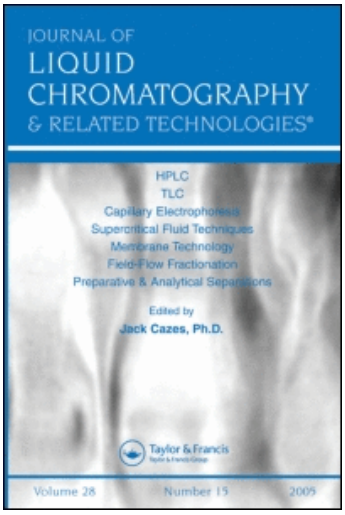

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Trevor P. Castor[a]; Theodore A. Tyler[a]
[a] Bio-Eng, Inc., Arlington, Massachusetts

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DETERMINATION OF TAXOL IN *TAXUS MEDIA* NEEDLES IN THE PRESENCE OF INTERFERING COMPONENTS

TREVOR P. CASTOR AND THEODORE A. TYLER
Bio-Eng, Inc.
216 Sylvia Street
Arlington, Massachusetts 02174

ABSTRACT

Usual methods for the analysis of taxol incorporate an extraction with organic solvents, partitioning into methylene chloride, and determination on a phenyl column using an acetonitrile-methanol-water mobile phase. Although these systems work well for extractions from bark samples, needle samples contain compounds which co-elute with taxol in this reversed phase system.

We have developed an analytical method using methanol extraction, methylene chloride partitioning, and minicolumn cleanup. The influence of co-eluting compounds on taxol purity was quantified from absorbance data at 228 nm and 280 nm.

INTRODUCTION

Taxol, a potent anti-cancer agent [1-7] is currently extracted from the bark of the Pacific yew, Taxus brevifolia. The quantity of taxol in the bark is low and therefore large quantities of bark are needed to meet the demand for taxol [8]. Since removal of the bark kills the tree, alternative sources of taxol are needed. One such source is yew leaves or needles, which is much preferable on ecological grounds since the needles are a renewable resource.

There are several published methods for the determination of taxol in yew needles and the bark of Taxus brevifolia [9-14]. Herein we report an analytical method involving a simplified extraction technique, cleanup using a silica minicolumn, and final quantitation using absorbance data at 228 nm and 280 nm. This method succeeds in correcting for a co-eluting impurity which is not determined by previously published methods.

EXPERIMENTAL

General

The HPLC system consisted of ISCO ternary gradient instrumentation (Lincoln, NE) incorporating an ISCO V4 detector for routine analytical work, an ISCO S 500 detector for simultaneous quantitation at 228 and 280 nm, and a Spectra Physics Spectra Focus Model SF101-0122 detector for generating 3- dimensional spectral scans. The silica minicolumns (01-00 SPICE Cartridges) were obtained from Rainin Instrument Company, Woburn, MA. The silica used in column chromatography was supplied by EM Science, Cat. No. 10180-3 Silica Gel 40, 70-230 mesh.

Sample Preparation

Samples of Taxus branches were dried at 60 C for 16-20 hours. The needles were removed from the stems, ground in a Waring blender and sieved to pass through a 70 mesh screen. Material that did not pass was reblended until a total of 90% of the sample had passed through the screen.

Extraction Procedure

A ground sample (200 mg) was weighed into a 40 mL vial. Methanol (30 mL) was added and the sample was stirred overnight using a magnetic stirrer. The extract was filtered through #40 Whatman paper into a 250 mL separatory funnel containing

with 30 mL of methylene chloride and this rinsing was filtered into the separatory funnel.

The methanol-water phase was extracted with three volumes of methylene chloride (ca 50 mL each) and these extracts were transferred to a 250 mL rotary evaporation flask. Additional water or methanol was added as needed to produce or to clarify the phase separation. The extraction was continued until a total volume of 200 mL had been collected. The extract was evaporated to apparent dryness. Traces of residual water were removed by adding 20 mL of acetone followed by 25 mL of hexane and again bringing the extract to dryness. The sample was then redissolved in 8 mL of methylene chloride.

Silica Minicolumn Cleanup

The methylene chloride solution of the extract was slowly passed through a silica minicolumn and the column was washed with an additional 4 mL of methylene chloride. This washing removed waxy, non-polar substances; the taxol was contained within a green band near the top of the column. The eluant was changed to 4% acetone in methylene chloride and the elution was stopped just as the green band began to exit from the column. The eluant was then changed to 20% acetone in methylene chloride and the taxol containing band was eluted from the column using two 10 mL portions of this solvent. The extract was taken to dryness using rotary evaporation and the residue was taken up in 2.00 mL of methanol.

HPLC Analysis

The HPLC column was a Rainin 80-D15-C5 Microsorb 5 micron, 4.6 mm x 15.0 cm phenyl bonded phase column with a 1.5 cm phenyl guard column. The mobile phase was 12% acetonitrile and 55% methanol - the remainder being water. The flow rate was 1.0 mL/min.

