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Isocratic high-performance liquid chromatographic assay of taxol in biological fluids and tissues using automated column switching

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Abstract

This report describes the analysis of taxol in human plasma, cell culture medium, and dog bladder tissue by isocratic high-performance liquid chromatography (HPLC) with automated column switching. Cephalomannine was used as the internal standard. Biological samples were extracted with ethyl acetate, with a recovery of >80%. Sample extracts reconstituted in 37.5% acetonitrile were stable in polypropylene tubes at room temperature for 22 h. The HPLC stationary phase consisted of a clean-up column (Nova-Pak C_8, 75 × 3.9 mm I.D., 4 μm particle size) and an analytical column (Bakerbond octadecyl, 250 × 4.6 mm I.D., 5 μm particle size). Taxol and cephalomannine were monitored at 229 nm. Samples were injected onto the clean-up column and eluted with the clean-up mobile phase (37.5% acetonitrile in distilled water) at 1 ml/min. Concurrently, the analytical mobile phase (49% acetonitrile in distilled water) was directed through the analytical column at a flow-rate of 1.2 ml/min. A heart-cut fraction from 8 to 15 min was transferred from the clean-up column onto the analytical column. Loading of a second sample onto the clean up column while the first sample was eluting from the analytical column reduced the HPLC analysis time to about 15 min per sample. This method has a lower detection limit of 5 ng/ml and intra- and inter-day variations of <5%.

1. Introduction

Taxol is an important anticancer agent with significant activity against breast, ovarian, lung, and head and neck cancers. It has a unique mechanism of action, mediated by stabilization of cellular microtubules [1].

Analysis of taxol in biological samples has been performed using high-performance liquid chromatographic methods with UV detection (HPLC-UV) or mass spectrometry (HPLC–MS), and immunoassay, with lower detection limits of 10, 0.2, and 0.3 ng/ml, respectively [2–12]. Although the HPLC–MS method gives greater sensitivity and higher specificity than HPLC-UV methods, its utility for routine analysis of taxol is limited partly because of the lack of general availability of the highly specialized instrumentation. The immunoassay also provides high sensitivity but lacks the specificity of HPLC methods. For these reasons, analysis of taxol is still most commonly performed using the HPLC-UV method. Taxol is usually monitored at a wavelength near its UV absorption maximum of

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about 230 nm where many endogenous compounds also absorb. In order to decrease the interferences from biological matrices such as plasma, most studies used solid-phase extraction (SPE) alone [4,7], SPE coupled with liquid–liquid extraction (LLE) [2] or with protein precipitation [5], or gradient elution coupled with LLE [3] or with protein precipitation [6].

Column-switching techniques for HPLC analysis of drugs in biological samples have recently attracted considerable interest. These can be used for on-line sample clean-up and sample preconcentration, resulting in significant advantages such as increased separation power, reduction of analysis time, and reduced costs [13]. The present study was performed to establish a rapid and sensitive column-switching HPLC method to analyze taxol in plasma, culture medium and dog bladder tissue.

2. Experimental

2.1. Materials and reagents

Taxol was a gift from Bristol-Myers Squibb (Wallingford, CT, USA). Cephalomannine was obtained from the National Cancer Institute (Bethesda, MD, USA). All HPLC solvents were of HPLC grade and were filtered and degassed before use. Fetal bovine serum (FBS), minimum essential medium (MEM), Dulbecco's modified Eagle medium (D-MEM), non-essential amino acids, gentamicin, L-glutamine, and MEM vitamins were obtained from Gibco Life Technologies (Gaithersburg, MD, USA) and cefotaxime sodium was obtained from Hoechst-Roussel Pharmaceuticals (Somerville, NJ, USA).

2.2. Solutions and standards

Stock solutions of taxol with final concentrations of 1000, 100, 10, 2, 1, 0.1 μg/ml and of the internal standard, cephalomannine, with a concentration of 8.7 μg/ml were prepared in methanol. Portions of the taxol stock solutions of appropriate concentrations were pipetted into 15-ml polypropylene tubes and evaporated to dryness under a nitrogen stream. One ml of blank plasma or culture medium with 9% FBS, or about 40 mg of dog bladder tissue, were added and mixed by vortex-mixing. Calibration curves were prepared for taxol in the concentration range of 10 ng/ml to 10 μg/ml. The absolute recovery was determined by comparing the peak height of taxol ($n = 4$) of the extracted samples and those derived from unextracted stock solutions.

