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Short Communication

Separation of taxol from related taxanes in *Taxus brevifolia* extracts by isocratic elution reversed-phase microcolumn high-performance liquid chromatography

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ABSTRACT

Previous attempts to resolve the closely related taxanes cephalomannine and taxol by reversed-phase high-performance liquid chromatography (HPLC) on octadecyl silica columns have not been successful. In the present study, high-resolution HPLC on microcolumns packed with octadecyl silica was evaluated for the separation of the aforementioned taxanes. Due to the enhanced separation efficiency of this technique, baseline resolution between cephalomannine and taxol was readily achieved under isocratic elution conditions. Chromatographic profiles of *Taxus brevifolia* twig and needle foliage extracts are presented that illustrate this separation.

INTRODUCTION

Taxol is a taxane diterpene amide produced by the Pacific yew (*Taxus brevifolia*) [1]. This natural product shows promise for the treatment of a variety of human cancers, including leukemia and certain ovarian, breast and lung tumors [1–3]. The mode of action of taxol stems from the unique propensity of this compound to promote microtubule formation and inhibit post-mitotic spindle disassembly [4]. Phase II clinical tests of taxol, which are currently in progress, will require kilogram quantities of pure compound [5]. Presently, taxol is available only from limited natural sources. Efforts to synthesize taxol have not been fruitful, primarily because of difficulties encountered in forming the taxane skeleton [6]. For this reason there has been a great deal of interest in isolating other taxanes from *T. brevifolia* for use as intermediates in the synthesis of taxol. Taxol is isolated from the bark of *T. brevifoolia* by extraction, solvent partitioning and preparative-scale chromatography [7]. Taxol constitutes less than 0.01% of the bark on a dry weight basis [5]. The processing of bark from 2000 to 3000 trees is required to produce approximately 1 kg of taxol [3]. The sheer magnitude of required plant material has prompted ecological concerns about the poten-

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tial impact of massive harvesting on Pacific yew populations. The limited geographical distribution, the slow maturation, and specific niche requirements of this species all contribute to these concerns. Bark stripping holds no advantage over lumbering, as the bark-stripped trees eventually perish. Isolation of taxol from portions of the tree that are capable of regrowth (such as the needle foliage) would be particularly attractive, since harvesting would not decimate the stock. It seems likely that future research will focus on genetic methods aimed at maximizing taxol yield as well as detailed analytical studies focusing on taxol distribution within the plant.

Analysis of taxol in crude bark extracts of *T. brevifolia* presents a challenge for the separation scientist because a bewildering array of indigenous components is present. Analysis of needle foliage extracts becomes even more difficult due to the presence of large amounts of photosynthetic pigments and cuticular waxes. Taxol is one of a series of compounds contained in *T. brevifolia* extracts that share the taxane ring system. One of these taxanes, cephalomannine, is present in significant quantities and is difficult to separate from taxol [5]. The structures of taxol and cephalomannine are shown in Fig. 1. Past work by Witherup and co-workers investigated the utility of high-performance liquid chromatography (HPLC) for the analysis of taxol in bark [5] and needle foliage extracts [8]. Chromatography on 25 cm × 4.6 mm I.D. columns packed with 8-μm particles of octadecyl-, cyanopropyl- or phenyl-bonded silica was studied under either isocratic or gradient reversed-phase elution conditions. These authors found that the added selectivity of the phenyl or cyanopropyl silica was necessary to provide baseline resolution between taxol and cephalomannine. Baseline resolution between these two compounds was not achieved on the octadecyl silica stationary phase [5].

Although cyanopropyl- and phenyl-bonded silica columns provide the requisite resolution between taxol and cephalomannine, there are several shortcomings associated with the separation of crude extracts on these columns. Many extract components elute after taxol when separated on phenyl or cyanopropyl silica columns. As a consequence, extended gradient runs are required to ensure elution of strongly retained components. Additionally, elution of taxol and cephalomannine occur on a background that is significantly above the initial baseline. This baseline offset is most likely caused by elution of numerous unresolved matrix components. Further development of separations on octadecyl silica has not been pursued due to the initial failure of conventional columns to provide resolution between cephalomannine and taxol. However, provided that the column efficiency is sufficient to allow resolution between taxol and cephalomannine, separations on octadecyl silica may prove advantageous over separations performed on cyanopropyl- or phenyl-bonded silica.

