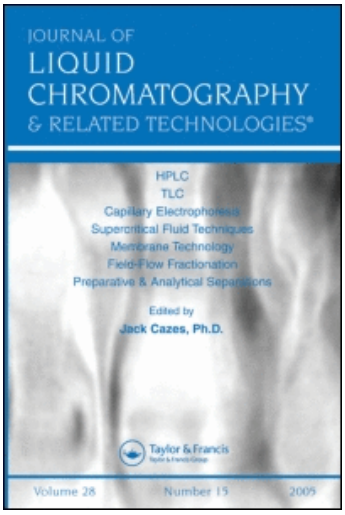

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Rapid Isocratic Reversed Phase HPLC of Taxanes on New Columns Developed Specifically for Taxol Analysis

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RAPID ISOCRATIC REVERSED PHASE HPLC OF TAXANES ON NEW COLUMNS DEVELOPED SPECIFICALLY FOR TAXOL ANALYSIS

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Abstract

We describe a new HPLC method for detection of taxanes from crude extracts, using new specialty columns developed for HPLC analysis. Taxol [**1**] and cephalomannine [**2**] were separated in 13 minutes using an isocratic elution. Additionally, there is baseline separation between cephalomannine [**2**], 7-epi-10-deacetyl-taxol [**3**], and taxol [**1**]. Elution of 7-epi-taxol [**4**] occurs in 20 minutes. We compared chromatography of taxanes on specialty, C18, and phenyl columns. This isocratic HPLC method permits rapid analysis of large numbers of crude extracts, with excellent resolution of taxanes, enabling the detection of 7-epitaxol [**4**] in 20 minutes.

Introduction

As the anti-mitotic, chemotherapeutic drug, taxol, continues to show promise in clinical trials, current estimates are that worldwide demand for the will be 500 kg per year (M. Suffness, personal communication). It will require the harvest of 1.5 **million** trees to produce this amount of the drug from *Taxus brevifolia* bark, based on current yield estimates of three trees per gram of taxol (2). In 1989, it was estimated that there were enough trees in the Pacific and Rocky Mountain Northwest to meet taxol demand for 5-10 years (2). Those estimates, however, were based on a worldwide demand of 50 kg of taxol per year. Clearly alternative sources for taxol must be found.

Among the promising alternatives for obtaining taxol are isolation of taxanes from the regenerable stems and needles of *T. brevifolia* (3,4) and partial or complete synthesis of taxol similar to the approach used to produce taxusin from patchouli alcohol (5). A third strategy is to produce taxanes from living cells isolated from *Taxus* species through plant cell tissue culture (6,7). One prerequisite for using plant tissue culture to produce taxol or other taxanes is the ability to screen a large number of genetically distinct cell lines and tissue samples for their taxane content. The HPLC method described here allows us to screen up to 400 samples per week, on a single instrument, for taxane content.

MATERIALS

Taxane Standards

Authentic samples of baccatin III [**5**], 10-deacetylbaccatin III [**6**], 10-deacetyltaxol [**7**], 7-xylosyl-10-deacetyltaxol [**8**], cephalomannine [**2**], 7-epi-10-deacetyltaxol [**3**], taxol [**1**], and 7-epi-taxol [**4**] were very generously provided by Dr. David T. Bailey, Hauser Chemical Research, Inc., Boulder, CO. Taxanes were provided as methanolic solutions with approximate concentrations provided. Additional powdered samples of baccatin III [**5**], cephalomannine [**2**], and taxol [**1**] were provided by Dr. Kenneth M. Snader, NCI, Bethesda, MD. A test mixture containing all of the taxanes at an approximate concentration of 1 µg/ml in methanol was used to evaluate the performance of the HPLC columns. A test mixture using just baccatin III, cephalomannine, and taxol was used at higher concentrations (approximately 100 µg/mL) in some cases. All solvents used were HPLC-grade and filtered through 0.2 µm Nylon 66 filters (Gelman) prior to use (1).

HPLC Columns

A number of specialty columns produced by Phenomenex (Torrance, CA) were evaluated for taxane separation. These Taxsol™ columns were available in varying lengths, and particle sizes, and proprietary bonded phases optimized for taxane analysis. The columns evaluated were: Phenomenex 4 µm, 250 x 4.6 mm Taxsol™ F; Metachem 5 µm, 250 x 4.6 mm Taxsil™; Phenomenex 5 µm, 250 x 4.6 mm IB-Sil™ phenyl; Phenomenex 3 µm, 250 x 4.6 mm Ultremex™ phenyl; and Supelco 5 µm, 250 x 4.6 mm Supelcosil™ LC-18. Analyses were performed without guard columns.

