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Quantitation of paclitaxel in micro-sample rat plasma by a sensitive reversed-phase HPLC assay

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Abstract

A sensitive high-performance liquid chromatographic (HPLC) method was developed for the determination of paclitaxel in micro-samples of rat plasma in order to study the mechanism of enhanced systemic exposure of paclitaxel co-administered with P-glycoprotein inhibitors. The assay involved solid-phase extraction procedures using 2′-methylpaclitaxel as the internal standard. Chromatographic separations were achieved using a ZORBAX ODS C18 column and mobile phase consisting of acetonitrile, methanol and ammonium acetate buffer (10 mM, pH 5.0) (48.5:16.5:35) pumped at 0.8 ml/min. The effluents were measured for UV absorption at 227 nm, with retention times of 8.5 and 11.0 min for paclitaxel and 2′-methylpaclitaxel, respectively. The chromatographic separation was excellent, with no endogenous interference. The standard curves showed a good linearity ($r = 0.9994$) over the concentration ranges of 10–1000 ng/ml. At 1000 ng/ml, the absolute recoveries of paclitaxel and 2′-methylpaclitaxel are 89 and 90%, respectively. The intra- and inter-day variabilities of paclitaxel were both less than 15%. This validated method for the assay of paclitaxel in micro-sample rat plasma made it feasible to study the pharmacokinetics of the drug in a single rat.

Keywords: Paclitaxel; Reversed-phase HPLC; Rat plasma

1. Introduction

Paclitaxel is a natural product isolated from the Pacific yew tree, *Taxus brevifolia* and the lead compound of a class of new antitumor drugs, which acts by stabilizing microtubules [1]. It was approved by the United States Food & Drug Administration (FDA) in 1992 and launched 1 year later in USA, Canada, Sweden and Austria because of its promising clinical activity against a variety of human solid tumors such as ovarian, breast, lung, head and neck cancers and melanoma [2,3].

The development of a sensitive assay for the drug is warranted to obtain a clear picture of the pharmacokinetic behavior, as paclitaxel exerts its

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cytotoxic activity at concentrations as low as 50 nM [4]. To date, several approaches have been used to develop assays for the determination of paclitaxel in biological fluids. These include HPLC-UV [5–9], immunoassay [10,11], and, LC-MS [12,13] with lower detection limits of 10, 0.3, and 0.2 ng/ml, respectively. Among these, the immunoassay lacks the specificity of the high-performance liquid chromatographic (HPLC) although it possesses higher sensitivity. LC-MS was demonstrated to be more sensitive and specific than HPLC-UV, but it is not affordable at most laboratories because of high equipment cost. HPLC methods have been extensively used for clinical studies of paclitaxel since 1987. These methods are mainly designed for human biological samples in a relatively large volume, typically 1–2 ml human plasma. However, in the cases of mouse/rat studies, usually only one or two blood samples can be taken from each animal for measurement by current HPLC methods. This situation makes it impossible to get a whole set of blood samples from one small animal to perform a pharmacokinetic study. Even though capillary electrophoresis needed only a small amount of sample, [14] its sensitivity is still not sufficiently high for the determination of paclitaxel in micro-samples (0.1 ml).

In this study, a very sensitive HPLC-UV assay has been developed for the determination of paclitaxel in rat plasma samples. Unlike the traditional methods that require at least 0.5 ml of plasma for each measurement, the present method uses only a 0.1 ml sample volume. Since blood loss is minimal, repeated blood sampling from a single rat becomes practicable. This validated method has been used to investigate the systemic exposure of paclitaxel after oral and i.v. administrations in Sprague–Dawley rat.

2. Experimental

2.1. Chemical and reagents

Paclitaxel was obtained from the Institute of Materia Medica of Chinese Academy of Medical Sciences, Beijing, PRC. Cremophor EL® was purchased from Sigma–Aldrich, Germany. 2′-methylpaclitaxel were provided by Bristol–Myers Squibb. Acetonitrile and methanol (all HPLC grade) were obtained from Merck Darmstadt, Germany. Water was deionized by the Milli-Q Plus system (Millipore).

2.2. Instrumentation

The HPLC system consisted of a Hewlett Packard (HP) 1050 quaternary pump equipped with a (HP) 1050 auto-sampler injector with a 100 μl loop, HP 1100 variable wavelength UV detector and HP ChemStation for data analysis. Solid-phase extraction was processed with Supelco VISIPREP cartridges (Supelco, Bellefonte, USA).

