Journal of Chromatographic Science 2012;50:259–270
doi:10.1093/chromsci/bmr054

Article

A Simple and Rapid 3D View Method for Selective and Sensitive Determination of Paclitaxel in Micro Volume Rat Plasma by LC–Diode Array UV and Its Application to a Pharmacokinetic Study†

Sekar Vasantha Kumar[1], Selladurai Srinath[1] and Ranendra N Saha[1*]

[1]Birla Institute of Technology and Science (BITS), Formulation Development and Pharmacokinetic Laboratory, Pharmacy Group, Pilani, Rajasthan, India

*Author to whom correspondence should be addressed. Email: vasanthceutics@yahoo.co.in

†Note: Authors have presented the initial work of 'Using Propylparaben has Internal Standard in the Estimation of Paclitaxel by HPLC-UV Detector Method' in 2009 AAPS Annual Meeting and Exposition, LA, USA.

A simple, highly repeatable and reproducible method for the estimation of Paclitaxel (TAX) in micro volume amounts of rat plasma is successfully developed and validated. The extraction procedure using 800 μL of ice-cold acetonitrile is very simple and economical with high sensitivity. The rectangular ratiograms and purity curve demonstrate the selectivity of the method. The validation and stability results show that propylparaben (PP) is a suitable internal standard (resolution 7.70 ± 0.15 min) for the estimation of TAX in micro volume rat plasma. TAX and PP are separated by isocratic reversed-phase high-performance liquid chromatography with diode array UV method with a retention time of 8.0 ± 0.25 and 5.3 ± 0.15 min, respectively, with a total run time of 10 min. The system suitability results show that the method has good reproducibility. The stability of TAX is well studied in rat plasma, and the % RSD of all stability studies of TAX are well within the acceptable range of ± 20 % at the lower limit of quantitation (LLOQ) and ± 15% at all quality control levels. The limit of detection (LOD) and LLOQ of the method are 5 and 10 ng/mL, respectively. This rapid method is successfully used to study the i.v pharmacokinetic of TAX at 10 mg/kg in wistar rats, and drug concentration is detected up to 24 h.

Introduction

Paclitaxel (TAX) is a unique natural diterpene pseudoalkaloid, isolated from the bark of pacific or western yew tree (*Taxus brevifolia*). It is one of the most effective antitumor agents developed in the past three decades. TAX is one of the preferred anticancer drugs because of its unique mechanisms of action: (*i*) promoting the formation of microtubules from tubulin dimmers (Polymerization), even in the absence of factors that are normally required for microtubule assemble (e.g., guanine triphosphate) and (*ii*) stabilizing the microtubules by binding to the N-terminal 31 amino acid of the beta-tubulin subunit in the microtubule rather than to tubulin dimmers as in the case of other drugs; hence it prevents the depolymerization of microtubules. The described cascade stops cell replication by TAX in the late G2 and M phase of the cell cycle. Thus, TAX is used to treat a range of malignancies, including breast, ovarian, head, neck, and lung tumors (1, 2). Due to its unique pharmacological action, TAX is subjected to numerous preclinical investigations, and it exerts cytotoxic activity at concentration as low as (50 nM) 43 ng/mL (3, 4).

TAX has shown tremendous therapeutic benefit in the treatment of various tumors; hence, there is an increase in the demand for a formulation of a novel drug delivery system for TAX. There are several problems associated with TAX production and its administration to patients as in dosage forms. There is a need for a analytical method which is rapid, selective, and sensitive and can be performed routinely.

Many analytical methods have been developed to determine TAX in biological samples (e.g., plasma, serum, and tissue) over the past decade, such as high-performance liquid chromatography (HPLC) with UV (4–14), immunoassays (14, 15), capillary electrophoresis (CE) (16), and liquid chromatography tandem mass spectrometry (LC–MS–MS) (17–20). The immunoassay methods lack the specificity of the HPLC ones; although it possesses higher sensitivity. CE method needs only a small amount of samples, but it does not have the sensitivity of the HPLC–UV or LC–MS, which can use micro-samples. LC–MS–MS is more sensitive and selective in the determination, but this method is costly and not suitable for routine and simple analysis (13). There are some HPLC–UV methods (Table I) reported in literature for the estimation of TAX in plasma with sophisticated and tedious sample preparation procedures. There is no extensive method with peak purity determination by ratiograms and purity curve. Therefore there is a need for simple and sensitive HPLC method for analysis of TAX in rat plasma for routine preclinical study.

In the present study, an attempt has been made to develop a new, simple, rapid, sensitive, accurate, and reproducible HPLC–diode array UV method for the quantification of TAX in micro-volume rat plasma by using protein precipitation with ice-cold acetonitrile as extracting solvent. PP is used as the internal standard (IS) for the first time in the estimation of TAX, which is a commonly used preservative (21, 22) and readily available, which is an advantage over those used in previously published methods (Table I). PP, chemically propyl 4-hydroxybenzoate, is poorly soluble in water and very commonly available in laboratories as oppose to other parabens. PP is readily available in high purity and has a detection response at 233 nm as like TAX. The validated method (23, 11, 7) has been successfully applied to the i.v. bolus pharmacokinetic investigation of TAX in wistar rats. The advantages of the present method has been compared with the previously published methods as shown in Table I.

© The Author [2012]. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com

Table I

Comparison of Published Method to the Present Method

Extraction procedure and % Recovery (TAX and IS)	Sample volume (plasma/serum) (μL), LOD and LLOQ	IS	Validation parameters	Retention time (TAX and IS) and total run time	Disadvantage compared to our method	Reference
LLE* (2 mL of dichloromethane) 90–100%	250 0.012 μg/mL (S/N* = 3) 0.18 μg/mL (% RSD 11.7)	Glafenine	The precision of the method ranged from 0.61 to 9.98 % for 0.18 to 1.44 μg/mL	10 and 15 min Total run time is not given	Total run time is more than 15 min and hence not more suitable for the preclinical study Plasma volume is high hence not suitable for preclinical PK studies High LOD and LLOQ Special IS	Coudore et al., (12)
LLE (0.8 mL of tert-butylmethylether and and 0.6 mL of dipotassium hydrogen phosphate) and washing with 1 mL of n-Hexane.	200 10 ng/mL (% CV 14.2)	Butyl paraben	The coefficient of variation is 14.2 %	6.7 and 4.8 min	Mixture of solvents as mobile phase with 1.3 mL/min flow rate. Complicated extraction procedure In our method with 100 μL, the LOD is 5 ng/mL High volume of plasma was used hence not suitable for preclinical PK studies Total run time was not given Peak purity is not determined	Li et al., (10)
LLE (double extraction with 1 mL tert-butylmethyl ether) 78.9–88.3 and 55.4 %	100 7.5 ng/mL (S/N = 3)	4- Hydroxybenzoic acid n-hexyl ester	The intra-day and inter-day precision ranged from 2.7 to 9.2 % and 3.3 to 6.0 % for concentration ranged from 0.15 to 15 μg/mL	11.2 and 20.4 min. Total run time 25 min	Stability of TAX checked in human plasma at two higher concentrations and its % bias is not given between the cycles. Method was intended for the estimation of TAX in human plasma and cannot be used for the preclinical study. Low extraction efficiency of IS and drug Time consuming double extraction Total run time is very high and hence not more suitable for the preclinical study Validation is not done for rat plasma In our method with 100 μL the LOD is 5 ng/mL (S/N = 3) Peak purity is not determined	Yonemoto et al., (6)
LLE (with 10 mL ethyl acetate) 93.7 and 96.9%	100 5 ng/mL (S/N ≥ 3) 10 ng/mL	Dimethyl-4-4′-dimethoxy 5, 6, 5′, 6′-dimethylene dioxy diphenyl- 2, 2′ dicarboxylate (DDB)	The intra-day and inter-day precision ranged from 0.78 to 9.35 % and 0.55 to 3.75 % for concentration ranged from 0.1 μg/mL to 20 μg/mL	18.0 and 13.7 min and total run time is 30 min	Gradient analysis Total run time is high and routine analysis is not easy Specially synthesized IS High volume of extracting solvent and hence may be costly. Concentration dependent recovery with LLE Peak purity is not determined	Kim et al., (7)
LLE and SPE * (double extraction with total of 8 mL of diethyl ether and with multiple steps of modified solid-phase extraction) Concentration independent 76 to 85 % recovery	200 15 ng/mL (S/N ≥ 3) 25 ng/mL (within-day precision less than 20%)	2′-Methylpaclitaxel	The within-day precision ranged from 1.2 to 3.4 % and between-day precision ranged from 1.6 to 3.2 % for 50-3000 ng/mL respectively	Around 10 and 15 min (interpreted from the chromatogram) Total run time 30 min	The tedious procedure in extraction, aqueous layer was frozen in ethanol-solid carbon dioxide Costly IS Very sophisticated extraction procedure and not suitable for preclinical study Peak purity is not determined	Sparreboom et al., (4)
LLE (mixture of (1:4, v/v) acetonitrile–n-butyl chloride) 89.6 and 93.7%,	1000 (1 mL) 5ng/mL (outside 20%) 10ng/mL (RSD 4%)	Docetaxel	The within-run and between-run precision ranged from 0.47 to 2.99 and 0.58 to 2.76 for 10 ng/mL to 15000 ng/mL respectively	7.5 and 8.5 min and total run time 30 min	Large volume of biomatrix hence not suitable for preclinical PK studies Complicated mobile phase Costly IS Multiple extraction solvent Total run time is high and not suitable for routine analysis in preclinical study Peak purity is not determined	Sparreboom et al., (8)