The complete culture medium consisted of 250 ml of MEM, 250 ml of D-MEM, 5 ml of non-essential amino acids, 5 ml of gentamicin (10 mg/ml), 0.5 ml of cefotaxime (0.1 g/ml), 5 ml of glutamine (200 m*M*), 5 ml of MEM vitamins (100 ×), with pH adjusted to 7.4 with 1 *M* NaOH. Culture medium with 9% FBS was prepared by adding 50 ml FBS to 500 ml complete medium.

2.3. Sample preparation procedures

To 0.2–1 ml of human plasma, culture medium with 9% FBS, or dog bladder tissue (about 40 mg), 100 μl of cephalomannine in methanol (8.7 μg/ml) was added. The mixtures containing plasma or culture medium were extracted twice with 3 volumes of ethyl acetate. The mixtures containing dog bladder tissue were homogenized in 3–4 ml acetonitrile or ethyl acetate with a homogenizer (Tekmar Co., Cincinnati, OH, USA) for 1 min. The probe of the homogenizer was then washed with another 3–4 ml of the same organic solvent for 15–20 s. The 2 fractions of the organic solvent extracts of samples containing plasma, culture medium or tissue were combined in a 15-ml polypropylene centrifuge tube and centrifuged at 2000 *g* for 5 min. The supernatant was transferred to another 15-ml polypropylene centrifuge tube and evaporated to dryness under nitrogen. The dried extracts were reconstituted in 100–150 μl of 37.5% acetonitrile. A 10–60 μl aliquot was injected onto the HPLC system.

2.4. Instrumentation and chromatographic conditions

The HPLC system was operated isocratically at ambient temperature (Fig. 1). The system

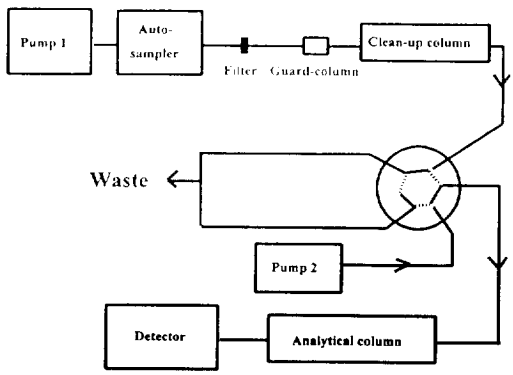

Fig. 1. Chromatographic system used for taxol analysis. Valve positions: 0–8 min (—); 8–15 min (- - - -). Arrows indicate the flow of the eluents.

included two columns: a reversed-phase Nova-Pak C_8 column (75 × 3.9 mm I.D., 4 μm, Waters Assoc., Milford, MA, USA) was used for sample clean-up, and a Bakerbond octadecyl column (C_{18}, 250 × 4.6 mm I.D., 5 μm, J.T. Baker, Phillipsburg, NJ, USA) was used for analytical separation. The HPLC system consisted of a Consta Metric IIIG metering pump (LDC/Milton Roy Co., Riviera Beach, FL, USA), a Spectroflow 400 solvent delivery system (Applied Biosystems, Foster City, CA, USA), an AS-4000 Intelligent autosampler with the thermo-electric heating/cooling accessory (Hitachi Instruments, Naperville, IL, USA), an electrically actuated multifunctional 10-port HPLC valve (Valco Instruments Co., Houston, TX, USA), a Model 680 automated gradient controller (Waters Associates), and a HP 3966A integrator (Hewlett-Packard, Wilmington, DE, USA). A Model LP-21 pulse dampener (Scientific Systems, College, PA, USA) was used to maintain a constant pressure on the clean-up column to ascertain the consistency of drug retention times. A 0.5-μm precolumn filter (Upchurch Scientific, Harbor, WA, USA) and a Nova-Pak C_8 guard column (Waters Assoc.) were placed in sequence before the clean-up column to remove particulates and strongly retentive components in samples.

