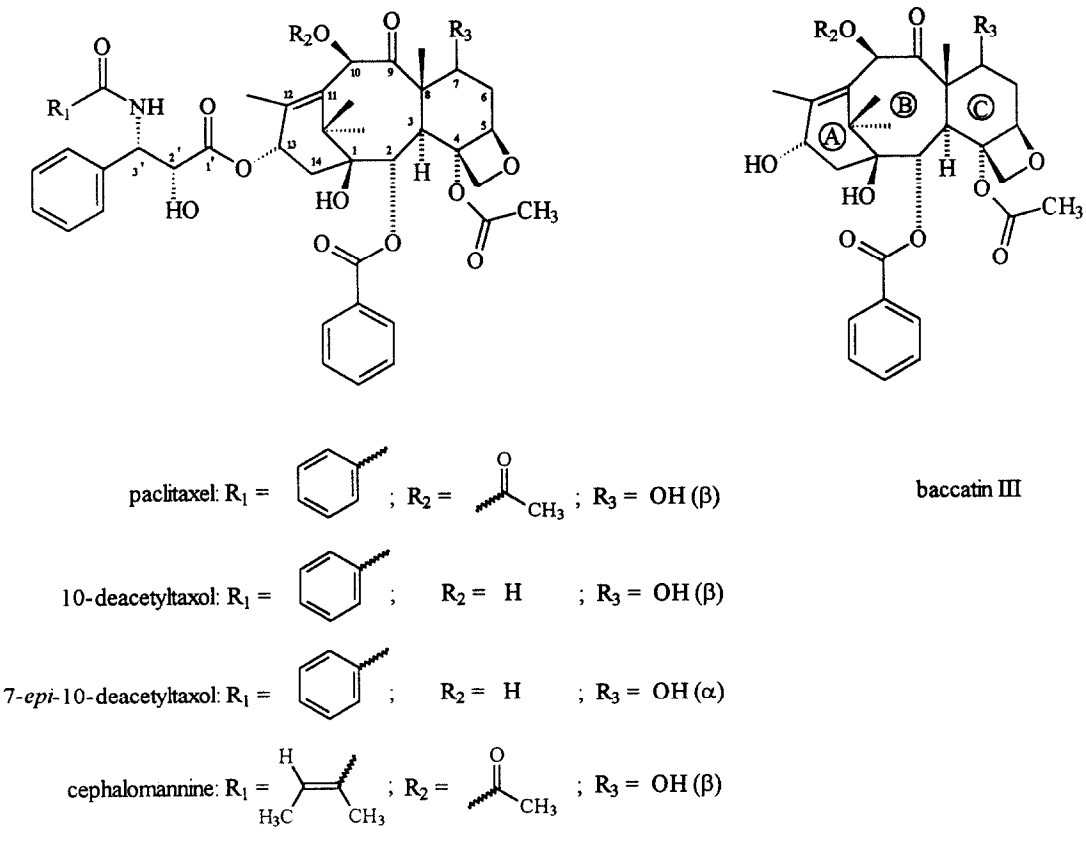

Fig. 1. Structures of taxane diterpenoids.

typically found in Pacific yew bark (i.e., 400 ppm). The development of taxanes as agrochemicals must not compete with supplies of these compounds destined for application as chemotherapeutic anticancer agents. In addition there is considerable expense entailed in the purification of a single, pure taxane, a necessary procedure for pharmaceuticals, which may be unnecessary in the production of taxane agrochemicals. Annual prunings of *Taxus* shrubs, which are a waste product of commercial nursery operations, can serve as the source of yew needles destined for extraction to produce bio-fungicides. Our studies [13] suggest that the fungitoxic efficacy of a mixed-taxane fraction may exceed that of a single pure taxane. These studies on potential taxane bio-fungicides extracted from yew needles require that we improve our methods for obtaining large quantities of the partially purified taxanes. Solid-phase extraction membrane technology permits us to achieve this objective rapidly and economically.