2.3. Chromatographic conditions

Chromatographic separations were achieved using a ZORBAX ODS C18 column (4.6×250 mm, 5 μm) and a ZORBAX ODS C18 guard column cartridge (4.6×12.5 mm, 5 μm). The mobile phase consisting of acetonitrile, methanol and ammonium acetate buffer solution (10 mM, pH 5.0) (48.5:16.5:35) was passed through a 0.22-μm membrane filter and degassed by ultrasonication under vacuum before use. The flow rate was 0.8 ml/min, and the effluent was monitored for UV absorption at 227 nm. All separations were performed at ambient temperature.

2.4. Sample pretreatment procedure

The determination of paclitaxel in rat was modified according to the previous HPLC method published by Willey et al. [5] Briefly, 0.4 ml of 0.2 M ammonium acetate buffer solution pH 5.0 was added into 0.6 ml Eppendorf tubes containing 0.1 ml of rat plasma. The buffered plasma was spiked with 10 μl of 2′-methylpaclitaxel working solution (10 μg/ml) in methanol. The purification was processed through solid-phase extraction (SPE). First of all, Cyano Bond Elut columns (1 ml, Supelco) were first conditioned with consecutive washings with 2.0 ml of methanol and 0.2 M ammonium acetate buffer solution pH 5.0, respectively; then, the diluted plasma-buffer mixture was

loading onto the SPE column; next, the columns were washed with 1 ml of 0.01 M ammonium acetate buffer solution pH 5.0, 1 ml of methanol 0.01 M ammonium acetate buffer solution pH 5.0 (2:8, v/v), consecutively. The columns were dried under maximum vacuum for 30 s (10 mmHg). Finally, the analytes were eluted from the columns with 0.8 ml of a mixture of acetonitrile–triethylamine (1000:1, v/v) twice in 2.0 ml Eppendorf tubes. The eluent was evaporated to dryness under a nitrogen stream at ambient temperature. The residue was reconstituted with 80-μl mobile phase by vortex-mix for 30 s. Then, the reconstituted samples were transferred to auto-sampler vials (250 μl) containing limited-volume inserts and 50 μl was injected from each sample onto the HPLC column.

2.5. Preparation of stock solutions

Stock solutions of paclitaxel and 2′-methylpaclitaxel were prepared in methanol at 1 mg/ml and were kept at −20 °C. These stock solutions were diluted with methanol to obtain the concentrations required for preparation of standard working solutions. For paclitaxel, its working solutions were 0.1, 0.5, 1, 5, 10 μg/ml. For 2′-methylpaclitaxel, its working solution was 10 μg/ml.

2.6. Assay validation

2.6.1. Linearity

Standard calibration curves were prepared by adding 10 μl of pure paclitaxel at concentrations of 0.1, 0.5, 1, 5, 10 μg/ml and 10 μl of pure 2′-methylpaclitaxel (10 μg/ml) to 0.1 ml drug-free rat plasma. Thus, the corresponding plasma calibration standards were 10, 50, 100, 500, 1000 ng/ml, respectively. These plasma samples were extracted as described above. Calibration curves of paclitaxel were computed using the ratio of the peak area of paclitaxel and that of 2′-methylpaclitaxel as a function of the paclitaxel concentration in plasma.

2.6.2. Recovery

For the determination of the extraction yield, 10 μl of the working solutions of paclitaxel in concentrations of 0.1, 5, 10 μg/ml were added separately to 0.1 ml of control plasma to yield concentrations of 10, 500, 1000 ng/ml. The internal standard, 2′-methylpaclitaxel, was evaluated at the spiked concentration of 1000 ng/ml. The spiked plasma samples were then subjected to extraction procedure as described above. The absolute recovery was calculated by comparing the peak areas of compounds after solid-phase extraction with those obtained on direct injection onto the column of the same amount of paclitaxel, and 2′-methylpaclitaxel dissolved in mobile phase [15]. Each measurement was made in triplicates.

$$\text{Recovery (\%)} = \frac{\text{peak area of extracted standard}}{\text{peak area of unextracted standard}} \times 100$$

2.6.3. Intra- and inter-day variability

Intra-day variability was tested on five different rat plasma samples using the same calibration curve. Inter-day variability was tested on five different days and each day a new calibration curve was constructed.