LLE (35mM ammonium acetate buffer (pH 5) and 7 ml of diethyl ether) 82 % for 100 ng/mL	1000 (1 mL) 10 ng/mL (S/N ≥ 3) 25ng/mL (C.V 57.72%)	Docetaxel	The Intra-assay variability (precision) was 6.34% (44ng/m/L), 2.84% (440ng/mL) and 1.54% (750ng/mL), respectively. inter-assay at the same concentrations were 11.18%, 2.97% and 3.02%, respectively	7.7 and 6.7 min	Stability was done only in -20^{0}C and in higher concentration in human plasma Costly IS Large volume of plasma is used hence not suitable for preclinical PK studies Complicated extraction procedure Large volume of plasma to get 25 ng/mL as LLOQ Peak purity is not determined	Martin et al., (11)
SPE (multiple steps of extraction) 82 and 90%	100 5 ng/mL (S/N ≥ 3) 10 ng/mL (R.S.D-14%, Deviation: -6%)	2′-Methylpaclitaxel	The intra-day and inter-day precision ranged from 2.9 to 6.7 % and 3.3 to 14.1 % for concentration ranged from 10 ng/mL to 1000 ng/mL	8.5 and 11.0 min and the overall run time lasted 30 min.	Complicated mobile phase Costly extraction technique and IS Sophisticated and multiple step extraction procedure The technique is not widely used in laboratories Peak purity is not determined	Wang et al., (13)

* LLE-Liquid liquid extraction; SPE-Solid phase extraction; S/N-Signal to noise ratio

Experimental

Materials and methods

TAX (assay 99.95%) was obtained as gift sample from Good Well Pharmaceuticals (New Delhi, India). PP and dimethyl sulfoxide (DMSO) were purchased from Spectrochem (Mumbai, India). HPLC-grade acetonitrile was purchased from Merck (Mumbai, India). Analytical grade EDTA-2Na was obtained from SRL Lab (New Delhi, India).

Instruments

The Prominence ultra-fast liquid chromatography (UFLC) system consisted of a pump (LC-20AD) with integrated system controller auto sampler (SIL-20AC) and variable wavelength UV detector/diode array detector (SPD-M20A) was purchased from Shimadzu (Shimadzu, Kyoto, Japan). Data acquisition and analysis was performed using LC solution software (Shimadzu). LichroCART 250-4, HPLC-Cartridge, Lichrospher 100 RP-18 e (5 μm), Lot. L57062233 double end-capped RP-HPLC columns (Merck, Darmstadt, Germany) fitted with guard column of the same material were used for the separation. All calculations were performed using a peak-area ratio ($R_U = r_u/r_{is}$), where r_u is peak area of TAX obtained from a chromatogram and r_{is} is peak area of IS obtained from a chromatogram, by using Microsoft Excel (MS Office, 2003). The pharmacokinetic data were obtained using WinNonlin (Scientific Consultants, Mountian View, CA).

Method development

Preparation of stock and standard solution

A master stock solution of 2 mg/mL was prepared in DMSO and stored in glass ampoules at −20°C. Secondary stock solution of 60 μg/mL was prepared by taking an aliquot from the primary stock, and this stock solution was further serially diluted to get working standard solutions in the range of 30−0.1 μg/mL; all the dilutions were made in 50:50% v/v acetonitrile and water. The secondary stock and working solution were prepared fresh daily. The master stock solution of the IS was prepared at a concentration of 100 μg/mL in acetonitrile and stored at −20°C between uses. The working standard of 0.125 μg/mL was prepared from the master stock daily in acetonitrile. The precipitation solvent (acetonitrile) containing PP is made ice cold at −80°C for sample processing. The concentration of IS in all samples were maintained at 1 μg/mL. All the stock solutions were prepared freshly on the day of validation and care was taken to store TAX stock in glass ampoules.

Sample collection and preparation

Rat blood samples, approximately 1.5 mL from each animal, were withdrawn from more than 15 healthy animals by cardiac puncher under diethyl ether anaesthesia with a disposable syringe (Dispovan, India) with 26 G needle. After blood collection, all animals were recovered from anaesthesia. The blood was collected into 2 mL polypropylene microtubes (Tarsone, India) containing EDTA-2Na (0.1 mL of 10% solution for 5 mL of plasma) for preparation of plasma, and care was taken to mix

the blood with EDTA-2 Na. After centrifugation at 12000 rpm for 15 min at 4°C, the plasma was pooled into one tube and stored at −20°C and thawed before analysis.

Sample processing

To 100 μL plasma samples (drug spiked/real time sample), 800 μL of ice cold acetonitrile containing IS (0.125 μg/mL) was added. This solution was vortexed for 2 min in multi-holder vortex mixer (SPINIX Multilab, Mumbai, India) and centrifuged in cooling compufuge CPR 24 (REMI, Mumbai, India) at 12000 rpm for 10 min at 4°C. The total sample processing time before evaporation was 12 min. The plasma protein precipitates were collected as pellets at the bottom of the tube. The supernatant was taken, dried using a vacuum concentrator (MAXI dry lyo, Heto vacuum centrifuge, Germany), and the dry residue was reconstituted with 100 μL of 50:50% (v/v) acetonitrile and water. The reconstitution solution is close to that of mobile phase, to avoid possible baseline drifts that might occur. An injection volume of 75 μL was injected into the HPLC.

System suitability parameters

The column efficiency for the separation of TAX and the PP was evaluated using the following formula:

$$N = 16\,(t/\mathrm{W})^2 \, or \; N = 5.54\,(t/\mathrm{W}_{h/2})^2$$

where, N is the number of theoretical plates, t is the retention time of TAX or IS, and W the peak width of TAX or IS at the baseline or $\mathrm{W}_{h/2}$ the width of TAX or IS peak at half height.

The capacity factor (k') for TAX and IS were calculated as:

$$k' = (t - t_0)/t_0$$

where t and t_0 are the retention time of TAX or IS and the non-retained sample (solvent front), respectively.

The tailing factor (T) for TAX and IS were calculated as follows

$$T = \mathrm{W}_{0.05}/2f$$

where $\mathrm{W}_{0.05}$ is the width of peak (TAX or IS) at 5% height and f is distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline.