The present study evaluates high-resolution microcolumn HPLC on octadecyl silica, as described by Novotny [9,10], Yang [11], and others [12–14], for the separation of taxol in crude *T. brevifolia* extracts. This technique utilizes fused silica tubing (1 m × 250 μm I.D.) that is slurry packed with 5-μm particles to provide columns that approach separation efficiencies of 100 000 theoretical plates [15]. High-resolution microcolumn HPLC has been applied to a variety of complex environmental [16,17] and biological [11,18] samples allowing for separations that cannot be achieved within the resolution constraints imposed by columns of conventional (25 cm × 4.6 mm I.D.) dimensions. In addition to

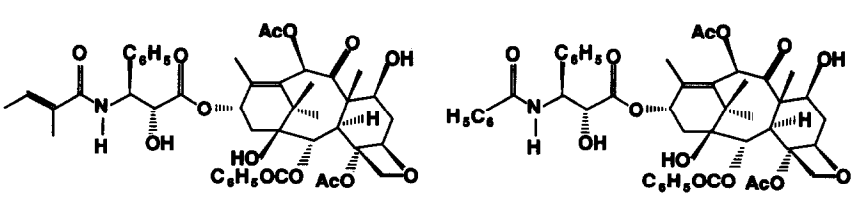

Fig. 1. Structures of cephalomannine and taxol. Ac = Acetyl.

the enhanced resolution, microcolumn separations offer a number of inherent advantages including minute sample volume requirements, enhanced mass flow detection sensitivity, and the feasibility of utilizing expensive "exotic" mobile phases [9]. Importantly, the low flow-rates characteristic of microcolumn HPLC greatly simplify introduction of the column eluate to mass spectrometric analyzers [19,20].

The objective of the present study was to evaluate high-resolution microcolumn HPLC as applied to the analysis of taxol in crude twig and needle foliage extracts of *T. brevifolia*. With over five times the available separation efficiency, as compared to columns of conventional dimensions packed with 8-μm particles, it becomes possible to resolve taxol and cephalomannine on octadecyl silica under isocratic elution conditions.

EXPERIMENTAL

Cephalomannine and taxol standards

Standards of taxol were donated by Dr. D. Ellis of the Department of Horticulture, University of Wisconsin, USA, and the National Cancer Institute (NCI), Bethesda, MD, USA. Cephalomannine was donated by NCI.

Tissue collection and extraction

T. brevifolia selected for analysis were located on the Oregon State University, McDonald Experimental Forest, near Covallis. In October 1990, branches from five trees were harvested and returned immediately to laboratory where twigs from 1989 and 1990 and their needles were separated for each tree. Tissues were placed in an oven maintained at 50°C to dry overnight. Dry tissues were ground with a Wiley mill to pass a 20-mesh screen and stored for less than a week at room temperature until extracted.

Extraction of tissue samples was in accordance with the procedure set forth by Witherup *et al.* [5]. Before weighing, each sample was redried for 1 h at 50°C and cooled to room temperature. Approximately 2 g were accurately weighed into a flask, covered with 20 ml of methanol, and placed on a shaker at room temperature for 1 h. The supernatant was decanted and centrifuged to remove large particulates. A 10-ml aliquot of the methanol extract was evaporated to dryness at 40°C under vacuum. The residue was next partitioned between methylene chloride and water (10 ml each). A measured aliquot of the methylene chloride layer was then dried under vacuum. Samples were reconstituted with methanol and filtered through a 0.45-μm nylon 66 filter (Alltech, Deerfield, IL, USA) immediately before analysis.

Microcolumn instrumentation and modifications

The microcolumn HPLC system consisted of an Isco μLC-500 syringe pump (Lincoln, NE, USA) and Schoeffel Model 770 detector (Westwood, NJ, USA) that was modified to meet the technique's stringent low-dead-volume requirements. The modified flow cell was centered within a screw-mounted aluminum block that contained a 345-μm machined slit. This block housed a length of 250 μm I.D. fused silica (345 μm O.D.) that had a 1.0-cm section of polyimide coating removed to serve as a low-dead-volume quartz cell. A section of 50 μm I.D. (189 μm O.D.) fused silica was inserted into the 250-μm tube up to the point of the quartz window. This insert was secured to the 250-μm tube with epoxy and further minimized the detector dead volume.

Column preparation and evaluation

Fused-silica tubing of 344 μm O.D. × 250 μm I.D. and 189 μm O.D. × 50 μm I.D. was obtained from Polymicro Technologies (Phoenix, AZ, USA). Columns (1 m × 250 μm I.D.) were slurry packed with Spherisorb 5-μm ODS-2 (Phase Separations, Norwalk, CT, USA) and evaluated according to the methods described by Borra *et al.* [15]. The column bed was held in place by a porous PTFE frit, which in turn was held stationary by a length of 50-μm fused silica inserted into the 250-μm column and cemented with epoxy to prevent leaking [21]. The column was joined to the detector cell by butting the ends of the 50-μm fused-silica tubes together within a snug-fitting PTFE sheath [22].