2.6.4. Determination of LOD and LLOQ

The limit of detection (LOD) and the lower limit of quantification (LLOQ) were measured according to the FDA's guidance for bioanalytical method validation in 2001 [16]. The LOD was defined as the lowest concentration of paclitaxel that this assay can reliably differentiate from background noise (Signal/Noise greater or equal to three). The LLOQ was determined by spiking an aliquot of blank rat plasma with paclitaxel at the concentration of the lowest calibrator with a precision of 20% and accuracy of 80–120%. The LLOQ was repeated on 5 different days.

2.7. Application of the method

The method described has been used to quantitate paclitaxel concentrations in rat plasma microsamples (100 μl) in a pharmacokinetic study to investigate drug interaction between verapamil, a known cytochrome P450 3AP substrate and P-

glycoprotein inhibitor, and paclitaxel. All experiments were performed with male Sprague–Dawley rats (270±40 g). Animals were housed and handled according to institutional guidelines. For blood sampling experiments, an external catheter was cannulated surgically under anesthesia with the cocktail prepared by mixing Hypnorm, Dormicum and saline (1:1:2; v/v/v) from jugular vein 2 days before the study. The cannulated rats needed 1 day to recover from the cannulation operation. The rats were fasted overnight before oral or i.v. drug administration.

For each rat, blood sampling (0.25 ml) from the cannulation tube was performed at 0, 0.33, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 h after oral administration of 10 mg/ml paclitaxel. For the control group, paclitaxel was administered with saline. For the treatment group, paclitaxel was administered with verapamil (100 mg/kg).

3. Results

3.1. Chromatographic separation

Fig. 1 illustrates representative chromatograms of a blank rat plasma, a control plasma spiked with 500 ng/ml paclitaxel and 1000 ng/ml 2′-methylpaclitaxel, and plasma sample at 6 h after i.v. bolus of paclitaxel at 10 mg/kg (body weight). Paclitaxel and 2′-methylpaclitaxel were eluted with retention times of 8.5 and 11.0 min, respectively. The overall run time lasted 30 min.

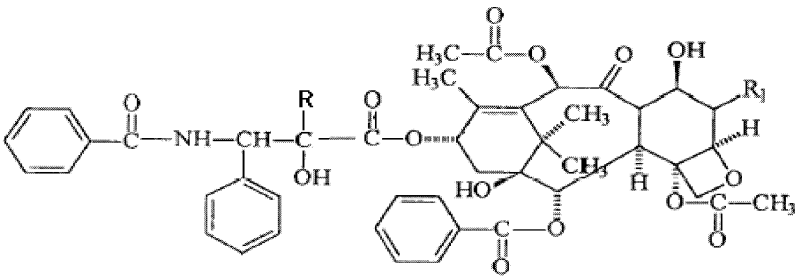
3.2. Validation characteristics

A standard curve was constructed for paclitaxel by plotting the ratio of the peak area of paclitaxel and that of 2′-methylpaclitaxel as a function of the plasma paclitaxel concentrations (10, 50, 100, 500, 1000 ng/ml). There is an excellent linearity over the range of 10–1000 ng/ml. The typical equation describing the calibration curve in rat plasma was $y = 0.0008x + 0.0029$ where y is the peak area ratio of paclitaxel against 2′-methylpaclitaxel and x is the concentration of paclitaxel, with a mean correlation coefficient of 0.9994.

The recovery of paclitaxel after solid-phase extraction procedures was evaluated at three concentrations of 10, 500, 1000 ng/ml. The average efficiency of extraction from rat plasma is 82%. The recovery of internal standard, 2-methylpaclitaxel, is 90% when its spiked concentration in rat plasma is 1000 ng/ml (Tables 1 and 2).

The limit of detection for paclitaxel in rat plasma defined as a minimum signal-to-noise of three was 5 ng/ml. The LLOQ for paclitaxel in 0.1 ml rat plasma was 10 ng/ml with an acceptable precision and accuracy (R.S.D.: 14%, Deviation: −6%, $n = 5$).

The intra- and inter-day assay accuracy and precision values are given in Table 2. The intra-day



R:	Compound:
H	paclitaxel
CH_3	2'-methylpaclitaxel

Fig. 1. Chemical structures of paclitaxel and 2′-methylpaclitaxel.