As shown in Fig. 1, only 5 ports of the 10-port valve were connected, the remaining 5 ports were closed. The eluent from the analytical column was monitored at 229 nm with a Model 441 absorbance detector (Waters Assoc.). The switching valve and the integrator were controlled by a Model 680 automated gradient controller (Waters Associates). To reduce the analysis time, a second sample was injected onto the clean-up column while the first sample was eluting from the analytical column. The HP3966A integrator was started at 15 min, when the second injection was made.

3. Results and discussion

3.1. Extraction methods

Taxol is highly protein bound with a bound fraction of 95% in human plasma at the clinically relevant concentration range of 0.1–6 μ*M* [14]. For highly protein-bound drugs, separation of drug molecules from binding sites is important for a high recovery and reproducibility of the assay. Proteins upon precipitation may or may not release drugs from their binding sites. We first compared the extraction efficiency of LLE with ethyl acetate to that of protein precipitation with trichloroacetic acid (TCA, 5% in final solution) followed by LLE with ethyl acetate. Protein precipitation with TCA caused a dramatic decrease in extraction recovery of taxol at a concentration of 7.2 μg/ml from 90.3 ± 0.67% to 16.4 ± 0.26% ($n = 3$ each) in culture medium with 9% FBS, and from 76.3 ± 0.51% to 6.5 ± 0.34% ($n = 3$ each) in human plasma. The recovery of the internal standard (cephalomannine, 2.19 μg/ml) decreased from 95.5 ± 1.9% to 31.2 ± 1.0% ($n = 3$ each) in culture medium with 9% FBS, and from 82.4 ± 1.3% to 5.7 ± 0.9% ($n = 3$ each) in human plasma. Therefore, protein precipitation with TCA appeared to cause a co-precipitation of taxol and cephalomannine with the plasma proteins and gave an unacceptably low recovery. Direct LLE, because it gave a high recovery and consisted of one instead of two steps, was the method of choice.

Extraction of dog bladder tissue with ethyl acetate gave an extract that contained less endogenous substances than after extraction with acetonitrile. Ethyl acetate was therefore selected for tissue extraction. Table 1 shows the extrac-

Table 1
Extraction recovery of taxol with ethyl acetate

Sample	Concentration	Recovery (%)	C.V. (%)
Plasma	100 ng/ml	83.6	1.8
	500 ng/ml	80.5	1.7
Medium	100 ng/ml	95.6	0.94
	500 ng/ml	93.7	0.96
Tissue	100 ng	95.7	1.2
	500 ng	94.4	1.7

$n = 4$ for each condition. C.V. is the coefficient of variation.

tion recovery of taxol after ethyl acetate extraction. The extraction yields of taxol from plasma, culture medium and tissues ranged from 80 to 96%. The recovery of taxol from plasma samples was slightly lower than from culture medium and tissue samples. This may be due to the affinity of taxol to plasma proteins, thus lowering its partitioning into the organic phase.

3.2. Selection of reconstitution solvent

Optimally, for reversed-phase HPLC, a solvent more hydrophilic than the mobile phase should be used to reconstitute the sample in order to concentrate the sample at the column inlet and therefore minimize the band-broadening and increase the efficiency. Other considerations are the solubility of the drug and the interferences from the biological matrix. An ideal reconstitution solvent should dissolve the drug completely to achieve a high recovery, but dissolve a minimum amount of interferences. Furthermore, the solvent should prevent drug loss for an extended time period to facilitate the use of an autosampler.