Method validation

Selectivity and peak purity

The selectivity of the method was studied by investigating the interference from various endogenous matrix components (mainly proteins) and exogenous substances that may come in contact with the sample (intentional or accident) during the process. Blood from six different rats were collected for this study, and the blood samples were processed for plasma and stored at −20°C until analysis. Six individual samples of drug and IS free plasma (blank sample), samples with IS (zero samples), and lower limit of quantitation (LLOQ) (10 ng/mL) samples were processed individually and analyzed by the proposed method. The obtained chromatograms of blank samples were compared against analytical, calibration standards, and real time i.v. pharmacokinetic samples to investigate possible interference in the determination. The conditions for LLOQ require that the peak area of compounds co-eluting with the TAX or IS should not exceeds 20% of the TAX peak area at LLOQ or 5% of the IS area.

The selectivity is further confirmed by peak purity determination by using the ratiograms and purity curve. The ratiograms is constructed by plotting the ratio of absorbance/response of TAX at two different wavelengths (231 and 235) over a retention time (8.0 ± 0.25). The rectangular ratiograms show that the peak is pure.

Linearity and quality control samples

Calibration standards in drug free rat plasma were prepared at concentrations of 10, 25, 50, 100, 250, 500, 750, and 1500 ng/mL of TAX from respective working stocks. Calibrator samples were prepared by spiking 95 μL of blank rat plasma with 5 μL of the respective TAX working stock solutions in 1.5 mL polypropylene microcentrifuge tubes. This satisfies the limit of 5% addition of organic solution to plasma. The calibration samples consist of a blank sample (matrix sample processed without IS), a zero sample (matrix sample processed with IS), and seven non-zero samples including LLOQ.

Quality control (QC) samples were prepared at concentrations of 10, 25, 50, 500, and 1500 ng/mL from respective working stock solutions similar to the the calibration sample. On each day of validation, calibration standards and QC samples were prepared fresh and analyzed.

Determination of LLOQ and limit of detection

The LLOQ determination was performed on five different days ($n = 5$), by spiking an aliquot of blank rat plasma (95 μL) with TAX (5 μL) at a concentration of the lowest calibrator with a precision less than 20%, accuracy of 80−120%, and signal-to-background noise ratio greater than 6:1. The limit of detection (LOD) was defined as the lowest concentration of TAX that the method can detect with a signal-to-noise ratio greater than or equal to 3 (i.e., the response: peak height/area or height/area ratio of peaks in the case of IS method of the TAX concentration should be equal to or greater than 3 times of the base line noise of the instrument).

Recovery

The determination of the processing method efficiency was done by calculating the recovery of TAX in spiked plasma samples. The recovery was calculated by comparing the TAX peak area of the spiked plasma samples (extracted sample) with their respective aqueous samples. By the same method, recovery of the IS was calculated. The concentration used to study the recovery of IS was 1 μg/mL. TAX recovery study was carried out in all calibration points. All the prepared calibration standards were subjected to sample processing and analyzed by the proposed method.

Recovery (%) = area of extracted standard /area of aqueous standard x 100.

Intra-day and inter-day precision and accuracy

The precision and accuracy were determined by taking five concentrations (QC samples) in the range of calibration curve

(all measurement were five determinations per concentration). The intra-day precision and accuracy were determined by analyzing the spiked QC plasma samples prepared within a day on three different occasions. The inter-day precision and accuracy were determined by analyzing the spiked QC samples prepared on three different days. On each day of validation separate calibration curves were constructed to determine the calculated concentration or actual concentration of the prepared samples. After the concentrations were calculated by using the regression equation, the % relative standard deviation (RSD) was calculated using the mean value and the standard deviation (SD); the % Bias was calculated from the calculated concentration and known concentration (concentration prepared); the % recovery was calculated by using the standard formula at each concentration of the QC samples. The limit for precision is reached when the % RSD value does not exceed 15%, except for LLOQ, where it should not exceed 20%. A low percent relative error shows the accuracy of the proposed method.

Stability

During the pharmacokinetic study, the collected blood samples were processed to separate the plasma and then stored in respective storage condition (−20°C). Hence, it was necessary to determine the stability of TAX and PP in the biological samples at these respective storage conditions. The stability studies are conducted as follows: freeze-and-thaw stability, short-term stability, long-term stability, post-preparative stability, and stock solution stability. On each day of the stability study, separate calibration standards were processed and analyzed with the stability samples. All stability studies were conducted as per US FDA guidelines in five QC standards.

Freeze-and-thaw stability

The five QC standards were prepared in plasma and stored at −20°C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 18 h under same conditions. The freeze-and-thaw cycles were repeated four more times, after which the samples were analyzed with the proposed method on the fifth cycle. Hence, TAX and IS stability in plasma were determined for five freeze-and-thaw cycles. The stability of TAX was determined by calculating % Bias and % recovery. Each concentration was measured in triplicate.

Short- and long-term stability

The short term stability was conduced up to 24 h at room temperature (25°C ± 0.5), based on the expectation that TAX in plasma will be maintained at this temperature for a maximum of 24 h. The selected QC standard were thawed at room temperature, and then processed and analyzed at 1, 6, 12 and 24 h.

The long-term stability time points were selected by considering the time between the date of first sample collection and the date of last sample analysis. The time points chosen for long-term stability were 7, 15, and 30 days. On the respective time points the samples were thawed unassisted at room temperature, processed, and analyzed by using the proposed method. The concentration of all stability samples were compared to the mean of back calculated values of the fresh QC standards at the appropriate concentration. As in the freeze-and-thaw stability study, five QC standards were analyzed in triplicates.

Post-preparative stability

The aim of this study was to determine the stability of TAX and the PP in the reconstitution solution (50:50% v/v acetonitrile and water) during the time the sample rests in the autosampler (18°C ± 0.2). The time points were selected based on the anticipated run time for the batch size in validation samples. The time points selected for this study were 1, 3, and 5 days. The QC samples were prepared by spiking respective aliquots from working stock to the plasma, then all the samples were processed and loaded into the autosampler, and the analysis was done as per the time points.

Stock solution stability

The stability of TAX and PP in the stock solution is evaluated at room temperature and at −20°C for one month. TAX stability in dimethyl solfoxide (DMSO) is monitored at two QC standard concentrations (500 and 1500 ng/mL). PP stability in acetonitrile was evaluated at 100 μg/mL. Each determination was performed in duplicate.

Application of the method

Preparation of TAX solutions

TAX is a poorly water soluble drug (less than 1 μg/mL) and hence TAX solution for i.v. injection was prepared by dissolving 90 mg of pure TAX in a mixture of 7.5 mL of ethanol and 7.5 mL of Tween 80. Before injection, this solution was diluted with sterile saline (0.9% of sodium chloride) to a final concentration of 3 mg/mL, such that the volume of drug solution injected into the rat was below 1 mL. TAX in saline solution was used within 3.5 h (23).

Pharmacokinetic study

The pharmacokinetic studies of TAX were performed on male wistar rats (160–220 g) as described elsewhere with slight modification (10, 13). The study protocol was approved by the Institute Animal Ethical Committee (protocol no: IEAC/RES/5/6/ rev 01) before beginning the experiment. Throughout the experiment, animals housed in polypropylene cages filled with sterile padded husk and maintained at: 22 ± 2°C, 50–60% relative humidity, and under a 12:12 h light–dark cycle. The rats were housed three per cage and kept under these conditions for at least one week before the experiment was initiated. The rats were fasted overnight before i.v. drug administration and had access to water ad libitum. The animals were separated according to body weight in five cages, with each cage containing three animals, and they were marked by the head, tail, and body method with picric acid. The dosing (10 mg/kg) through tail vain was began after diluting the stock solution with saline. The tail vain is dialated using xylene to avoid accumulation of the drug in muscles, which may cause necrosis. Blood samples (300 μL) were collected into EDTA-2Na containing prelabeled 1.5 mL polypropylene microtubes through cardiac puncher under mild diethyl ether anaesthesia. Immediately after collection, the microtubes were gently inverted several times to ensure complete mixing with the anticoagulant. The sample

collection time points were 30 min, 1, 1.5, 3, 6, 9, 12, 24, and 48 h after TAX administration, and at each time point samples were taken from three animals. The blood samples were centrifuged at 12000 rpm for 15 min at 4°C, and collected plasma samples were frozen at −20°C and thawed every time before analysis. During the processing of the real-time samples, IS was spiked at 1 μg/mL concentrations and analyzed by the developed method. Along with this, one set of calibration standards to calculate the concentration of the sample was incorporated. To accept the assay run, five sets of QC standards were prepared and analyzed with the real-time sample. The pharmacokinetic parameters were obtained by a noncompartment model.