A taxol standard at a concentration of 0.050 mg/mL was prepared in methanol. The injection volumes were 10 microliters for both standards and samples. Peak heights were measured in dual channel mode at both 228 nm and 280 nm and taxol was quantified using the following equation:

$$\% \text{ Taxol} = 100 \times \frac{C(\text{Standard}) \times H(\text{Sample})}{C(\text{Sample}) \times H(\text{Standard})} \times PF \qquad (1)$$

where C = concentration in mg/mL

H = peak height from the 228 nm channel

PF = Purity Factor of the taxol peak calculated from A(280)/A(228) absorbance ratio in Equation 2

RESULTS AND DISCUSSION

As part of an ongoing project on the development of improved methods for the isolation of taxol from Taxus needles, we prepared extracts of both Taxus needles and bark using classical organic phase extraction as well as supercritical fluid extraction. Analysis conducted in our laboratory using reversed phase HPLC on a phenyl column, and using single wavelength detection at 228 nm, yielded values in the range of 0.04% to 0.05% of the dry biomass for the taxol content. Portions of these extracts were then submitted to the National Cancer Institute at Frederick, Maryland for confirmatory analysis. Their analysis, done with an HPLC equipped with a photo-diode array detector, indicated the presence of a substance with a large absorbance at 280 nm for the needle samples. The bark samples, all of the compounds in the taxol reference mixture, and pure taxol did not show this co-eluting compound. The discovery of this impurity prompted us to attempt its isolation so that its presence within the taxol peak could be compensated for based on its spectral characteristics.

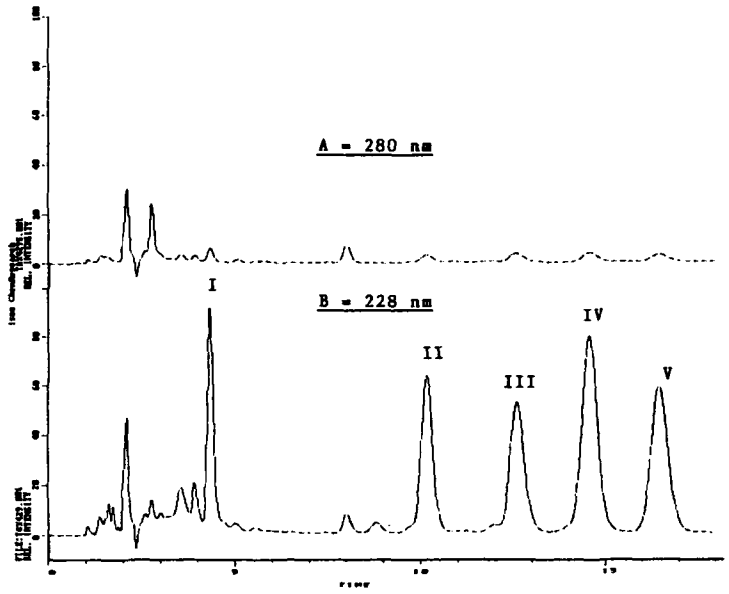

FIGURE 1. Separation of Taxus reference mixture on phenyl column using MeOH:H20:ACN (60:28:12) at 0.8 mL/min. UV detection at A = 280 nm and B = 228 nm. I = Baccatin III; II = 10-deacetyltaxol; III = Cephalomannine; IV = Taxol; V = 10-deacetyl-7-epitaxol.

Since the interfering compound co-eluted with taxol in the reversed phase system, we used a normal phase system to make the separation. A 27g sample of Taxus media "Hicksii" needles was extracted with methanol-toluene and chromatographed on a 22 mm x 250 mm column of silica gel. A rapid separation was made and 50% acetone in methylene chloride was used to strip the taxol fraction from the column. This extract was evaporated to dryness, redissolved in 9% acetone in methylene chloride, and placed on a second 22 mm x 250 mm column of silica gel which had been equilibrated with 9% acetone in methylene chloride. A slow acetone gradient was begun and the acetone concentration was monitored spectrophotometrically at 270 nm.

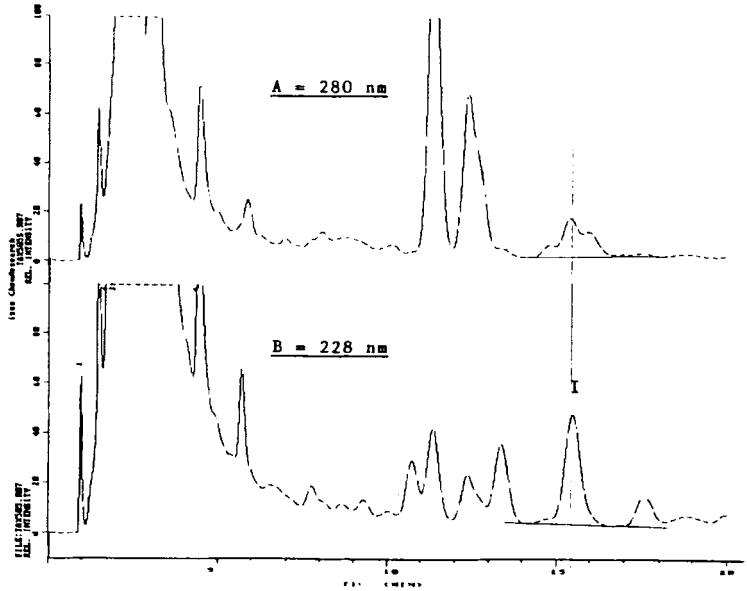

FIGURE 2. Chromatographic Scan of Taxus needle extract on phenyl column using MeOH:H2O:ACN (55:33:12). UV detection at A = 280 nm and B = 228 nm. Flow rate = 0.8 mL/min. I = Taxol