2. Experimental

2.1. Extraction

The extraction procedure has not been altered substantially since our initial report [12]. Clippings from ornamental yew cultivars, such as *Taxus* × *media* "Hicksi" and *T.* × *media* "Nigra" from commercial plantings in Connecticut and Rhode Island, USA, from field research plots at The Connecticut Agricultural Experiment Station's Lockwood Farm, and from greenhouse-rooted cuttings, were collected and stored in plastic bags at 4°C. Within one week of collection the needles were stripped from the twigs. A

small sub-sample of the whole needles was dried to constant mass (typically 16 h) in an oven at 95–100°C solely for moisture determination. The remaining needles were reduced to 3 mm or smaller in size using a laboratory blender. A 3–4-g sub-sample of the well mixed, ground needles was weighed, transferred to an Erlenmeyer flask, and extracted with 100 ml of methanol at ambient temperature for 16 h on a wrist-action shaker. We have observed that 4 g of biomass/100 ml of extraction solvent results in the quantitative extraction of the taxanes. The resulting solution was filtered through Whatman 1 or 2 filter paper, the needles were washed with 25 ml of methanol, and the combined methanolic solution was evaporated to dryness on a rotovap at 40–43°C. The dried residue was reconstituted in 10 ml methanol and 1 ml distilled–deionized water. All water used in our procedures is distilled prior to finishing in a NANOpure II four-cartridge system (Barnstead/Thermolyne Corporation, Dubuque, IA, USA), and is referred to as distilled–deionized (DI-DI) water.

2.2. SPE Partitioning

Three C_{18} SPE techniques were compared for their partitioning efficiency of the crude extract. The eluents for the SPE methods in their order of usage are DI-DI water, 20% methanol, 45 or 50% methanol, and 80% methanol. Most taxanes selectively elute in the methanol–water (80:20) fraction (hereafter referred to as the taxane fraction).

Method 1

High-capacity 6-ml C_{18} disposable extraction columns (7020-07; J.T. Baker, Phillipsburg, NJ, USA) containing 1000 mg of sorbent have been routinely used in our laboratory for clean-up of the crude extract. The cartridge was conditioned with 10 ml ethyl acetate, 10 ml methanol and 10 ml DI-DI water. It is critical that a minimum of 2 mm of water remain above the top frit in the cartridge at the time the crude extract is transferred to the cartridge. After transfer of 0.5 ml of crude extract, the cartridge was eluted with 5 ml DI-DI water, 10 ml 20% methanol, 10 ml 50% methanol and 10 ml 80% methanol.

Method 2

Ultra-high-capacity C_{18} columns have been prepared in our laboratory by packing 4 g of C_{18} 40 μm sorbent (7025-00, J.T. Baker) into a 6-ml disposable polypropylene filtration column (7121-06, J.T. Baker). The sorbent bed is fitted with 20-μm polyethylene frits at the top and bottom. Conditioning was accomplished with 40 ml ethyl acetate, 40 ml methanol and 40 ml DI-DI water. Once again a sufficient layer of DI-DI water must remain above the top frit at the time of addition of <4.0 ml of crude extract to the cartridge. The cartridge is then eluted with 20 ml DI-DI water, 40 ml 20% methanol, 40 ml 50% methanol and 40 ml 80% methanol.

Method 3

Empore (3M Corp., St. Paul, MN, USA) extraction disks, 47 mm size (7460-06, J.T. Baker) are used in conjunction with a Millipore all-glass filtration apparatus (XX15 047 00; Millipore, Bedford, MA, USA). To avoid clogging the Empore disk with particulate matter from the crude extract, a 47-mm polypropylene separator with 10-μm pore size (61757; Gelman Sciences, Ann Arbor, MI, USA) is placed on top of the Empore disk before the top reservoir is clamped in place. Conditioning is accomplished with 15 ml ethyl acetate, 15 ml methanol and 15 ml DI-DI water under gentle vacuum. Under very gentle vacuum (e.g., 1–2 mmHg; 1 mmHg = 133.322 Pa) 10 ml DI-DI water are added to the reservoir followed immediately by 7 ml of crude extract. Partitioning was accomplished under gentle vacuum with 15 ml DI-DI water, 15 ml 20% methanol, 15 ml 45% methanol and 20 ml 80% methanol.