Table 1
Recovery of paclitaxel and 2′-methylpaclitaxel from rat plasma

Concentration (ng/ml)	Peak area (mean ± S.D., $n = 3$)		Recovery (%)
	Extracted	Unextracted	
Paclitaxel			
10	1.1 ± 0.1	1.6 ± 0.1	73
500	52.5 ± 2.4	61.4 ± 1.9	85
1000	109.2 ± 4.6	122.7 ± 3.5	89
2′-methylpaclitaxel			
1000	133.2 ± 6.3	148.5 ± 4.5	90

accuracy (deviation) was within 6.4% for all values, and the intra-day precision (R.S.D.) varied between 2.4 and 6.7%. The inter-day accuracy and precision were within 11.2 and 14.1%, respectively.

4. Discussion

4.1. Method validation

Determination of paclitaxel in blood samples using reversed-phase HPLC coupled with UV detector has been regarded as a challenge in biopharmaceutical analysis, especially at very low concentration (e.g. 10 ng/ml) with the use of a 24 h infusion. The analysis of paclitaxel in biological samples remains a challenge, even though Taxol® has been used in clinics for 10 years. The difficulties are not related to the UV absorption of paclitaxel. In fact, paclitaxel possesses a very strong UV absorption at 227 nm and its ε value is as high as 29 800 (in methanol) [17]. A compound is usually regarded as having a strong UV absorption when its ε value is more than 10^4. However, its second UV maximum at 273 nm is very weak with the ε value of 1700 in methanol. Hence, 227 nm is the optimal wavelength for UV detection. Unfortunately, there are many compounds from plasma constituents with similar lipophilicity as that of paclitaxel. These endogenous substances also have UV absorption at 227 nm and severely interfere with the quantitation of paclitaxel. In order to develop a sensitive assay for determination of paclitaxel in micro-sample rat plasma, the endogenous interfering peaks were avoided by selecting an appropriate HPLC column, optimizing the mobile phase and using a suitable sample pretreatment.

For HPLC column selection, we compared the difference between column of 15 and 25 cm lengths with the same C18 filling material and particle size. The longer column is preferred for getting a better resolution between paclitaxel and endogenous interfering compounds.

Although a single-step liquid-liquid extraction (LLE) has been reported in several publications, the interfering peaks cannot be tolerated for the determination of paclitaxel in case of low concentrations. Initially, we followed the procedure

Table 2
Precision and accuracy of HPLC assay for paclitaxel from rat plasma

Spiked concentration (ng/ml)	Calculated concentration (ng/ml, mean ± S.D., $n = 5$)	R.S.D. (%)	Deviation (%)
Intra-day ($n = 5$)			
10	10.3 ± 0.7	6.7	3.0
50	48.6 ± 3.1	6.3	−2.9
100	106.4 ± 4.0	3.8	6.4
500	471.3 ± 11.4	2.4	−5.7
1000	996.7 ± 28.7	2.9	−0.3
Inter-day ($n = 5$)			
10	9.4 ± 1.3	14.1	−0.6
50	46.2 ± 3.8	8.4	−7.5
100	111.2 ± 9.0	8.1	11.2
500	483.5 ± 26.9	5.6	10.1
1000	1018.2 ± 33.9	3.3	1.8

reported by Lee et al. [18] with different organic solvents such as tert-butyl methyl ether, diethyl ether, or ethyl acetate. All these extractions failed to satisfy the criteria of the assay's sensitivity. Then, solid-phase extraction was selected to clean the rat plasma samples. After treatment with SPE, the baseline of blank rat plasma was much cleaner than that of LLE. At the same time, the recovery of paclitaxel was also achieved at 82% on average by twice-elutions with 0.8 ml of acetonitrile each time, and this guaranteed a high sensitivity of the assay. Although the SPE method was similar to that of Willey et al. [5], we were able to reduce the plasma sample volume from 500 to 100 μl.

The mobile phase played a very important role in obtaining a good resolution between paclitaxel and the endogenous compounds. Using 48.5% acetonitrile shortened the elution time for paclitaxel which is one of the key points to achieve a sharp peak. The addition of 16.5% methanol to the mobile phase improved the separation of paclitaxel from interfering compounds and increased the sensitivity.

The excellent separation is demonstrated in the chromatograms (Fig. 1). No interfering peaks from the endogenous substances were observed in any of the plasma samples. The method was good linear over the concentration range of 10–1000 ng/ml. The method was accurate and reproducible with deviation and coefficients of variation less than 11.2 and 14.1%, respectively.