Thirteen 200 mL fractions were taken as the acetone concentration was gradually increased from 9% to 15%. Fraction #4 contained a pattern of peaks in the taxol retention time region that exactly matched the pattern of peaks seen at 280 nm in the chromatographic scan of needle samples (Figure 2). Spectral scans of these interfering peaks showed several common features. The interferences had absorption maxima very close to 280 nm, absorption minima close to 235 nm, and a response ratio of approximately 3.5 for A(280)/A(228).

Fraction #5 showed an absorption pattern similar to fraction #4; however, the quantity of material in this fraction was 38% that of fraction #4. Fractions #6, #7, and #8 showed decreasing mass with the same chromatographic pattern. Fraction #8 has essentially no mass. Taxol first appeared in fraction #9 as a large peak with only a

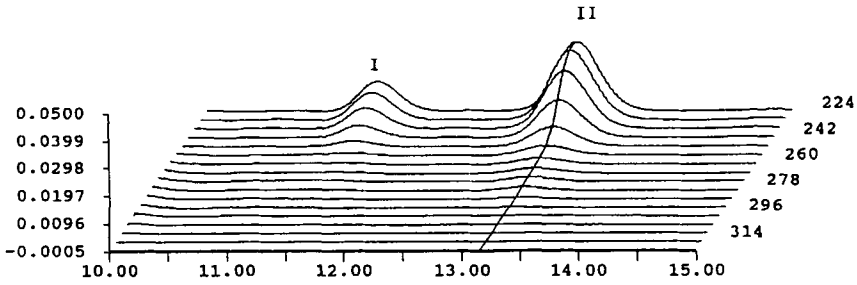

FIGURE 3. 3-Dimensional projection of cephalomannine (I) and Taxol (II) using the same mobile phase and conditions as in Figure 1, but at a flow rate of 0.9 mL/min.

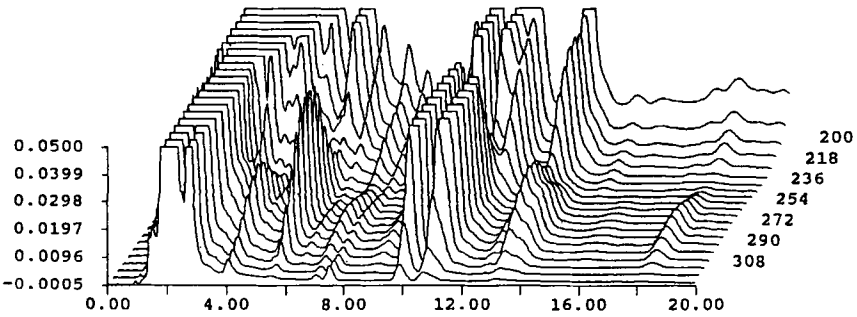

FIGURE 4. 3-Dimensional projection of Taxus needle extract using the same mobile phase and conditions as in Figure 3.

small quantity of cephalomannine. Fraction #10 contained large peaks of both taxol and cephalomannine. These taxol fractions showed spectral scans identical to that of pure taxol and yielded a value of approximately 0.05 for A(280)/A(228). Simultaneous equations were set up to calculate the PF (Purity Factor) of the taxol peak:

Let A(280) = AT(280) + AU(280)

A(228) = AT(228) + AU(228)

where A(280) = Total absorbance at 280 nm

AT(280) = Absorbance due to Taxol at 280 nm

AU(280) = Absorbance due to the Unknown at 280 nm

Let K1 = AU(280)/AU(228) = ca 3.50

K2 = AT(280)/AT(228) = ca 0.05

and K3 = A(280)/A(228)

Then $PF = \frac{AT(228)}{A(228)} = \frac{K1 - K3}{K1 - K2}$

Substituting in our measured values for K2 and K1, we obtained:

$$PF = 1.0145 - 0.29 \times A(280)/A(228) \qquad (2)$$

We used the above formula to correct our previous results. Most of our samples gave an absorbance ratio of approximately 0.35 which yielded 0.91 as the purity factor. Thus, values of the taxol content of the needles of the ornamental yew, Taxus media "Hicksii," measured to be 0.050 % by reversed-phase HPLC on a phenyl column with absorbance at 228 nm were corrected to 0.046 % on a dry biomass weight basis.

We recommend that other workers who analyze for taxol using a phenyl bonded column be aware of the possibility that their taxol peaks may contain underlying interferences and test for these interferences by running a spectral scan of the taxol peak and/or monitoring at 280 nm in addition to monitoring at 228 nm.

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