Results and Discussion

Chromatographic separation

During the chromatographic separation, in order to obtain good resolution for the TAX and PP peaks, different aqueous phases were tried while keeping the organic phase (acetonitrile) constant. Acetonitrile (55:45 % v/v) with ammonium acetate buffer or millipore water as aqueous phase was selected with respect to peak symmetry (T = 1.02). Based on the ease of preparation, water was selected as the aqueous phase. TAX does not have any ionizing group (no reported pKa); hence the mobile phase pH does not greatly influence the separation. The mobile phase used in this method is very simple to prepare when compared with those previously reported (10, 13, 24) and the extraction procedure is also very simple when compared to the procedure of Fruscio et al. (25), which has multiple extraction steps. The optimized mobile phase was acetonitrile and millipore water (55:45 v/v), which provided a moderate and quick retention time, with better peak properties, resolution, and selectivity for TAX and PP. The retention times for TAX and IS were 8.0 ± 0.25 and 5.3 ± 0.15 min respectively, with 7.70 ± 0.15 min resolution.

Estimation of TAX in biological samples requires several chemicals as IS, such as *N*-cyclohexy benzamide, cephalomannie, d5-TAX, 2'-methyl TAX, docetaxel, butyl paraben, *n*-hexyl *p*-hydroxy benzoic acid, dimethyl-4-4′-dimethoxy 5, 6, 5', 6'-dimethylene dioxy diphenyl- 2, 2' dicarboxylate (DDB), and glafenine free base. However, most of these chemicals are expensive, lack commercial availability, must be synthesized especially for analysis purpose, have specificity problem, or are difficult to reproduce (4–13). In general, the solubility of PP in organic solvents is less (TAX is highly lipophilic drug) than the butylparaben, and PP is the most commonly available pharmaceutical excipient than the hexyl ester of 4-hydroxybenzoic acid (22). Hence PP was selected as the IS among the parabens. For the first time, PP was used as the IS for the extraction of TAX from 100 μL rat plasma and its determination by HPLC-diode array UV method. PP is adequately separated from the other components of plasma (Figure 1).

TAX and IS were separated by isocratic reversed-phase HPLC with diode array UV detection at 233 nm. TAX lacks strong UV absorption, and detection at the more permissive wavelength of 227 nm requires the use of large volumes (0.4–1 mL) of sample (Table I). There are many endogenous substances from biological matrix with similar lipophilicity as that of TAX, which also have a strong absorption at 227 nm, and they are also strongly retained on the C18 column (13, 20). Because of the reasons listed TAX was determined at 233 nm, where endogenous substance absorption is decreased (Figure 1A) and was, thus, estimated with high accuracy and precision. In this method, only 800 μL of ice cold acetonitrile was used as extracting solvent (average % recovery 100.6 ± 3.2) to estimate TAX (LLOQ 10 ng/mL) from micro volume rat plasma. The extraction procedure described is very economical (only 800 μL of extracting solvent) when compared to the reported method using solid-phase extraction (SPE) and liquid–liquid extraction (LLE). In the reported LLE (6, 10) and SPE (4, 13) methods for the estimation of TAX in biological matrix, more than 1 mL of extracting solvent is used; however, in the present method, only 800 μL of ice-cold acetonitrile [less than this volume results in a reduction in extraction efficiency (% recovery)] is used for extraction and estimation of TAX from plasma. The sample processing (before evaporation step) takes less time (12 min), and there are no time-consuming step, such as freezing the aqueous layer in the freezer as in the LLE method and multiple extraction steps as in the SPE method (Table I). In the reported method, there are many complicated and sophisticated steps before the supernatant is concentrated by evaporation (Table I). By using LLE, it has been reported that the liquid solvents causes interference of endogenous compounds with TAX and with insufficient efficiency of extraction to permit routine application. It is well know that the LLE method has more inconsistent variation in extraction between samples (11).

In our method, the simple protein precipitation purified the matrix and helped to estimate TAX and PP with high sensitivity (low signal-to-noise ratio to obtain LOD of 5 ng/mL and LLOQ of 10 ng/mL) and specificity (Figures 1 and 2) at 233 nm. There was no interference from the matrix during total analysis time of 10 min, and there were no high interfering peaks up to 30 min.

Most of the methods developed for the separation of TAX from biological samples used multiple steps of LLE with a large volume of sample required (2–10 mL) and mixture of extracting solvents. Some of the method have pre- and post-washing steps with *n*-hexane, double extraction, complicated reconstitution solution, and there are more assay methods that used SPE (Table I). All of the described methods make the process more complicated and difficult for routine analysis. In the present method, a very simple and rapid method was used to estimate TAX in micro volume rat plasma.

Yonemoto et al. developed a simple LLE method for the separation of TAX and 4-hydroxybenzoic acid *n*-hexyl ester in plasma. This HPLC–UV method offers the separation of TAX and IS in 25 min with a retention time of 11.2 min for TAX and 20.4 min for IS on an ODS-3 column (6). In the presented method, TAX and IS are estimated with in 10 min with LLOQ of 10 ng/mL and LOD of 5 ng/mL, which makes the developed method sensitive, rapid, and simple. Hence this method can be used for routine analysis of TAX in rat plasma.

Method validation

Selectivity and peak purity

Figure 1A–1F are 3D representative chromatograms of a blank rat plasma sample, zero sample, control plasma spiked with 10, 500,