Apparatus

Instrumentation consisted of a Beckman Model 126 binary pump and gradient mixer, Beckman Model 168 Diode Array Detector, and Beckman Model 507 autosampler. Data acquisition, processing, integration, and instrument control was with a Gateway 2000 computer with 80486 microprocessor running Beckman System Gold v. 6.07 software.

METHODS

Callus Extracts

In this analysis, a 4.295 g (fresh weight) sample of *Taxus brevifolia* callus cell line 4aYAC was ground in a glass tissue homogenizer with 5 mL $CHCl_3$. This slurry was centrifuged at 1800 x g for 5 min to facilitate separation of the two phases. $CHCl_3$ layer was removed and the remaining cell slurry was ground, again, with an additional 5 mL $CHCl_3$. Organic phases were combined, water was removed over anhydrous $MgSO_4$, and the extract was evaporated to dryness on a Büchi rotary evaporator. The residue was extracted with four 100 µL washes of MeOH. This final solution was filtered through a 0.2 µm filter prior to HPLC analysis of a 10 µL sample.

Column Conditions

For both the Phenomenex 5 µm IB-Sil™ phenyl and Supelco 5 µm Supelcosil™ LC-18 isocratic reversed phase analyses, modifications of the methods of Witherup, *et al.* (8) were used. A 10 µL sample of a taxane standard solution containing 100 µg/mL baccatin III [**5**], 130 µg/mL cephalomannine [**2**], 110 µg/mL taxol [**1**] in methanol was injected on the column with 60:40 MeOH:H_2O as the mobile phase, flow rate of 1 mL/min, temperature was 30° C, and detection at 228 nm.

Gradient elution of taxanes from the Phenomenex 5 µm IB-Sil™phenyl column was also a modification of the Witherup, *et al.* (8) method. The taxane standard mixture consisted of 119 µg/mL baccatin III [**5**], 115 µg/mL cephalomannine [**2**], 112 µg/mL taxol [**1**] in methanol. A 10 µL sample of these standards was injected on the 5 µm phenyl column and eluted with a linear gradient. Mobile phase flow rate was 1.0 mL/min, temperature was 30° C. Peaks were detected at 227 nm. The gradient profile began at 12.5 mM sodium acetate buffer, pH 4.5: acetonitrile (85:15). The gradient progressed linearly to NaOAc:CH_3CN

(65:35) over 20 min. At 20 min the gradient changed linearly to NaOAc:CH_3CN (52.5:47.5) over 10 min and remained there for an additional 15 min. At 45 min the gradient returned to NaOAc: CH_3CN (85:15), linearly over 1 min, where the column equilibrated for 10 min before another injection.

The Phenomenex 3 µm Ultremex™ phenyl column also used a modification of the Witherup, *et al.* (8) method. Taxane standard mixture was the same as that used on the 5 µm phenyl column. A 10 µL sample of these standards were injected on the phenyl column and separated with gradient elution. Column flow rate was 0.75 mL/min, temperature was 30° C. Peaks were detected at 232 nm. Gradient profile began at acetonitrile:water (25:75) and increased linearly to CH_3CN:H_2O (60:40) over 35 min. At 45 min the gradient changed to CH_3CN:H_2O (25:75) over 1 min and equilibrated there for 9 min before the column was ready for another injection.

Both the Metachem 5 µm Taxsil™ column and the Phenomenex 4 µm Taxsol™ F column were operated under identical conditions. A 10 µL sample of a taxane standard solution containing approximately 3 µg/mL each of baccatin III [**5**], cephalomannine [**2**], and taxol [**1**] and 1 µg/mL each of 10-deacetylbaccatin III [**6**], 10- deacetyltaxol [**7**], 7-xylosyl-10-deacetyltaxol [**8**], 7-epi-10-deacetyltaxol [**3**], and 7-epi-taxol [**4**] in methanol was injected on a column with acetonitrile:10 mM sodium acetate (47.5:52.5) as the mobile phase, temperature 30°C, flow rate of 1 mL/min, and detection at 228 nm.

RESULTS AND DISCUSSION

Absorption spectra of all of the taxane standards that were available to us were remarkably consistent in the presence of an absorption minimum at 210-212 nm and an absorption maximum at 228-232 nm and virtually no other details in the spectrum up to 600 nm - the limit of our diode array detector (Figure 1A). The combination of retention time and absorption spectrum provided us with a rapid means of accurately detecting and quantifying taxanes in our crude extracts. It was particularly valuable in distinguishing between authentic taxanes and unknown contaminants that occasionally coeluted with the taxanes. These contaminants had similar spectra to the authentic standards but with the addition of a second absorption maximum at 280 nm.