Reconstitution of the evaporated ethyl acetate extracts with 100% acetonitrile yielded chromatograms with unacceptably large numbers of UV absorbing peaks. Reconstitution with pure distilled water yielded the cleanest chromatograms but a low recovery. In addition, storage of sample extracts in water in autosampler vials (polypropylene 100-μl inserts) overnight at 4°C or room temperature resulted in a 50% reduction in taxol peak height. An additional experiment examined whether the drug concentration decline during storage was due to drug instability and/or drug binding to polypropylene inserts. The results showed that the concentration of taxol (3.6 μg/ml) in a 1% methanolic solution (0.5 ml), upon storage in 1.5-ml polypropylene tubes, declined to about 67% of the initial concentration in 2 h. About 50% of the lost taxol was recovered by washing the empty tube with 1 ml of methanol suggesting that taxol binding to polypropylene was the major reason for the taxol concentration decline during storage, and that a higher strength solvent may minimize the binding. Similar observations have been reported for propranolol, a lipophilic and highly protein bound drug, which is also unstable in water but is stable in plasma samples [15]. A subsequent experiment examined the stability of taxol in 10–40% of acetonitrile in water during storage for 22 h in polypropylene inserts at room temperature. Fig. 2 shows that the taxol concentration was stable in 40% acetonitrile, but declined to 84.5 and 55.2% in 20 and 10% acetonitrile, respectively. Therefore, a 35–40% acetonitrile reconstitution solvent was used to achieve maximum solubility and stability with minimal interferences.

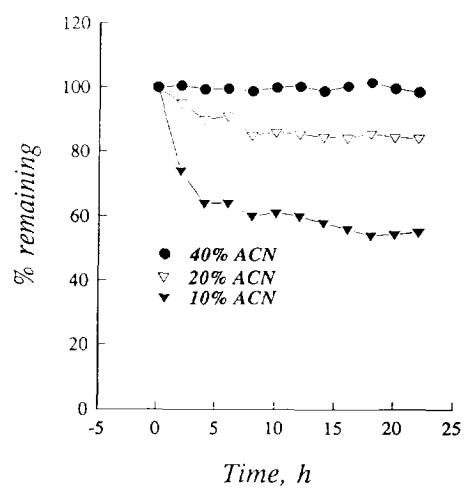

Fig. 2. Stability of taxol (10 μg/ml) in different solvents in 100-μl polypropylene autosampler inserts at room temperature. Single determination for each data point.

3.3. Column-switching method

The next step was to eliminate the interferences in plasma after LLE. A previous study utilized SPE after the LLE step to remove some of the endogenous interferences [2]. We first tried to eliminate the SPE steps by direct injection of the sample after LLE onto the Curosil-G analytical column (250 × 3.2 mm I.D., 6 μm, Phenomenex, Torrance, CA, USA) coupled to a Curosil-G guard column (30 × 3.2 mm I.D., 6 μm, Phenomenex) with isocratic elution. We found that the column can separate taxol and cephalomannine from the endogenous compounds. But after 3 injections, the peak shape deteriorated, possibly because of the build up of highly lipophilic endogenous components in the column (Fig. 3). This suggested the potential benefit of using column-switching where samples are eluted in sequence from a clean-up column prior to an analytical column, in order to protect the analytical column and increase the separation of drugs from endogenous components.

The principle of column switching for sample clean-up is to trap the analytes in a primary column or clean-up column, while the unwanted endogenous compounds in the biological matrix are eluted to waste. Subsequently, the fraction containing the analytes in the beginning (front-cut technique), middle (heart-cut technique), or end (end-cut technique) of the eluent from the clean-up column is diverted to a second column or analytical column for separation. The column-switching method is analogous to an on-line SPE method. The fine-tuning of the heart-cut time will eliminate most endogenous interferences even when a similar stationary phase is used for the clean-up column and analytical column.

The heart-cut fraction of the eluent from the clean-up column (8–15 min) containing taxol and cephalomannine was diverted to the analytical column. Fig. 4 shows a representative chromatogram of extracts of culture medium with 9% FBS, with or without taxol and cephalomannine. No interfering peaks were observed in the blank sample. Similar chromatograms were obtained for dog bladder tissue and human plasma samples (data not shown).