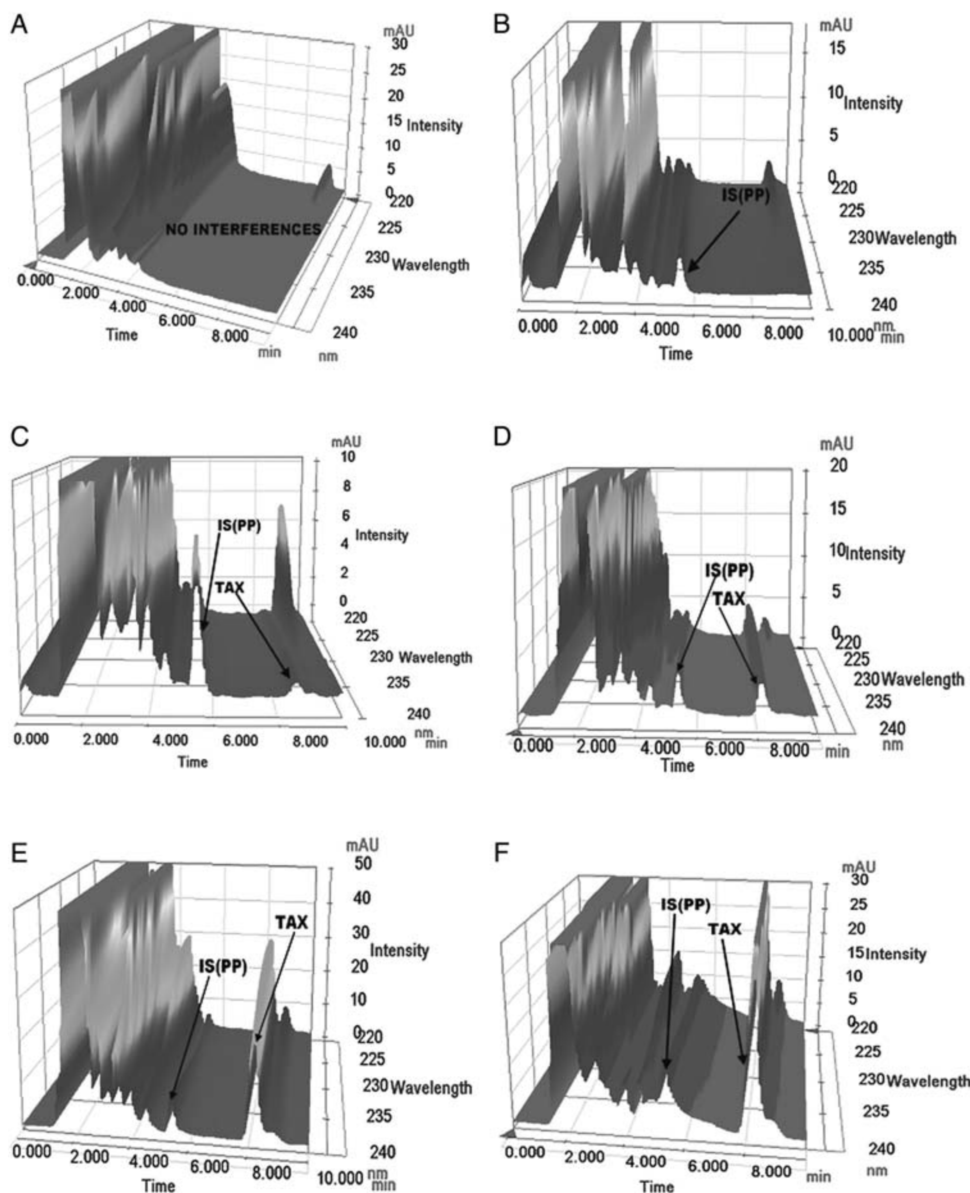

Figure 1. Representative 3D (Time, Wavelength and Intensity) chromatograms of TAX and PP: (A) Blank sample, (B) Zero sample, (C) Control plasma spiked with 10 ng/mL, (D) Control plasma spiked with 500 ng/mL, (E) Control plasma spiked with 1500 ng/mL, (F) Plasma sample at 6 hr after i.v. bolus injection of TAX.

and 1500 ng/mL of TAX, and a plasma sample (at 6 h) of an i.v. bolus pharmacokinetic study. Chromatogram of blank sample and zero samples revealed that there was no interfering peak present in the eluting window of TAX and IS. Further, the real time i.v. pharmacokinetic chromatogram confirms that there was no interference from metabolites or degradation products or other exogenous xenobiotics in the near vicinity of TAX and IS. There was no co-eluting peak, $>$ 20% of the TAX at LLOQ and $>$ 5 % of the area of IS at their respective retention time (Figure 1A–1C). These results confirm the selectivity of the developed method for extracting TAX from micro-volume rat plasma.

The chromatograms recorded at the elution time at different wavelengths (229-235 and 256) show the peaks homogeneity of TAX and PP (Figure 2A and 2B) with good overlay of peak shape and retention match. There is no interfering, co-eluting, or co-migrants endogenous or exogenous or metabolite substance at the selected wavelength, this shows the efficiency of the precipitation method and selectivity of determining TAX at 233 nm. This is the reason, in addition to extraction efficiency, for the lower limit of quantification (10 ng/mL).

In the obtained rectangular ratiograms, the ratio of response (area/absorbance) at two selected wavelengths (231 and 235) are less than one (Figure 2C–2E) and it is constant across the elution time. The rectangular ratiograms shows that the TAX is quantified with high selectivity at 233 nm. The peak purity index and single point threshold (Figure 2F) value is always close to one (1.0000 and 0.987749). The positive minimum peak purity index value (12250) also shows the purity of peak (TAX) and it is selective. The obtained purity curve data shows selectivity of the method in determining the TAX.

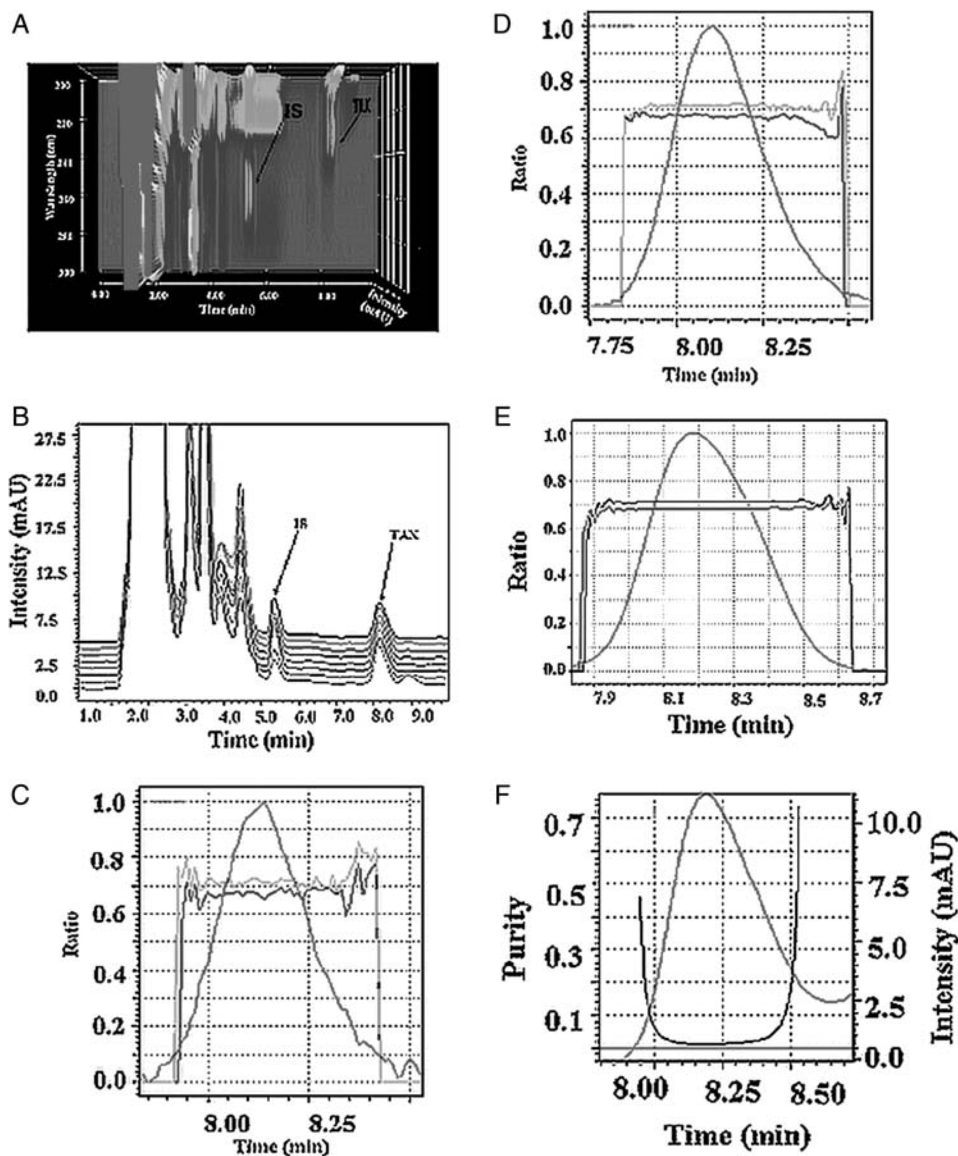

Figure 2. Representative 3D chromatogram for selectivity and sensitivity: (A) Top 3D view chromatogram of 500 ng/mL, (B) Chromatogram of 500 ng/mL recorded at the same time at different wavelength, (C) Ratiograms of 10 ng/mL, (D) Ratiograms of 500 ng/mL, (E) Ratiograms of 1500 ng/mL, (F) Peak purity.