Our early work with HPLC separation of taxanes concentrated exclusively on baseline separation of cephalomannine and taxol. While this was achieved on a 5 µm C18 column with a methanol:water solvent system (Figures 2A and 2B), we ran into difficulties with irreversible high backpressure on the column after relatively few samples analyzed (<200), similar to problems described by Witherup, et al. (8).

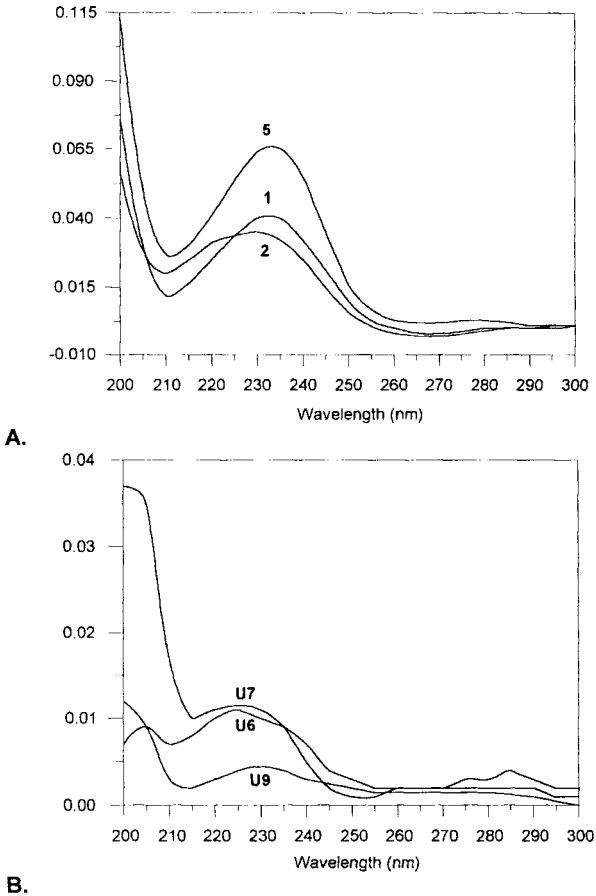
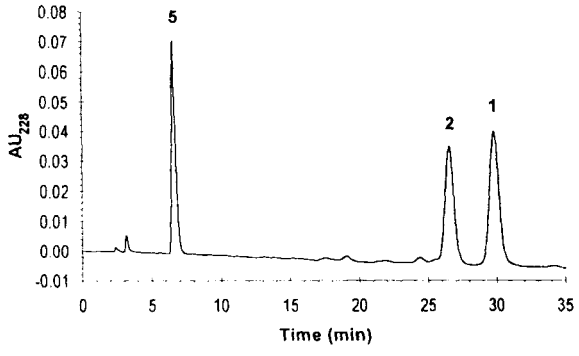
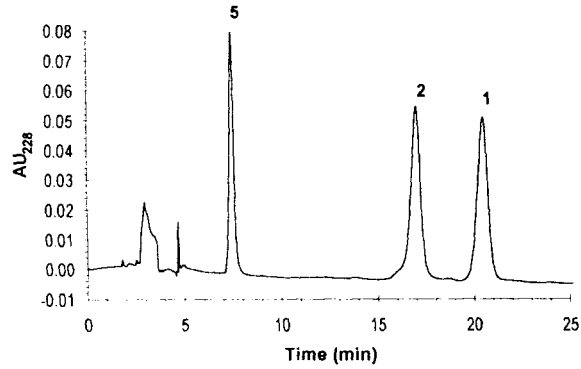


Figure 1. Diode array scans of taxanes over the 200 to 300 nm range of the absorption spectrum. For the figure, absorbance was measured at 5 nm intervals and the data was graphed with an interpolated line drawn through the data points. **1A**. Diode array scans of authentic taxane standards, 4 µg of each taxane injected (40 µL injection of ~100 µg/mL standard mixture), separated on C18 column. **1B**. Diode array scans of unknown possible taxanes in 4aYAC callus extracts, analyzed on Taxsol ® F column. Column conditions as described in text. **1** = taxol; **2** = cephalomannine; **5** = baccatin III; **U6**, **U7**, **U9** = unknown, possible taxanes eluting at approximately 6, 7, and 9 minutes, respectively.



