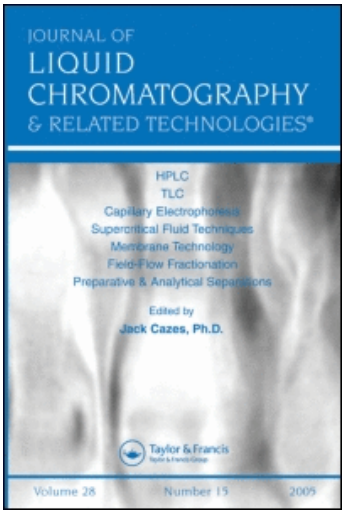

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Khisal A. Alvi[a]; Songjie Wang[a]; Guillermo Tous[a]
[a] Phyton Biotech Inc., New Jersey, USA

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New and Rapid Ultra-Performance Liquid Chromatography Assay of Paclitaxel

Khisal A. Alvi, Songjie Wang, and Guillermo Tous

Phyton Biotech Inc., New Jersey, USA

Abstract: A new Ultra-Performance Liquid Chromatography (UPLC) method for assaying paclitaxel and related compounds in plant tissue cultures is described. The method has been shown to be rapid, economical, accurate, linear, precise, and specific, and can be used to assay bulk drug substance (BDS) and determine its chromatographic purity, as well as to assay paclitaxel in process and biomass samples.

Keywords: Paclitaxel, 10-Deacetyl baccatin III, Baccatin III, UPLC

INTRODUCTION

Paclitaxel was discovered in the 1960's by researchers at the National Cancer Institute in an extract from the bark of the rare Pacific yew tree. In 1991, NCI awarded Bristol-Myers Squibb (BMS) the rights to develop and produce paclitaxel. The drug was quickly approved as Taxol®, a second-line therapy for ovarian cancer. Label extensions to other cancers followed. Obtaining bulk paclitaxel was a problem from the beginning. Paclitaxel comprises only about 0.03% by weight of yew tree bark and is found, along with numerous other taxanes and compounds, in *Taxus* biomass. Therefore, harvesting *Taxus* trees for production of paclitaxel commercially poses a serious problem in the U.S. from the environmental point of view.

As alternative sources, plant tissue and cell cultures have been investigated in many research groups. Phyton Biotech, Inc. has successfully established a plant cell culture process to produce paclitaxel, the active ingredient in Taxol at a commercial scale. Currently, Phyton Biotech, is the largest producer of paclitaxel via plant tissue culture.

Correspondence: Khisal A. Alvi, Phyton Biotech Inc., East Windsor, NJ 08520, USA. E-mail: khisal.alvi@phytonbiotech.com

Analysis of paclitaxel and related compounds in plant material is performed by reversed-phase high-performance liquid chromatography (RP-HPLC),[1,2] and often represents a challenge, due to interferences from the complex matrix. There are several reversed phase HPLC assays for paclitaxel in the literature,[3–7] but all of them employ relatively long gradients (15–35 minutes).

We have developed a method with a reduced run time that does not compromise accuracy, resolution, or efficiency (Figure 1). The method is very rugged and was used for screening, in-process support, and BDS analysis (Figure 2). This was made possible by the recent introduction of Ultra-Performance Liquid Chromatography, or UPLC instrumentation.

HPLC can be extended to new limits by using smaller particles. This column feature increases speed and peak capacity (number of peaks resolved per unit time in gradient separations), and allows the HPLC system to be run at higher pressures in the UPLC mode. The technology takes full advantage of chromatographic principles to run separations using columns packed with smaller particles and/or higher flow rates for increased speed, with superior resolution and sensitivity. This report describes a new and fast assay for analysis of paclitaxel in plant (*Taxus*) tissue culture fermentation using UPLC.