Linearity and quality control samples

Calibration curves were prepared on each day of analysis by an IS addition method for known concentrations of TAX in rat plasma samples. Calibration curves were constructed by plotting peak area ratio versus the concentrations. The typical best-fit linear regression equation for the calibration curve in the range of 10–1500 ng/mL: peak area ratio = 0.0084x concentration of TAX (ng/mL) − 0.1208, $r^2 = 0.9999$. Goodness of fit of regression equation for TAX in rat plasma was linear with a higher mean correlation coefficient of 0.9997 ± 0.0003, the standard deviation of slope and intercept were found to be 0.0007 and 0.0525, respectively (Table II), with low standard error of estimate (4.66). ANOVA test (one-way) was performed for peak area ratio obtained at individual concentration levels and calculated F-value was lower than critical F-value at 95% level of significant, which supports the best-fit linear equation.

Determination of LLOQ and LOD

The lowest standard (LLOQ) on the calibration curve (10 ng/mL) was accepted as the limit of quantification (LOQ) as its response was more than six times the blank response or background noise. The peak obtained for the LLOQ was identifiable, discrete, and reproducible with precision (% RSD) of 1.79% and accuracy (% recovery) of 100.76. The repeatability is very high as compared with the method of Li et al. (10). The method was found to be sensitive with a high signal-to-noise ratio and acceptable precision and accuracy. By using a micro-volume amount (100 μL) of rat plasma, the LOD was found to be 5 ng/mL, with signal-to-noise ratio greater than three.

Table II
Slope and Intercept of Calibration Curve of TAX in Micro-Volume Rat Plasma

Calibration curve*	Slope	Intercept	Correlation coefficient
1	0.0084	−0.1208	0.9999
2	0.0083	−0.0560	0.9999
3	0.0074	−0.1151	0.9998
4	0.0083	−0.1215	0.9998
5	0.0070	0.0093	0.9999
6	0.0085	−0.0974	0.9997
7	0.0069	−0.1002	0.9992
8	0.0085	−0.1663	0.9993
Mean	0.0079	−0.0960	0.9997
S.D.	0.0007	0.0525	0.0003
% RSD	8.741	-	0.028

* Each calibration curve is obtained using eight points

Table III
Recovery of TAX and PP From Micro-Volume Rat Plasma

Concentration (ng/mL)	Mean absolute recovery (%, ± SD)*	% RSD
TAX		
10	100.3 ± 4.7	4.73
25	106.5 ± 7.1	7.09
50	95.3 ± 5.0	5.24
100	100.3 ± 0.6	0.62
250	99.1 ± 5.3	5.36
500	102.5 ± 3.7	3.59
750	99.7 ± 2.1	2.06
1500	100.9 ± 1.2	1.20
Average recovery (± SD)	100.6 ± 3.2	0.003
IS		
1000	91.7 ± 2.9†	3.19

* Average of six determination (n = 3 on two days)
† Average of ten determination (n = 5 on two days)

This is lower than the reported method by Yonemoto et al. 7.5 ng/mL (6), Sparreboom et al. 15 ng/mL (4), and Martin et al. 10ng/mL (11) (see Table I). The LOD of present method is equal to the Wang et al. (13) who used sophisticated SPE (Table I). The clinical pharmacokinetic and cytotoxicity studies reviles that the analytical method with less than 43 ng/mL as quantification limit is required for the estimation of TAX in a biological matrix (4). The LOD and LLOQ of this study are satisfactory with respect to the 43 ng/mL of its cytotoxicity action. These suggest that the method is suitable for various pharmacokinetic investigations of TAX in rodents, which demands high sensitivity and repeatability. The developed method was able to detect TAX up to 24 h (26.42 ng/mL ± 2.55) in i.v. pharmacokinetic study with 10 mg/kg dose in rat.

Recovery

In general for protein precipitation, room temperature or ice-cold acetonitrile or any other protein precipitating agent is used to extract the drug from a biological matrix. In the present method, ice-cold acetonitrile resulted in high recovery (Table III) of both TAX and PP, respectively. The recovery of TAX after simple single protein precipitation with ice-cold acetonitrile was studied at all calibration standards in triplicates. The efficiency of extraction was found to be in the range of 95.3 ± 5.0 to 100.9 ± 1.2 %, with average recovery of 100.6 ± 3.2. The recovery of IS at 1 μg/mL was 91.7 ± 2.9%. This shows that the processing method is efficient in extracting the TAX and IS effectively in micro-volume of rat plasma than the SPE method (% recovery 85% and 89% for 500 and 1000 ng/mL, respectively) used by Wang et al. (13) and Caporossi et al. (27) (% recovery higher than 87%, using 0.5 mL of plasma) (Table III). The results show that the extraction efficiency of the method is consistent, precise, and reproducible (% RSD < 5.36).

Intra-day and inter-day precision and accuracy

Precision and accuracy data of TAX in rat plasma are shown in Table IV. The intra-day precision of TAX in micro-volume of rat plasma showed % RSD less than 1.94. The percent relative standard deviation (% RSD) in inter-day precision of all QC sample were less than 2.06 for TAX. The precision results (% RSD) are very low as compared Coudore et al. (12), Li et al. (10), Yonemoto et al. (6), Kim et al. (7), and Wang et al. (13) (Table I). These showed that the method is highly reproducible; hence, it can be used for routine analysis of TAX in rat plasma. The intra-day accuracy study showed % bias ranged from 0.03 to 0.78. The inter-day accuracy (% Bias) at all QC levels ranged from 0.05 to 0.76. The % recovery for intra-day and inter-day precision were in the range of 100.03 to 100.78 and 100.05 to 100.76.

The simple single step protein precipitation with ice-cold acetonitrile method showed consistent and high recoveries at all five concentrations. The average % recovery (with low and uniform standard deviation) was normally distributed around the mean with uniform % RSD, suggesting the homoscedastic nature of the data. Thus, the statistical data concluded that there was no interference from the endogenous or exogenous substance, and the method was found to be accurate with low % Bias. This suggested that the developed method is accurate for extraction of TAX from micro-volume rat plasma samples every time. This shows that the method has excellent reproducibility, and it can be used for routine analysis of preclinical pharmacokinetic samples.

System suitability parameters

The number of theoretical plates (N) of the column for separation of TAX and IS were 6400 and 4974, respectively. The capacity factor (k') for TAX and IS was 5.5 and 3.3, respectively. The tailing factor for TAX (1.00 ± 0.05) and IS (0.98 ± 0.06) approaches to unity, which shows that the peaks are perfectly symmetrical. The system suitability parameters show that the method is reproducible with good resolution.