Chromatographic conditions

All injections were accomplished by the stopped-flow technique [13]. Capillary injection volumes were kept below 200 nl. Mobile phases, prepared from Burdick & Jackson (Muskegon, MI, USA) HPLC-grade solvents, were filtered through

0.45-μm nylon 66 filters and degassed with helium flow prior to use. Columns were evaluated with a mobile phase consisting of acetonitrile–water (60:40). During evaluation, the mobile phase was delivered at a constant flow-rate of 1.5 μl/min and components detected by UV absorption at 254 nm. For analysis of *T. brevifolia* extracts, the mobile phase composition was methanol–water–acetonitrile (30:30:40) delivered at a constant pressure of 3500 p.s.i. This corresponded to a flow-rate of approximately 2.5 μl/min. The separated *T. brevifolia* components were detected by absorption at 225 nm.

RESULTS AND DISCUSSION

Column evaluation was conducted with a standard mixture containing acidic, basic, and neutral compounds as described previously [15]. The chromatogram of the test mixture is presented in Fig. 2. A theoretical plate calculation performed on the phenanthrene peak indicated a separation efficiency of 85 000 plates. This separation efficiency falls within the range of 70 000 to 90 000 theoretical plates that is typically realized for 1-m columns packed with 5-μm particles. This efficiency was judged adequate for the ensuing taxol separations. Separations performed on the microcolumn system described above offered at least 5.44 times the separation efficiency compared to the columns containing 8-μm material that were utilized by Witherup *et al.* [5].

A variety of chromatographic conditions were evaluated for the separation of cephalomannine and taxol standards. The objective was to obtain baseline resolution between these taxanes under isocratic elution conditions within a reasonable analysis time. Injections of pure standards with a methanol–water–acetonitrile (30:30:40) mobile phase, delivered at 3500 p.s.i., revealed that cephalomannine eluted with a retention time of 57.5 min, whereas taxol eluted at 61.2 min. The chromatogram shown in Fig. 3 illustrates a separation of a mixture containing cephalomannine and taxol standards. These taxanes were separated by 3.7 min under the chromatographic conditions employed. The ability of microcolumn HPLC to provide baseline resolution between these compounds is a direct consequence of the higher resolution available from the technique. Previous studies have shown that the related taxanes 10-deacetylcephalomannine, baccatin III, and 10-deacetylbaccatin III are readily resolved and elute well before cephalomannine on columns packed with octadecyl silica [5].

Analysis of *T. brevifolia* extracts proceeded under the chromatographic conditions described above. A representative chromatogram of twig extract is presented in the top of Fig. 4. The peaks labeled 1 and 2 correspond to the retention times of cephalomannine and taxol, respectively. The identity of these peaks was further established by co-elution of the respective taxane standards with these peaks. The bottom of Fig. 4 presents a chromatogram resulting

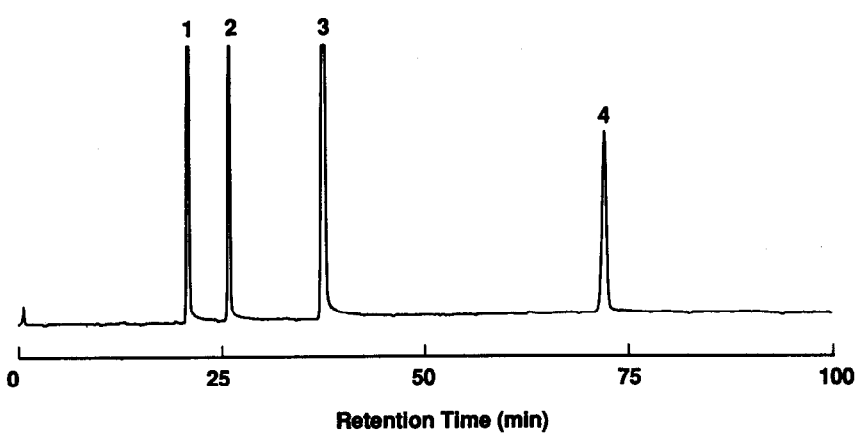

Fig. 2. Chromatogram of column evaluation test mixture. Peaks: 1 = *m*-cresol; 2 = nitrobenzene; 3 = diphenylamine; 4 = phenanthrene.

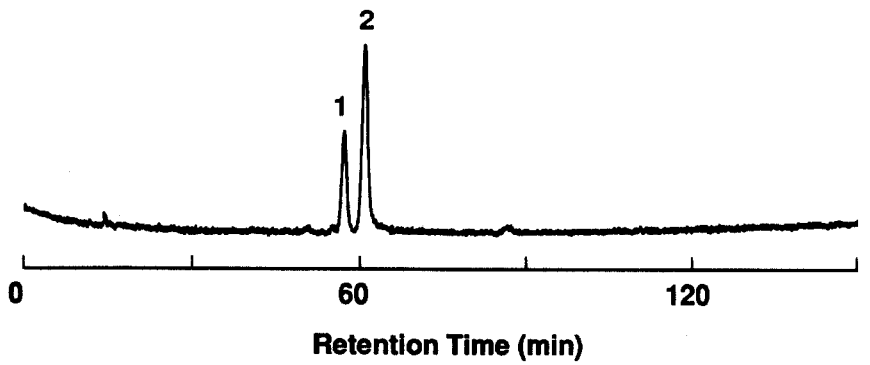

Fig. 3. Separation of cephalomannine (1) and taxol (2) standards.