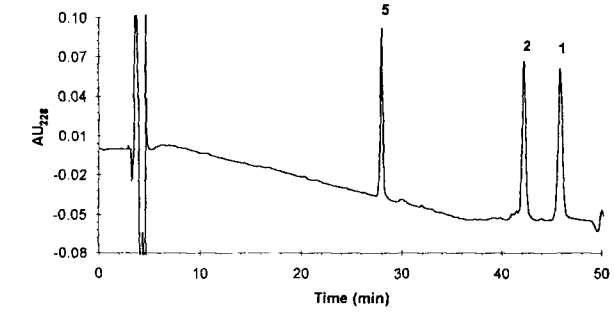
A.



B.

Figure 2. Isocratic, reversed-phase, separation of taxane standards on 5 µm columns. **2A**. Separation on Supelco Supelcosil™ 5 µm, 250 x 4.6 mm LC-18 column. **2B**. Separation on Phenomenex IB-Sil™, 5 µm, 250 x 4.6 mm phenyl column. Mobile phase for both columns was MeOH:H_2O (60:40), 1 mL/min flow. UV detection at 228 nm. **1** = taxol; **2** = cephalomannine; **5** = baccatin III.

Unlike those investigators, however, we did not see backpressure problems when injecting purified taxane standards. It was only after injection of relatively crude leaf, bark, and callus extracts that backpressure became a problem. It may not, therefore, be the taxanes precipitating on the column but, rather, other compounds in the extracts. The other problem with the C18 method is that it could not resolve the 2 to 3 additional peaks that we see eluting between cephalomannine [**2**] and taxol [**1**] (Figures 4 and 5).



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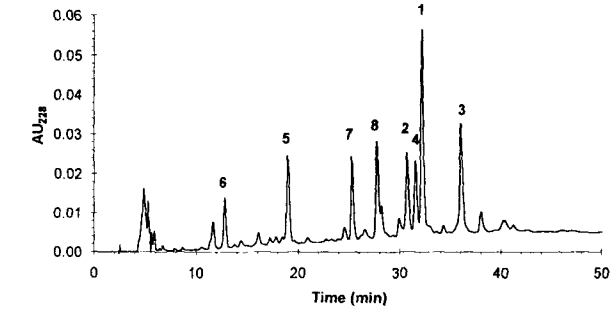
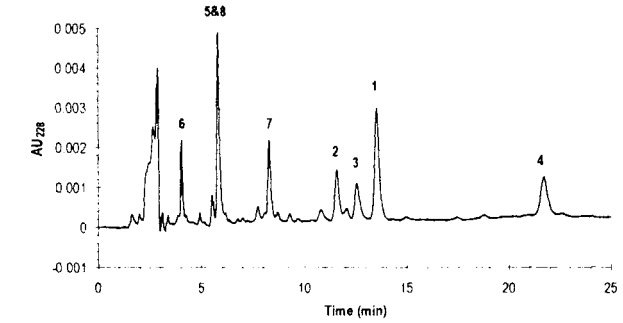


B.

Figure 3. Gradient elution of taxane standards on 5 µm (A) and 3 µm (B) phenyl columns. **3A**. Separation on Phenomenex IB-Sil™, 5 µm, 250 x 4.6 mm phenyl column. Linear gradient starting with 12.5 mM NaOAc, pH 5.0:CH_3CN (85:15) changing to NaOAc:CH_3CN (65:35) in 20 min. then changing linearly to NaOAC:CH_3CN (52.5:47.5) over 10 min. Mobile phase remained at 52.5:47.5 for 15 min. 1 mL/min flow. UV detection at 228 nm. **3B**. Separation on Phenomenex Ultremex™, 3 µm, 250 x 4.6 mm phenyl column. Gradient profile began at acetonitrile:water (25:75) and increased linearly to CH_3CN:H_2O (60:40) over 35 min. At 45 min the gradient changed to CH_3CN:H_2O (25:75) over 1 min and equilibrated there for 9 min before the column was ready for another injection. 0.75 mL/min flow. UV detection at 228 nm. **1** = taxol; **2** = cephalomannine; **3** = 7-epi-10-deacetyltaxol; **4** = 7-epi-taxol; **5** = baccatin III; **6** = 10-deacetylbaccatin III ; **7** = 7-xylosyl-10-deacetyltaxol; **8** = 10-deacetyltaxol.