Stability

The results of freeze-and-thaw stability at all QC levels demonstrated that TAX and the IS were stable in rat plasma up to five freeze-and-thaw cycles (Figure 3). Results are expressed in terms of % Bias and % recovery, which ranges from −0.45 to 0.99 and 99.55 to 100.99, respectively. Guo et al (26) showed that TAX is stable (% accuracy and % CV) up to three freeze-and-thaw cycles, and the present study result showed that TAX and IS are stable up to five freeze-and-thaw cycles. This confirms that TAX and IS is stable for five freeze-and-thaw cycles, making it suitable for subzero storage condition. The short- and long-term stability results showed that TAX and IS were stable up to 24 h in bench top conditions and for 60 days

Table IV
Intra-day and Inter-day Precision and Accuracy of TAX in Micro-Volume Rat Plasma (Each Value is Determination of Fifteen Values)

QC Levels (ng/mL)	Predicted concentration* (ng/mL)			% RSD		% Bias		% Recovery	
	Range	Intra-day (Mean ± SD)	Inter-day (Mean ± SD)	Intra-day	Inter-day	Intra-day	Inter-day	Intra- day	Inter-day
10	9.76–10.5	10.08 ± 0.20	10.08 ± 1.79	1.94	1.79	0.78	0.76	100.78	100.76
25	24.81–25.13	25.02 ± 0.11	25.02 ± *0.15*	0.42	0.59	0.08	0.07	100.08	100.07
50	49.66–50.33	50.12 ± 0.41	50.18 ± 1.04	0.83	2.06	0.25	0.36	100.25	100.36
500	499.08–502.75	500.85 ± 0.92	500.26 ± 0.95	0.18	0.19	0.17	0.05	100.17	100.05
1500	1498.22–1501.77	1500.49 ± 0.95	1500.70 ± 0.77	0.06	0.05	0.03	0.05	100.03	100.05

* Predicted concentration of TAX was calculated from linear regression equation

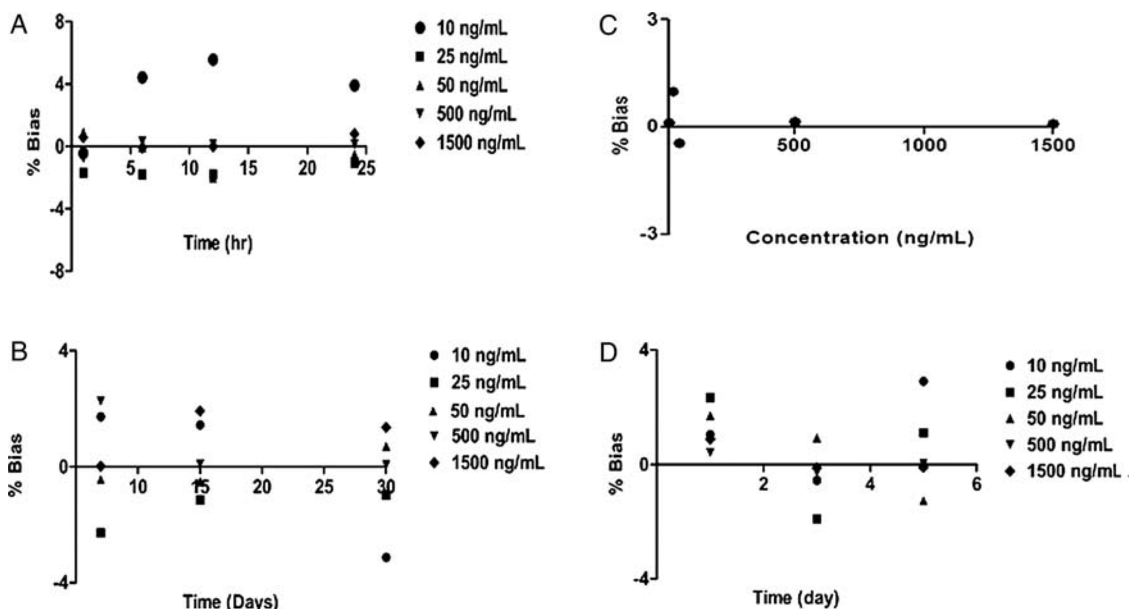

Figure 3. Stability study of TAX and PP in rat plasma: (A) Post preparative stability, (B) Short term stability, (C) Long term stability, (D) Freeze thaw stability.

at −20°C (Figure 3). The % Bias and % recovery for short-term stability ranged from −0.21 to 5.57 and 99.55 to 105.57, respectively. At all QC standards in long term stability the % Bias and % recovery ranged from −3.13 to 2.25 and 97.73 to 102.25, respectively. The post preparative study results demonstrated that TAX and IS can be stored in 50:50% (v/v) acetonitrile and water in an autosampler (18°C ± 0.2) for up to 5 days (Figure 3). The % recovery of post preparative study ranged from 98.15 ± 1.54 to 102.91 ± 0.56. Gardner et al. (27) showed that the TAX and d_5-TAX can be store in the auto sampler (4°C) for 24 h. It has been shown that the TAX and PP can be reanalyzed even after five days at 18°C ± 0.2. This allows the analyst to re-analyze the samples if required in situations like machine failure, which is very common for the researchers who work with HPLC. This post-preparative stability results gives confidence to reanalysis for TAX rat plasma sample up to five days. The % RSD calculated for all stability samples were well within the acceptable range of ± 20% at LLOQ and ± 15% at all concentration levels. These confirm that the TAX and IS were stable under various processing and storage conditions stated in the method. The stock solution stability data shows that TAX is stable at room temperature (mean % recovery 99.99 ± 0.57) and at −20°C (mean % recovery 99.95 ± 0.59) in DMSO for one month. IS was found to be stable in acetonitrile during a period of one month with mean percent recovery of 98.97 ± 1.32. After a complete survey of the literature, this is the first complete report with all stability study in micro volume rat plasma for TAX.

Estimation of TAX

i.v. bolus pharmacokinetic study

The validated method was applied to study the i.v. pharmacokinetic of TAX in wistar rats. Figure 4 showed the plasma concentration – time profile of TAX after i.v. bolus dose of 10 mg/kg. The plasma level of TAX was detected between 0.5 to 24 h. To ensure that the developed method continues to perform satisfactorily, QC standards were prepared and analyzed with the real time samples. The results showed that the four of the five sets of QC standards % RSD were less than 15%. Hence, the assay run was accepted for the real time sample analysis. The non-compartmental data analysis was presented in Table V. The AUC_{0-inf}, $t_{1/2}$ (h), and MRT (h) was high as compared with Yonemoto et al. (6) 1500 ng.h/mL, 1.3 x 102 min and 1.6 x 102 min. This difference in pharmacokinetic parameters is due to the nonlinearity and not because of amount of

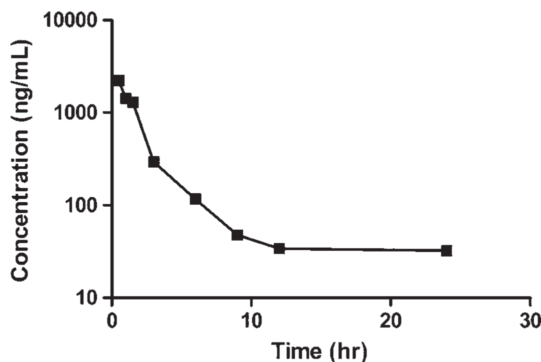

Figure 4. Plasma concentration-time profile of TAX.

Table V
Noncompartment-pharmacokinetic parameters of TAX

S.No	Parameters	Values ± SD
1	AUC_{0-inf} (ng.h)/mL	5787 ± 700.99
2	$AUMC_{0-inf}$ (ng.h^2)/mL	21484 ± 376.54
3	C_{max} (ng/mL)	3511 ± 59.43
4	$t_{1/2}$ (h)	3.77 ± 0.13
5	MRT (h)	3.71 ± 0.32
6	Cl (L/h/kg)	0.0018 ± 0.002

dose administered (29). The samples with concentrations greater than 1500 ng/mL were diluted with plasma and analyzed.

Conclusion

In this study, a new, simple, rapid, and sensitive HPLC-diode array UV method was developed for the estimation of TAX in micro volume (100 µL) rat plasma samples by simple single step protein precipitation with ice-cold acetonitrile (800 µL). PP was used as IS in the estimation of TAX in micro volume rat plasma. The validated method has been shown to be suitable for i.v. pharmacokinetic study of TAX (10 mg/kg) in wistar rats. TAX and IS are extracted efficiently with average % recovery of 100.6 ± 3.2% and 91.7 ± 2.9%, respectively. The intra-day and inter-day precision and accuracy showed % bias less than 0.78% and 0.76% and % recovery from 100.03% to 100.78% and 100.05% to 100.76%, respectively. All the stability study results shows that TAX and PP are stable in plasma with less % bias and high % recovery. The LOD and LLOQ of the developed method was 10 and 5 ng/mL. All the described results show that this method can be applied routinely in laboratories.