from an injection of needle foliage extract. The identity of cephalomannine and taxol in this extract was also verified by co-injection experiments. The higher complexity of the needle foliage extract is clearly evident by comparison of the two chromatograms in Fig. 4. Cephalomannine and taxol are baseline resolved in extracts from both twigs and needles and, judging from the symmetrical peaks, appear to elute free of perceptible interferences. It appears that microcolumn HPLC on octadecyl silica has the separation power necessary for quantification of taxol in crude extracts of *T. brevifolia*. Despite this capability, however, it remains desirable to develop an intermediate normal-phase clean-up of crude extracts prior to reversed-phase chromatographic analysis. An intermediate orthogonal separation would be expected to remove considerable matrix interferences.

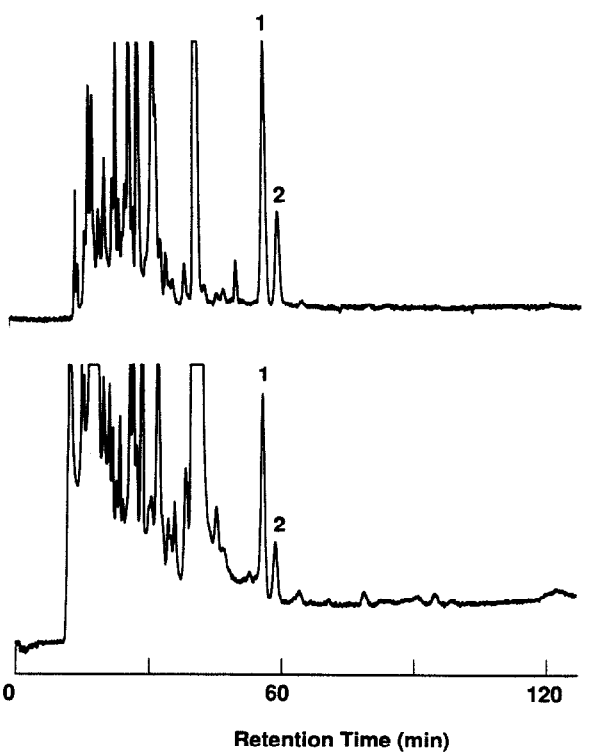

Fig. 4. Chromatographic profiles of *T. brevifolia* twig (top) and needle foliage (bottom) extracts. Peaks 1 and 2 correspond to cephalomannine and taxol, respectively.

The separations obtained in this study offer several unique attributes over those performed on cyanopropyl- and phenyl-bonded silica. Chromatograms in Fig. 4 were allowed to proceed for 2 h to demonstrate that very little material contained in the crude *Taxus* extracts elute after taxol. This elution pattern stands in sharp contrast to separations performed on cyanopropyl- and phenyl-bonded silica columns. High-resolution separations on octadecyl silica result in elution of taxol and cephalomannine within a clean retention window that is considerably removed from the majority of matrix components. Additionally, cephalomannine and taxol elute in a region that has a minimal baseline offset. Microcolumn sample analysis times of slightly over 60 min were approximately equal to previously reported gradient elution methods performed on conventional columns.

Although the objective of the above separations was the separation of taxol and cephalomannine, the same microcolumn techniques would be valuable for analysis of *T. brevifolia* extracts for other

known and hitherto unidentified taxanes. Toward this end, the implementation of high-resolution separations combined with either electrospray ionization [20] or continuous flow fast atom bombardment [19] mass spectrometry would be particularly powerful for structural elucidation. In addition, a variety of mass spectral techniques could be employed to minimize or eliminate matrix interferences during quantification. Our laboratory is presently engaged in this pursuit.

These studies have demonstrated the ability of microcolumn HPLC to provide resolution between closely related natural products that cannot be obtained on columns of conventional dimensions packed with the same octadecyl silica stationary phase. The conclusions of this study do not serve to undermine the sound approach of exploiting optimal selectivity available from different bonded stationary phases, but rather emphasizes that such an approach will always be more effective when used in conjunction with high-resolution chromatographic techniques. Recent advances in microcolumn HPLC separations include descriptions for preparing a variety of bonded-phase columns [23], applications of paired-ion mobile phase modifers [24], and further refinement of gradient elution strategies [25,26]. Progress in these directions ensures that the flexibility responsible for the popularity of conventional HPLC will also be available for high-resolution microcolumn techniques. This emerging technology can be expected to have a significant impact on the isolation and identification of natural products.

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