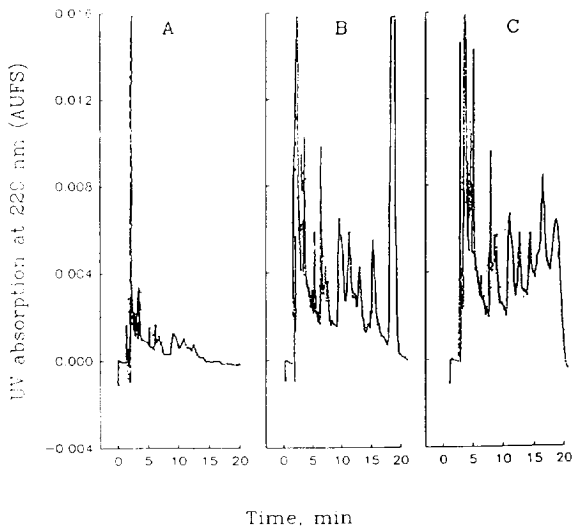

Fig. 3. Chromatograms of consecutive injections (5 μl) of blank human plasma (A, first injection) and human plasma spiked with taxol and cephalomannine (B: second injection; C: fifth injection). One ml of human plasma, with or without taxol and cephalomannine, was extracted with 3 ml of ethyl acetate twice, evaporated to dryness under nitrogen, and reconstituted in 100 μl of mobile phase. Isocratic HPLC conditions: analytical column, Curosil-G (250 × 3.2 mm I.D., 6 μm); guard column, Curosil-G (30 × 3.2 mm I.D., 6 μm); mobile phase, 45% acetonitrile in 3 m*M* ammonium acetate buffer adjusted to pH 5 with acetic acid; flow-rate, 0.7 ml/min. The retention times of taxol and cephalomannine were 17.5 and 14.3 min, respectively. Note the rapidly deteriorating sample separation.

3.4. Column and mobile-phase selection

Even though there are no ionizable groups on taxol and cephalomannine molecules, several previous studies used acetate [2,7] or phosphate buffer [4–6] in the mobile phase to maintain an acidic pH in the mobile phase, probably to improve the separation of taxol from endogenous interferences. In the present study, the use of ammonium acetate buffer to maintain the pH at 5 did not give a significant improvement in the separation and peak shapes of taxol and cephalomannine as compared to using an unbuffered mobile phase. On the other hand, the use of ammonium acetate buffer significantly increased the background absorption at 229 nm. Because endogenous interferences were no longer a sig-

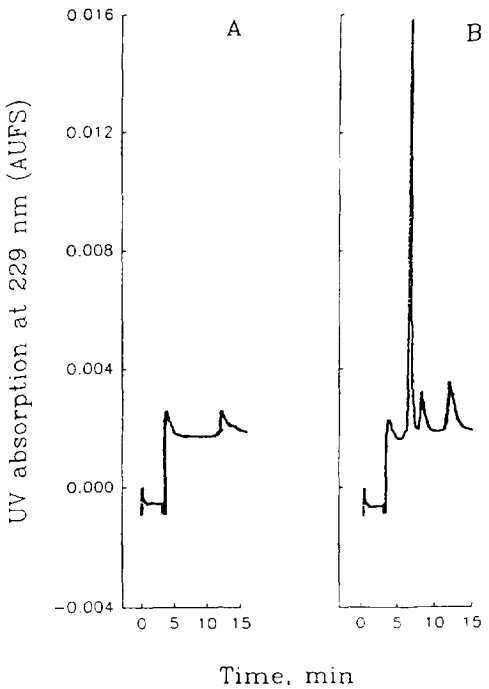

Fig. 4. Chromatograms of extracts of culture medium with 9% FBS: (A) blank sample, and (B) sample containing 20 ng/ml taxol and 870 ng/ml cephalomannine. Samples were injected onto the clean-up column and eluted with the clean-up mobile phase (37.5% acetonitrile in distilled water) at 1 ml/min. Concurrently, the analytical mobile phase (49% acetonitrile in distilled water) was directed through the analytical column at a flow-rate of 1.2 ml/min. The retention times of taxol and cephalomannine on the analytical column were 6.35 and 5.11 min, respectively. The integrator was started at 15 min after sample injection, or after the "heart-cut" fraction, i.e. eluent from the clean-up column at 8–15 min, was completely transferred to the analytical column.

nificant problem for the separation of taxol and cephalomannine with the use of the column-switching method, an unbuffered mobile phase was selected in order to simplify the mobile phase composition, minimize the background UV absorption and thereby increase the signal-to-noise ratio and the lower detection limit.