EXPERIMENTAL

Reagents and Material

Paclitaxel, cephalomannine, baccatin III, and 10-deacetyl baccatin III standards were obtained from LKT laboratories, Inc. (St. Paul, MN). Taxol

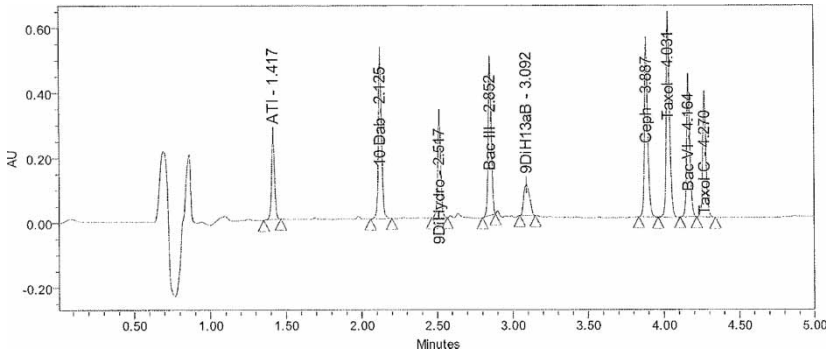

Figure 1. UPLC analysis profile of the taxane reference standard mixture. Nine different taxanes were separated using the newly developed UPLC method: advanced taxane intermediate (ATI, RT = 1.4 min), 10-deacetyl baccatin III (10-DAB III, RT = 2.1 min), 9-dihydrobaccatin III (9 DiHydro, RT = 2.5 min), baccatin III (Bac III, RT = 2.9 min), 9-dihydro-13-acetylbaccatin III (9DiH13aB, RT = 3.1 min), cephalomannine (Ceph, RT = 3.9 min), paclitaxel (Taxol, RT = 4.0 min), baccatin VI (Bac VI, RT = 4.2 min), and Taxol C (RT = 4.3 min).

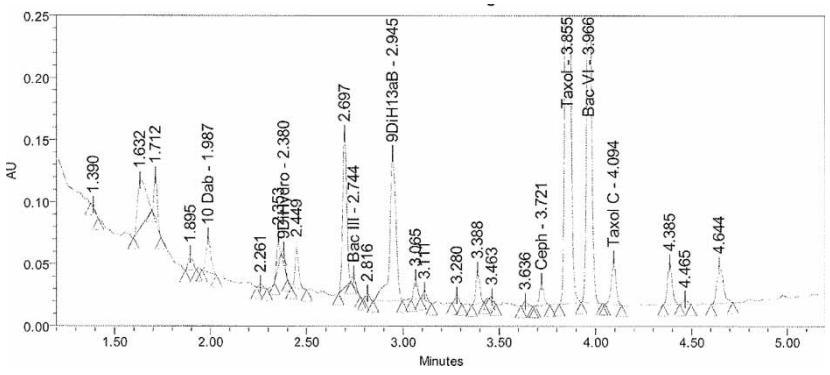

Figure 2. A new UPLC chromatographic profile method for the analysis of plant cell culture extracts from *Taxus chinensis.*

C was obtained from Hauser Chemical Research, Inc., (Boulder, CO), while all other remaining taxanes (9-dihydrobaccatin III, 9-dihydro-13-acetylbaccatin III, and baccatin VI) were isolated in-house. Advanced Taxane Intermediate (ATI) is an unidentified polar taxane. All chemicals used were of analytical grade.

Apparatus and Chromatographic Conditions

Separation was performed with a Waters Acquity UPLC system (Waters, Milford, MA) which consists of a binary solvent manager (BSM), a sample manager (SM) equipped with a 20 μL loop and a UPLC photodiode array detector. Linear gradient separation (Table 1) of eight compounds (and other taxanes) was achieved on a BEH C_{18} column (1.7 μm, 2.1 × 50 mm) at a flow rate 0.400 mL/min at 36°C. Injections of 5 μL were made using the Partial Loop Needle Overfill mode. To eliminate carryover, 500 μL of strong wash (MeOH), followed by weak wash (50:50 water/acetonitrile), was used. The ultraviolet spectra of paclitaxel and related taxanes were obtained in the range of 190–400 nm. All spectra were normalized.

Extraction of Whole Broth

The broth samples to be analyzed were prepared by adding 2.4 mL acidic methanol to 1.6 mL of broth sample. This mixture was solubilized and agitated ultrasonically for 60 minutes, and the remaining solids were removed by centrifugation. The remaining supernatant solution was then analyzed by UPLC.

Table 1. Gradient-elution program for the analysis of taxane mixtures

	Time (min)	Flow rate (mL/min)	A (%)	B (%)	Curve
1	Initial	0.350	70.0	30.0	6
2	0.10	0.350	70.0	30.0	6
3	3.80	0.350	20.0	80.0	6
4	4.40	0.350	0.0	100.0	6
5	4.60	0.350	0.0	100.0	6
6	4.80	0.350	70.0	30.0	6
7	5.00	0.350	70.0	30.0	11

Solvent A = 0.05% Formic acid in H_2O.
Solvent B = 0.035% Formic acid in acetonitrile/H_2O (86/14).