2.3. HPLC Analysis

The taxane fraction was well shaken and filtered through a 2-μm external filter tip (FT

4004; Centaur West, Sparks, NV, USA) into an automatic liquid sampler vial. The HPLC system consists of a Perkin-Elmer (Norwalk, CT, USA) binary LC250 pump, fitted with a ISS-100 autosampler and LC235 dual-channel photodiode array detector. The peaks recorded at 230 nm are used for quantitation; the LC trace at 280 nm is also recorded. Separation is achieved on a MetaChem (Torrance, CA, USA) 250 × 4.6 mm, 5 μm Taxsil analytical column (0335-250 × 046) preceded by a Taxsil guard cartridge (0335-CS). The column oven is maintained at 32°C, the flow-rate at 1 ml/min, reservoir A contains 100% acetonitrile (Omnisolv; EM Science, Gibbstown, NJ, USA), and reservoir B contains water–methanol (70:30) (Omnisolv, EM Science). The injection volume is 10 μl and the following linear gradient elution program is used (program A):

0 to 15 min: isocratic at 41% A
15 to 20 min: ramped to 65% A
20 to 30 min: isocratic at 65% A
30 to 35 min: ramped to 41% A
35 to 40 min: isocratic at 41% A

Alternatively, paclitaxel can be resolved from 7-*epi*-10-deacetyltaxol on the MetaChem Taxsil analytical column if the following parameters are employed: 100% acetonitrile in reservoir A, water–methanol (90:10) in reservoir B; and the following linear gradient elution program (program B):

0 to 15 min: isocratic at 43% A
15 to 20 min: ramped to 65% A
20 to 30 min: isocratic at 65% A
30 to 35 min: ramped to 43% A
35 to 40 min: isocratic at 43% A

Stock standard solutions in methanol (Omnisolv, EM Science) are prepared from authentic compounds received from the National Cancer Institute. Quantitation is by external standard using software on the PE Nelson 1020 personal integrator with 10-μl injections of either two or three standard solutions plus the origin. Day-to-day variation of the response factors for the three taxanes, paclitaxel, cephalomannine, and baccatin III, is typically < 10% and the correlation coefficient for the calibration curves is typically > 0.995.

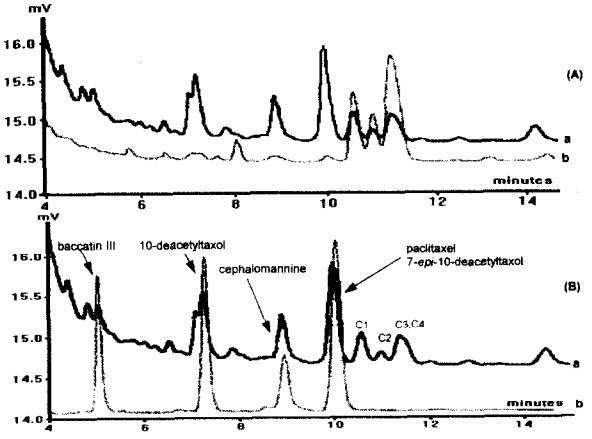

Fig. 2. LC traces using program A of (A) taxane fraction recorded at 230 nm (a) and 280 nm (b) and (B) taxane fraction (a) vs. 5-taxane standard (b) at the following concentrations: baccatin III, 16.06 ng/μl; 10-deacetyltaxol, 24.99 ng/μl; cephalomannine, 14.32 ng/μl; paclitaxel, 15.67 ng/μl; 7-*epi*-10-deacetyltaxol, 23.64 ng/μl. Peaks C_1, C_2, C_3, C_4 are assumed to be cinnamyl taxanes.

3. Results

It is well known that HPLC on C_{18} analytical columns fails to resolve paclitaxel (with λ_{max} = 230 nm) from cinnamyl taxanes (with λ_{max} = 280 nm) [14–16]. Fig. 2A shows the LC traces of the taxane fraction recorded at both 230 and 280 nm using program A. Fig. 3 shows the UV spectra of relevant peaks in the taxane fraction (peaks B and C) and the authentic paclitaxel standard

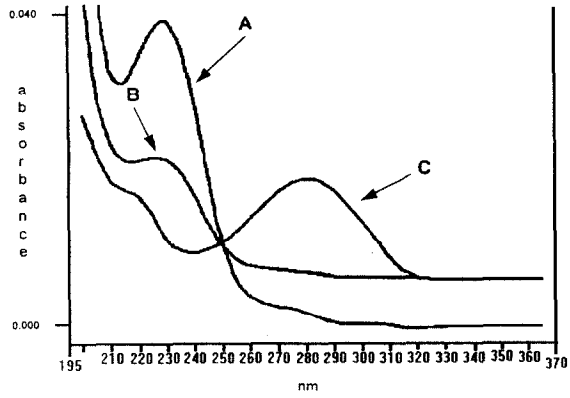

Fig. 3. UV spectra: A = paclitaxel from authentic standard; B = paclitaxel from taxane fraction; C = cinnamyl taxane from taxane fraction.