The retention of taxol and cephalomannine on the clean-up column determines the time to direct the heart-cut fraction to the analytical column as well as the time to load the next sample, and therefore determines the actual run time per sample. Furthermore, band-broadening can be minimized by using a lower strength mobile phase for the clean-up column than for the analytical column to re-concentrate the sample at the inlet of the analytical column. We selected a C_8 column as the clean-up column, with a particle size of 4 μm and a length of 75 mm. This column gave a lower retention of taxol and cephalomannine than the analytical column (C_{18}). Samples were injected onto the clean-up column and eluted with the clean-up mobile phase (37.5% acetonitrile in distilled water) at 1 ml/min. Concurrently, the analytical mobile phase (49% acetonitrile in distilled water) was directed through the analytical column at a flow-rate of 1.2 ml/min.

3.5. Sample throughput

The total elution time for each sample is 23 min; 15 min on the clean-up column and about 8 min on the analytical column. In order to increase the sample throughput, we used the "boxcar chromatography" technique first described by Snyder et al. [16], i.e. injecting the next sample onto the clean-up column while the previous sample was separated on the analytical column. We found that most endogenous substances eluted earlier than taxol and cephalomannine on the clean-up column. Hence, overlap of late-eluting compounds in the previous sample with taxol and cephalomannine in the current sample was minimal. Furthermore, because the next sample was injected onto the clean-up column after the "heart-cut" fraction of the current sample had been completely transferred from the clean up column to the analytical column, there was no interference from the early-eluting compounds in the next sample. The use of boxcar chromatography reduced the run time to 15 min per sample, hence increasing the efficiency of the system and minimizing mobile phase consumption.

3.6. Detection limit and reproducibility

The lower limit of detection for taxol was 5 ng/ml in plasma and culture medium samples, and 5 ng/injection in dog bladder tissue samples,

Table 2
Inter- and intra-day variations

Sample	Concentration	C.V. (%)	n
Inter-day variations			
Plasma	100 ng/ml	4.4	5
	500 ng/ml	2.8	5
Medium	100 ng/ml	3.7	6
	500 ng/ml	3.9	6
Tissue	100 ng	5.0	6
	500 ng	4.3	6
Intra-day variations			
Plasma	100 ng/ml	3.4	8
	500 ng/ml	2.1	8
Medium	100 ng/ml	1.8	8
	500 ng/ml	1.9	8
Tissue	100 ng	3.2	10
	500 ng	2.5	10

Plasma and culture medium samples containing taxol (100–500 ng/ml) and cephalomannine (870 ng/ml) were extracted twice with 3 volumes of ethyl acetate. Tissue samples were homogenized and extracted with 3–4 ml ethyl acetate. HPLC assay conditions were as described in text.

at a signal-to-noise ratio of 3:1. It is noted that the sensitivity limit was mainly due to the detector baseline noise rather than endogenous interferences. The taxol concentrations in plasma, culture medium, and dog bladder tissue were linearly correlated with taxol/cephalomannine peak-height ratios in the concentration range 10–10 000 ng/ml. The regression equation for this line was $y = 1.12x - 0.032$, where y is the concentration and x is the peak-height ratio, with coefficients of determination (r^2) > 0.999.

The inter-day and intra-day assay variations determined using samples prepared and measured either on the same day or on separate days over a period of 3 weeks, were < 5% (Table 2), indicating a high assay precision.

4. Conclusions

In summary, this report describes an isocratic HPLC-UV assay for taxol in biological samples using a column-switching method for sample clean up. This method requires LLE to recover the free and protein-bound taxol, has a relatively short HPLC analysis time (15 min per sample), and avoids the often-used second extraction of taxol from biological matrices by off-line SPE and the use of gradient elution. The lower detection limit and accuracy of this assay are better or equal to other HPLC-UV methods that required more extensive sample preparation.

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