RESULTS AND DISCUSSION

The retention times of nine taxanes that are commonly found in Taxus cell suspension extracts were determined by injecting authentic samples onto the UPLC. Baseline separation of all nine taxanes was achieved (Figure 1) with a gradient elution program of DI water with 0.05 percent formic acid (mobile Phase A), and water/acetonitrile (14/86) with 0.035% formic acid (Table 1). We prepared a mixture of these nine taxanes to achieve optimum chromatographic separation conditions for all the taxanes. These chromatographic conditions were found suitable for assaying paclitaxel in crude extracts (Figure 2). This method, when compared to our own HPLC method and other published methods, performed better in terms of accuracy, resolution, efficiency, and run time.

Identification of taxanes in cell culture extracts was based on retention time and ultraviolet spectral comparison. Quantification of paclitaxel (Taxol), 10-deacetyl baccatin III (10-DAB III), and baccatin III (Bac III) in our sample was based on response factors determined from authentic materials. Quantification of the remaining paclitaxel derivatives was based on the paclitaxel response factor.

Method Validation and Results

Preliminary assessment was made of the new assay and instrument specificity, linearity and linear range, precision, accuracy, and system suitability.

Specificity

The method specificity was determined by injecting authentic samples onto the UPLC and evaluating their UV spectrum using the peak purity parameters

that came with the system. The paclitaxel retention time (RT) and the related substances' retention time reproducibility were very satisfactory. Even after months of use and numerous injections of plants cell culture extracts, the retention time was consistent.

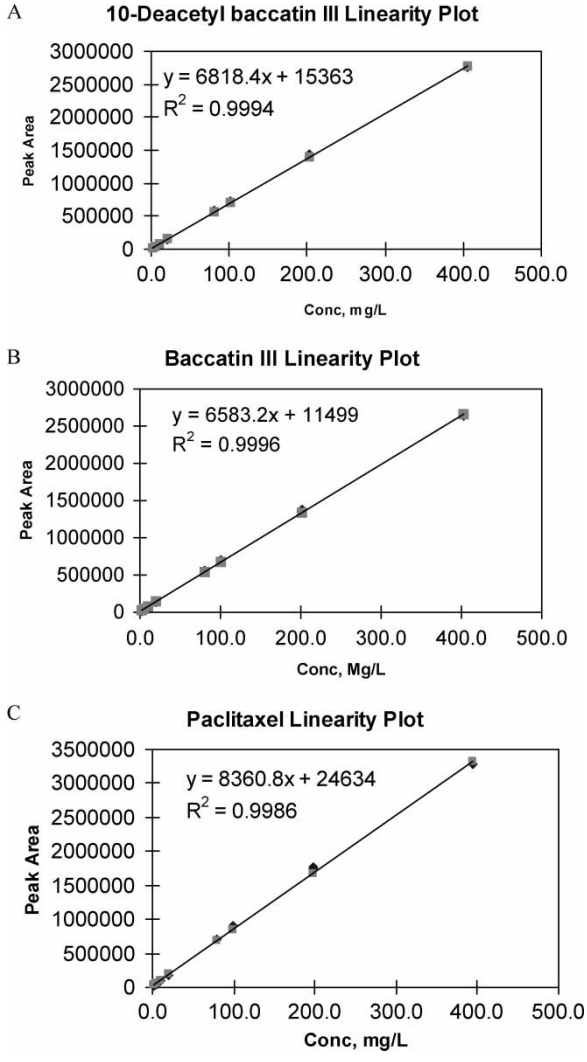

Figure 3. Linearity curve and linear correlation coefficient of authentic taxane related substances (Figure 3, A, B, and C). The plot shows the linear response factors of the taxanes of interest at concentrations ranging from 2.00 to 405.4 mg/L versus their peak area. (A) 10-DAB III: Concentration range: 2.0–405.4 mg/L, R^2: 0.9997. (B) Bac III: Concentration range: 2.0–402.7 mg/L, R^2: 0.9996. (C) Paclitaxel: Concentration range: 2.0–395.0 mg/L, R^2: 0.9986.