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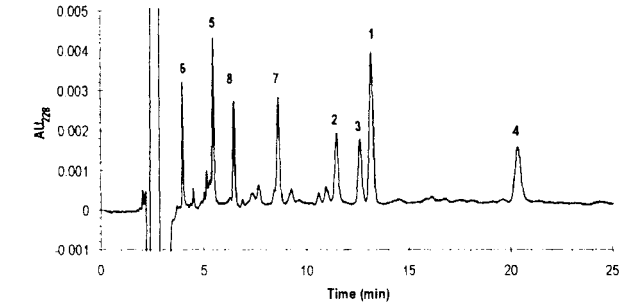
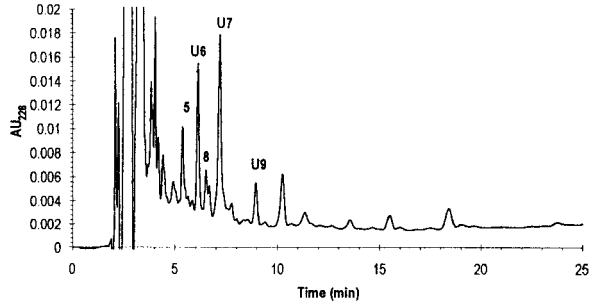


B.

Figure 4. Separation of taxane standards on columns specifically developed for taxane analysis. **4A**. Metachem 5 µm, 250 x 4.6 mm, Taxsil™ column. **4B**. Phenomenex 3 µm, 250 x 4.6 mm, Taxsol™ F column. Mobile phase was 12.5 mM NaOAc:CH_3CN (52.5:47.5), 1 mL/min flow rate. UV detection at 228 nm. **1** = taxol; **2** = cephalomannine; **3** = 7-epi-10-deacetyltaxol; **4** = 7-epi-taxol; **5** = baccatin III; **6** = 10-deacetylbaccatin III ; **7** = 10- deacetyltaxol ; **8** = 7-xylosyl-10-deacetyltaxol.

Some of the resolution problems associated with the C18 column were overcome by using a phenyl column (Figure 3). Gradient elution of taxanes and crude extracts greatly increased separation between cephalomannine [**2**] and taxol [**1**] (5 min separation on the 5 µm column). This method was particularly effective when separating taxanes from crude extracts. Baccatin III [**5**] and 10-deacetylbaccatin III [**6**] typically elute in the middle of a considerable mixture of compounds (Figure 5). The gradient elution allows for good separation of the early eluting taxanes from some of the other compounds in the crude extract. Unfortunately, the 65 minutes that was necessary for analysis of one sample and re-



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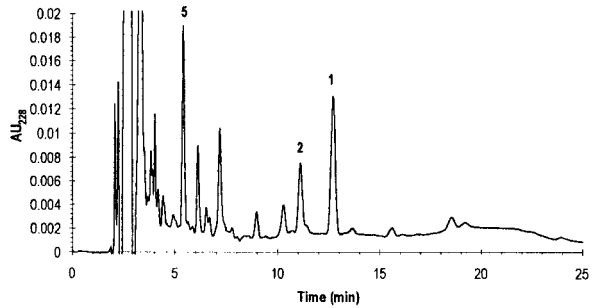

B.

Figure 5. Crude extract of *Taxus brevifolia* callus cell line 4aYAC (A) and spiked with 5,1, and 2 (B). Peak assignments and experimental conditions as in Figure 4B. **U6**, **U7**, **U9** = unknown, possible taxanes eluting at approximately 6, 7, and 9 minutes, respectively.

equilibration of the column prior to another injection made the gradient/phenyl column technique unsuitable for the rapid screening of large numbers of samples. It is important to note that this method was based on the assumption that the last taxane of interest to elute was taxol [**1**]. It is not known how long this method might require to elute 7-epitaxol [**4**].

The Metachem Taxsil™ column and the Phenomenex Taxsol™ are two specialty columns that have been developed for taxane analysis. In this investigation we used the 4 µm Phenomenex Taxsol™ F column and the 5 µm Metachem Taxsil™ column. The bonded phases of both of these columns are proprietary, so differences other than particle size are unknown. Examination of the chromatograms of the standard mixture on each of these columns (Figure 4)

demonstrates the advantages of using these columns for taxane analysis. Each column gives separation of taxanes within 25 minutes using isocratic elution consisting of acetonitrile/sodium acetate buffer. There are differences between the columns, however. The biggest problem with the Metachem Taxsil™ column (Figure 4A) was the difficulty in resolving baccatin III [**5**] and 7-xylosyl-10-deacetyltaxol [**8**]. These two compounds coeluted at 6 min on the Metachem column, but were clearly resolved on the Phenomenex column at 5.5 and 6.5 min, respectively. Other differences in the two columns can be seen in the elution order of two contaminants in the standard mixture that elute upstream and downstream of cephalomannine [**2**] on the Metachem column. These two peaks elute just prior to cephalomannine [**2**], at 10.5 and 11 min, on the Phenomenex column. The change in elution order of peaks on the Metachem Taxsil™ column and the Phenomenex Taxsol™ F column indicates that some of the differences between the two columns may be differences in the bonded phase of the packing material and not just differences in particle size.