Acknowledgments

One of the authors, Sekar Vasantha Kumar, would like to acknowledge the Council of Scientific and Industrial Research (CSIR: India) for providing Senior Research Fellow (SRF), and thanks are given to the Department of Science and Technology (DST) for providing the full travel grant and registration under Young Scientist International Travel Support Scheme (ITS) to present part of this work in 2009 AAPS Annual Meeting and Exposition, LA, USA.

References

1. Singla, A.; Garg, A.; Aggarwal, D. TAX and its formulations. *Int. J. Pharm.* **2002**, *235*,179–192.
2. Rowinsky, K. Donehower, R. Paclitaxel (Taxol). *N. Engl. J. Med.* **1995**, *332*,1004–1014.
3. Panchagnula, R. Pharmaceutical aspects of TAX. *Int. J. Pharm.* **1998**, *172*,1–15.
4. Sparreboom, A.; Tellingen, O.V.; Nooijen, W.J.; Beijnen, J.H. Determination of paclitaxel and metabolites in mouse plasma, tissue, urine and faeces by semi-automated reversed-phase high-performance liquid chromatography *J. Chromatogr. B.* **1995**, *664*,383–391.
5. Yang, T.; Cui, F.D.; Choi, M.K.; Cho, J.W.; Chung, S.J.; Shim, C.K.; Kim, D.D. Enhanced solubility and stability of PEGyalted liposomal paclitaxel: In vitro and in vivo evaluation. *Int. J. Pharm.* **2007**, *338*,317–326.
6. Yonemoto, H.; Ogino, S.; Nakashima, M.N.; Wada, M.; Nakashima, K. Determination of paclitaxel in human and rat blood samples after administration of low dose paclitaxel by HPLC-UV detection. *Biomed. Chromatogr.* **2007**, *21*, 310–317.
7. Kim, S.C.; Yu, J.; Lee, J.W.; Park, E.S.; Chi, S.C. Sensitive HPLC method for quantitation of paclitaxel (Genexol) in biological samples with application to preclinical pharmacokinetics and biodistribution. *J. Pharm. Biomed. Anal.* **2005**, *39*,170–176.
8. Sparreboom, A.; Bruijn, P.; Nooter, K.; Loos, W.J.; Stoter, G.; Verweij, J. Determination of paclitaxel in human plasma using single solvent extraction prior to isocratic reversed-phase high-performance liquid chromatography with ultraviolet detection. *J. Chromatogr. B* **1998**, *705*,159–164.
9. Huizing, M.T.; Sparreboom, A.; Rosing, H.; Tellinggen, O.; Pinedo, H.M.; Beijnen, J.H. Quantification of paclitaxel metabolites in human plasma by high-performance liquid chromatography. *J. Chromatogr. B* **1995**, *674*,261–268.
10. Li, X.; Choi, J.S. Effect of genistein on the pharmacokinetics of paclitaxel administered orally or intravenously in rats. *Int. J. Pharm.* **2007**, *337*,188–193.
11. Martin, N.; Catalin, J.; Blachon, M.F.; Durand, A. Assay of paclitaxel (Taxol) in plasma and urine by high-performance liquid chromatography. *J. Chromatogr. B* **1998**, *709*,281–288.
12. Coudore, F.; Authier, N.; Guillaume, D.; Beal, A.; Duroux, E.; Fialip, J. High-performance liquid chromatographic determination of paclitaxel in rat serum: application to a toxicokinetic study. *J. Chromatogr. B* **1999**, *721*,317–320.
13. Wang, L.Z.; Ho, P.C.; Lee, H.S.; Vaddi, H.K.; Chan, Y.W.; Yung, C.Y. Quantitation of paclitaxel in micro-sample rat plasam by a sensitive reversed-phase HPLC assay. *J. Pharm. Biomed. Anal.* **2003**, *31*,283–289.
14. Leu, J.G.; Chen, B.X.; Schiff, P.B.; Erlanger, B.F. Characterization of polyclonal and monoclonal anti-taxol antibodies and measurement of taxol in serum. *Cancer Res.* **1993**, *53*,1388–1391.
15. Grothaus, P.G.; Raybould, T.J.G.; Bignami, G.S. An enzyme immunoassay for the determination of taxol and taxanes in taxus sp.tissues and human plasma. *J. Immunol. Methods.* **1993**, *158*,5–15.
16. Hempel, G.; Lshmkuhl, D.; Krumpelmann, S.; Blaschke, G.; Boos, J. Determination of paclitaxel in biological fluids by micellar electrokinetic chromatography. *J. Chromatogr.* **1996**, *745*,173–179.
17. Mortier, K.A.; Verstrate, A.G.; Zhang, G.F.; Lambert, W.E. Enhanced method performance due to a shorter chromatographic run-time in a liquid chromatography-tandem mass spectrometry assay for paclitaxel. *J. Chromatogr. B* **2004**, *1041*,235–238.
18. Gardner, E.R.; Dahut, W.; Figg, W.D. Quantitative determination of total and unbound paclitaxel in human following Abraxane treatment. *J. Chromatogr. B* **2008**, *862*,213–218.
19. Vainchtein, L.D.; Thijssen, B.; Stokvis, E.; Rosing, H.; Schellens, J.H.M.; Beijnen, J.H. A simple and sensitive assay for the quantitative

analysis of paclitaxel and metabolite in human plasma using liquid chromatography/tandem mass spectrometry. *Biomed. Chromatogr.* **2006**, *20*,139–148.

20. Guo, P.; Ma, J.; Li, S.; Gallo, J.M. Determination of paclitaxel in mouse plasma and brain tissue by liquid chromatography–mass spectrometry. *J. Chromatogr. B* **2003**, *798*,79–86.
21. Sekar, V.; Selladurai, S.T.; Saha, R.N. A new, simple HPLC method for estimation of paclitaxel in micro-volume rat plasma. *The AAPS Journal* **2009**, *11*(S2):1953.
22. Ainley, W.; Paul, J.W. *Handbook of Pharmaceutical Excipients*, 2nd Ed. American Pharmaceutical Association, Pharmaceutical Press, Washington, DC, 1994, pp. 411–414.
23. Erni, F. Use of high-performance liquid chromatography in the pharmaceutical industry. *J. Chromatogr.* **1990**, *507*, 141–149.
24. Kemper, E.M.; Zandbergen, A.E.; Cleypool, C.; Mos, H.A.; Boogerd, W.; Beijnen, J.H.; Tellingen, O.V. Increased penetration of paclitaxel into the brain by inhibition of P-Glycoprotein. *Clin. Cancer Res.* **2003**, *9*, 2849–2855 .
25. Fruscio, R.; Lissoni, A.A.; Frapolli, R.; Corso, S.; Mangioni, C.; D'Incalci, M.; Zucchetti, M. Clindamycin-paclitaxel pharmacokinetic interaction in ovarian cancer patients. *Cancer Chemother Pharmacol.* **2006**, *58*,319–325.
26. Caporossi, L.; Rosa, M.; Pera, A.; Papaleo, B. Simple analytical method for the determination of paclitaxel (Taxol) levels in human plasma. *Chromatographia.* **2007**, *66*,921–924.
27. Guo, W.; Johnson, J.L.; Khan, S.; Ahmad, A.; Ahmad, I. Determination of paclitaxel in mouse plasma and brain tissue by liquid chromatography-mass spectrometry. *Anal. Biochem.* **2005**, *336*,213–220.
28. Gardner, E.R; Liau, C.T.; Chu, Z.E.; Figg, W.D.; Sparreboom, A. Determination of paclitaxel in plasma following the administration of Genaxol or Genetaxyl by liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2006**, *20*,2170–2174.
29. Tamura, T.; Sasaki, Y.; Nishiwaki, Y.; Saijo, N. Phase I study of paclitaxel by three-hour infusion: hypotension just after infusion is one of the major dose-limiting toxicities. *Jpn. J. Canc. Res. Gann.* **1995**, *86*,1203–1209.