Linearity and Quantification

A linear curve was built by linear dilution of paclitaxel and its related substances. The linearity of the method was evaluated in the range of 2 to 405.4 mg/L (Figure 3). The calibration curves showed a linear response over the studied range, with a 0.999 correlation coefficient (R^2) for the compounds of interest (Figure 3). The scope of the assay application was broadened to also address samples that could differ in concentration by several-fold with the potential of greater sensitivity by the UPLC methods (results not shown).

Accuracy

Triplicate injections were made at specified concentrations to assess the accuracy of the method. The accuracy of the method was assessed by back-calculation of the injection peak areas using the derived calibration curves to give the calculated concentration for each injection. These values were compared to the theoretical value derived from the linear curve and reported in terms of % deviation from the theoretical value (RSD, Table 2A, B, and C). The range of the method was established from 5 mg/L to 200 mg/L.

Precision

Precision was evaluated by the peak area relative standard deviation at three different concentrations. Inter-day reproducibility was evaluated with repeated analysis for 12 consecutive days and found to be satisfactory

Table 2A. UPLC method accuracy for 10-DAB III

Theoretical conc. (mg/L)	Calculated conc. (mg/L)	Accuracy (% deviation)
201.4	197.1	97.9
80.6	80.2	99.5
20.1	20.2	100.5
5.0	5.3	106.0

Table 2B. UPLC method accuracy for Bac III

Theoretical conc. (mg/L)	Calculated conc. (mg/L)	Accuracy (% deviation)
199.0	196.5	98.7
79.6	79.5	99.9
19.9	19.8	99.5
5.0	5.1	102.0

Table 2C. UPLC method accuracy for taxol

Theoretical conc. (mg/L)	Calculated conc. (mg/L)	Accuracy (% deviation)
200.2	192.7	96.3
79.6	78.1	98.0
20.0	19.6	98.0
5.0	4.9	98.0

(results not shown). Retention time reproducibility was also very consistent (results not shown), even after months of use and numerous injections of plants cell culture extracts.

System Suitability

Five replicate injections were made to evaluate system suitability. The results passed all the common USP acceptance criteria (see Table 3A, B, and C).

Table 3A. Evaluation of system suitability[a] for 10-DAB III

Injection	RT (min)	Peak area	Plate count	USP tailing
1	2.133	763048	55648	1.0
2	2.133	736596	55599	1.0
3	2.134	732300	55642	1.0
4	2.133	735448	55303	1.0
5	2.133	737074	55393	1.0
Mean peak area	2.133	740893	55517	1.0
%RSD	0.0	1.7	0.3	0.0

[a]Replicate injections of 101.4 μg/mL standard.

Table 3B. Evaluation of system suitability[a] for Bac III

Injection	RT (min)	Peak area	Plate count	USP tailing
1	2.860	722695	92808	1.0
2	2.860	697663	93052	1.0
3	2.860	693791	92876	1.0
4	2.860	699031	92802	1.0
5	2.859	700751	92712	1.0
Meak peak area	2.860	702786	92850	1.0
%RSD	0.0	1.6	0.1	0.0

[a]Replicate injections of 100.7 μg/mL standard.

Table 3C. Evaluation of system suitability[a] for taxol

Injection	RT (min)	Peak area	Plate count	USP tailing
1	4.032	924272	177668	1.1
2	4.032	901226	177355	1.1
3	4.032	891075	177427	1.1
4	4.032	896084	177447	1.1
5	4.031	896713	177336	1.1
Meak peak area	4.032	901874	177447	1.1
%RSD	0.0	1.4	0.1	0.0

[a]Replicate injections of 98.8 μg/mL standard.

CONCLUSION

A superior method for analyzing taxane samples has been developed. The method provides better resolution, and sensitivity than the current methods presented in the literature. The chromatographic peaks are narrower, which means that analytes are more concentrated and, thus, gives rise to a higher signal.

The scope of the assay application was broadened to address analytes which could differ in concentration by several-fold. The method compares well with other reported methods for paclitaxel and related compounds. The method also performed better in terms of resolution, accuracy, and efficiency (it provided base line separation of most of the taxanes including cephalomannine in only 5 minutes).

The UPLC method also showed that it is cost advantageous: solvent consumption and waste disposal charges should decrease by an order of magnitude, while UPLC column expense per analysis will be comparable to or slightly less than the current HPLC columns. Additionally, assay time was reduced by five-fold. This dramatically improves instrument return on investment and reduces the total number of instruments needed, if only HPLC is employed.

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