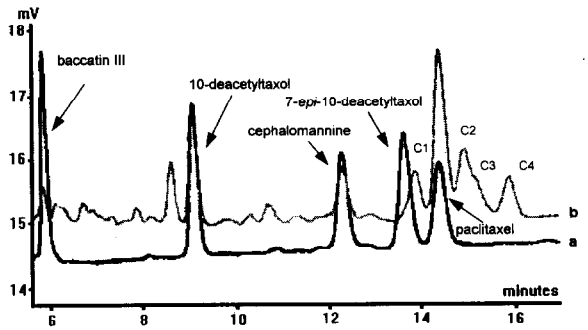

Fig. 4. LC traces using program B recorded at 230 nm of taxane fraction (b) and 5-taxane standard (a).

(peak A). The absence of any significant absorption at 280 nm in the paclitaxel peak in the taxane fraction (Fig. 2A; Fig. 3) confirms that the MetaChem Taxsil column together with program A not only provides rapid analysis times, but also resolves paclitaxel from the cinnamyl derivatives. Under these conditions, however, the retention times of paclitaxel and 7-*epi*-10-deacetyltaxol are equal (Fig. 2B). Fig. 4 shows that changing to the conditions given above in program B resolves these two compounds. Since the use of program B permits us to conclude that the amount of 7-*epi*-10-deacetyltaxol typically found in *Taxus* needles is quite small, our routine quantitation of the taxane fractions by means of program A gives accurate values of paclitaxel and taxane analogues. Program A is the quantitation program of choice since it permits baseline resolution of paclitaxel from the cinnamyl derivatives. Using the procedures above more than 1000 injections have been made on the system with only three changes of the Taxsil guard column.

Table 1 compares relevant parameters for the different SPE techniques. In our earlier studies [12] we showed by means of spiking that recovery of paclitaxel is quantitative on the high-capacity C_{18} cartridges within the limits given in the table. By using the ultra-high-capacity cartridges prepared in our laboratory, the volume of crude extract which may be partitioned is increased. Once again spiked recoveries and HPLC analysis of all eluates indicate that the less polar taxanes elute quantitatively from these ultra-high-capacity cartridges in the methanol–water (80:20) fraction within the limits given in Table 1, second column. The data in the third column of Table 1 show that the C_{18} Empore extraction disks significantly increase the volume of crude extract which may be partitioned, while maintaining quantitative recovery of the less polar taxanes.

4. Discussion

Quantitative extraction of the taxanes from *Taxus* needles must obviously be followed by

Table 1
Comparison of parameters for C_{18} SPE partitioning of crude extract from *Taxus* needles

	High-capacity cartridge	Ultra-high-capacity cartridge	47-mm Disk
Volume crude (ml)	0.5	3.5–3.9	7.0
Volume water (ml)	5	20	15
Volume 20% methanol (ml)	10	40	15
Volume 45% methanol (ml)	0	0	15
Volume 50% methanol (ml)	10	40	0
Volume 80% methanol	10	40	20
Paclitaxel recovery (%)[a]	104.0 ± 4.0 (n = 4)	103.5 ± 13.0 (n = 2)	115.8 ± 5.3 (n = 2)
Paclitaxel recovery (%)[b]			89.5 ± 0.1 (n = 2)
Estimated cost/ml of crude extract (US$)	5.70	2.80	1.00

[a] Authentic paclitaxel spiked directly into crude extract prior to partitioning on SPE.
[b] Recovery based on paclitaxel concentration calculated from partitioning on J.T. Baker 7020-07 high-capacity cartridge.

removal of co-extractives without loss of the target compounds. Although liquid–liquid partitioning of the crude *Taxus* extract has been used [14], fractional elution on SPE cartridges has distinct advantages, saving time and reducing solvent usage [17]. Our investigations into the effects of taxanes on non-mammalian cells demanded that we produce large quantities of the taxane fraction efficiently and economically. SPE membrane technology permits us to achieve these goals.