4.2. Application of the method

The applicability of the validated method was demonstrated in the study of pharmacokinetic drug interaction study of paclitaxel and verapamil in male Sprague–Dawley rats. The assay is sensitive enough to measure the plasma drug level after oral administration of 10 mg/kg paclitaxel even though the bioavailability was below 5%. Figs. 2 and 3 showed the plasma concentration-time profile of paclitaxel at the dose of 10 mg/kg after oral administration with or without oral coadministration of 100 mg/kg verapamil. With oral administration of paclitaxel only at the dose of 10 mg/kg, the plasma level can be detected between 0.33 and 8 h. However, the plasma level of paclitaxel at the same dose can be detected between 0.33 and 24 h with oral coadministration of verapamil at the dose of 100 mg/kg. This sensitive assay made it possible to use a single small animal to complete a full pharmacokinetic study.

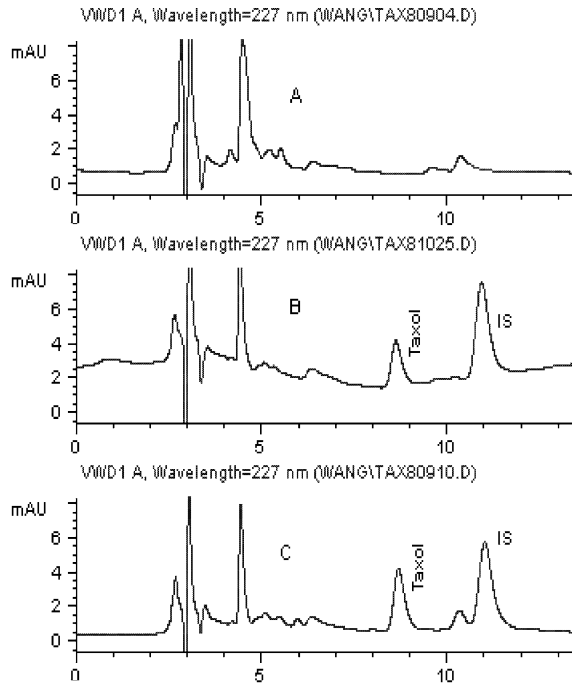


Fig. 2. The chromatograms: A, blank rat plasma; B, control plasma spiked with 500 ng/ml paclitaxel and 1000 ng/ml IS; C, plasma sample at 6 h after i.v. bolus injection of paclitaxel at 10 mg/kg.

5. Conclusions

In summary, a reproducible, accurate, and precise isocratic HPLC assay with UV detection was developed and applied to quantify 10–1000 ng/ml of paclitaxel in rat plasma samples with only 100 μl of sample volume. This assay has sufficient sensitivity to be used as demonstrated for PK studies of paclitaxel known to have low bioavailability after oral administration. The small sample volume required makes it possible to study the full PK profile in individual small animals, like the rat.

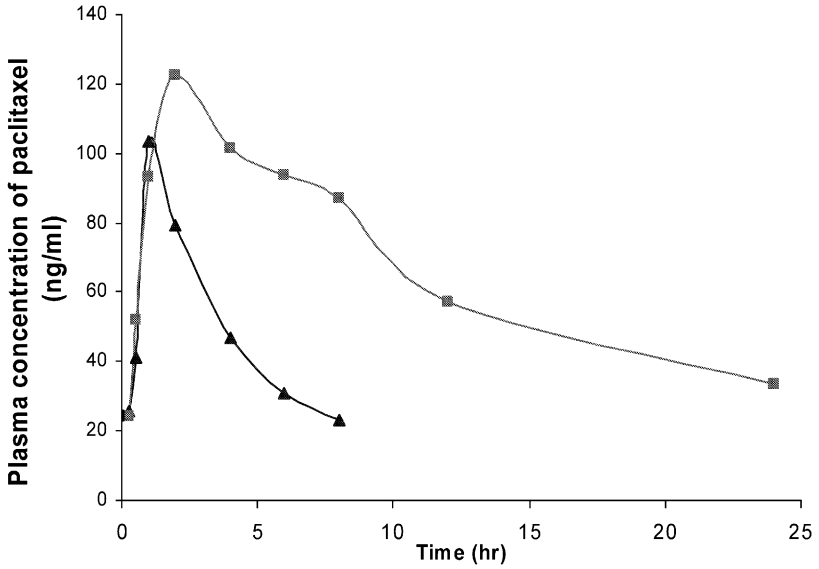


Fig. 3. Concentration-time curves of paclitaxel in a representative rat after oral Administration of 10 mg/kg paclitaxel: ▲ with saline (control group), ■ with 100 mg/kg verapamil, a known substrate of CYP 450 3A and inhibitor of P-gp (treatment group).

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