The analysis of an extract of a 4aYAC callus culture demonstrates the use of the Phenomenex Taxsol™ F column with crude cell extracts. 4aYAC callus was derived from cells isolated from *Taxus brevifolia* bark tissue. This cell line has not demonstrated an ability to produce taxol or cephalomannine, but does appear to produce baccatin III [**5**] and 7-xylosyl-10-deacetyltaxol [**8**] or similar compounds (Figure 5A). Peaks appearing at the retention times for baccatin III [**5**], and 7-xylosyl-10-deacetyltaxol [**8**], appear to be taxanes based on their characteristic spectra with an absorption minimum at 210 nm and an absorption maximum at 228 nm. Spiking the extract with a mixture of 2 µg/mL baccatin III [**5**] and cephalomannine [**2**], and 4 µg/mL taxol [**1**], confirms the possible presence of baccatin III [**5**] (Figure 5B). Furthermore, the diode array scans of peaks in the crude callus extract at 6 min, 7.25 min, and 9 min indicate taxane-like absorption spectra (Figure 1B). It is not known what taxanes these peaks might be if, indeed, they are taxanes. Current work is being done to characterize these compounds.

There are a few caveats to working with the Taxsol™ F column. Under the conditions described, typical operating pressures are 2500 psi at a flow rate of 1.0 mL/min. With a guard column, operating pressures on the Taxsol™ F column increased to 3000 psi. In contrast, under the same conditions the Metachem Taxsil™ column operates at just under 2000 psi. Another problem is gradual increase in backpressure due to precipitation on the column of compounds present in crude mixtures. This is by no means a problem unique to these columns (8,9). On one Taxsol™ F column, using 10 mM sodium acetate, pH 5.0, we were able to *analyze ~750 samples* before backpressure dramatically and irreversibly exceeded 5000 psi. The use of a more acidic buffer, either sodium or ammonium acetate,

causes the baseline on our HPLC system to become unstable in addition to creating excessive noise in the 190 - 220 nm range of the diode array detector.

We did not use guard columns for the analyses presented here because in one instance the precipitation of insoluble material occurred simultaneously on the guard column and on the analytical column necessitating the replacement of both columns. However, more recent work has demonstrated that the use of a guard column will significantly improve the life of the column. Replacing the guard column after 750 injections returned the operating pressure on the column to a normal (3000 psi) level.

We did attempt to increase column life by flushing the column with hot solvent after a series of analyses. After every set of samples that we ran we flushed the column with 50 column volumes of acetonitrile at 80 °C. After approximately 100 samples we flushed the column with 50 column volumes each of acetonitrile > methanol > ethyl acetate >acetonitrile at 60 °C. Using this washing method, however, did not increase the number of samples that we were able to analyze on the Taxsol F column. The column became unusable after approximately 750 analyses.

A number of recently published methods have concentrated on the preparative or semi-preparative separation of taxanes (8,10,11). The columns described in this paper are not suitable to semi-preparative isolation of taxol. The Curosil™ G column with 6 µm packing may be more appropriate for semi-preparative work, although we have not tested this column.

We have briefly described the use of new proprietary columns for taxol analysis. These columns allow the investigator to separate a complex taxane mixture in 25 minutes under isocratic conditions. Taxol is eluted in 13 minutes with complete baseline separation from cephalomannine (11.5 min). This method allows a more rapid analysis of a taxane mixture than the isocratic phenyl-bonded Si column method published recently by Wheeler, *et. al* (8). The elimination of a gradient to separate complex taxane mixtures greatly reduces the amount of time to analyze multiple samples by eliminating the need to equilibrate the column between sample runs. We have processed as many as 400 samples in one week using the described method. Gradient elution of crude taxane extracts on the Taxsol™ F column would enhance resolution of peaks, but the compromise for increased resolution would be the increased time necessary to analyze multiple samples.

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