SPE cartridges have been in use since 1978 [18]. In typical environmental applications 1 l of water can be extracted on the reversed-phase high-capacity C_{18} cartridges. When used in our laboratory to partition the crude *Taxus* extract, however, the optimum capacity of the high-capacity C_{18} cartridges for the methanolic crude extract is 0.5 ml. Larger volumes of the crude extract, up to 1 ml, may be partitioned on this cartridge. However, taxane breakthrough is possible close to this upper volume limit. Similarly, the ultra-high-capacity C_{18} cartridges prepared in our laboratories limit the volume of crude partitioned, are expensive, and even with vacuum filtration assist, tend to be slow because of the large amount of flocculate in the crude extract. Although, after adequate washing of the C_{18} sorbent, reloading of the crude extract on both cartridge types may be feasible, the additional time and solvent usage required reduces the practicality of this approach compared with the SPE membrane procedure.

Membrane SPE is a relatively new technique [19] introduced in 1989, which we decided to explore in our attempt to partition larger quantities of the crude *Taxus* extract more rapidly and economically. Some modifications of the cartridge approach were necessary to achieve quantitative recovery of the principal taxanes in the methanol–water (80:20) cluate from the Empore disk. First, a 10-μm pore size polypropylene separator was necessary to prevent the flocculate's clogging the 0.006-μm pores of the Empore disk. Rapid clogging of smaller pore size glass microfilters, e.g. Whatman GMF150 1 μm (1841-047; Whatman LabSales, Hillsboro, OR, USA) and glass bead filtration aids, e.g. 3M Empore filter aid 400 (Varian 1214-6005 or J.T. Baker 7467-01), by the crude extract rendered these products useless for our application. Although we are able to partition 7 ml of the crude extract on a single 47-mm disk without taxane breakthrough, it is essential that approximately 10 ml of water be maintained in the reservoir during the addition of the crude extract under very gentle vacuum. Furthermore, the 50% methanol eluent, which results in small but measurable breakthrough of the taxanes, must be replaced by a methanol–water (45:55) eluent. Under these conditions HPLC analysis of the loading solution eluate, the water eluate, the methanol–water (20:80) eluate, and the methanol–water (45:55) eluate shows no breakthrough of taxanes with retention times >6 min, namely, 10-deacetyltaxol, cephalomannine and paclitaxel. The HPLC analyses of these eluates did indicate, however, that small amounts of baccatin III are present, with the major amount in the methanol–water (45:55) or in the methanol–water (80:20) eluates. Finally, recovery of authentic paclitaxel spiked into the crude extract prior to partitioning on the Empore disk was quantitative in the taxane fraction (methanol–water, 80:20).

In summary, as may be seen in Table 1, there are several advantages of the SPE membrane versus cartridge methodology: total solvent volume/ml crude extract processed is reduced by more than 80%; a significant reduction in the cost of processing a given volume of crude, significant reduction in sample processing time, and a consistently high spike recovery are all achieved. Also, the LC traces of the taxane fraction from the cartridge and the Empore disk methods, shown in Fig. 5, are identical. Therefore, qualitatively and quantitatively SPE membrane technology provides the same taxane fraction as does the SPE cartridge.

We anticipate that this approach may be enhanced by stacking the 47-mm disks and/or using the 90-mm disks now available. Together with the three- and six-position filtration manifolds available from several manufacturers, SPE membrane technology will permit us to collect

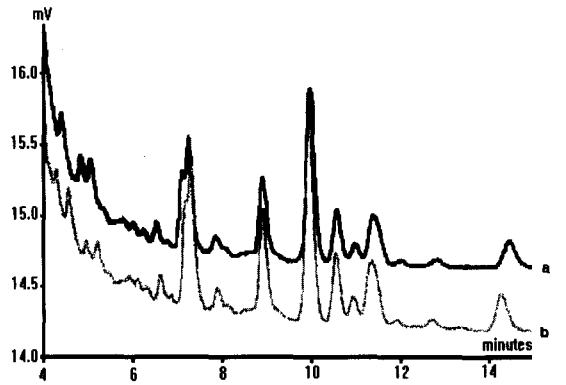

Fig. 5. Overlay of taxane fraction eluted from high-capacity C_{18} cartridge (a) with taxane fraction eluted from 47-mm Empore disk (b).

sufficient amounts of a multi-taxane fraction for examination of its effects on non-mammalian eukaryotic systems.

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