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Rapid and sensitive determination of paclitaxel in mouse plasma by high-performance liquid chromatography

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Abstract

This report describes a rapid, simple and sensitive isocratic high-performance liquid chromatography with diode array UV detection for micro-sample analysis of paclitaxel in mouse plasma. The analysis utilized a Capcell-pak octadecyl analytical column and a mobile phase consisting of acetonitrile–0.1% phosphoric acid in deionized water (55:45, v/v). Paclitaxel and *n*-hexyl *p*-hydroxybenzoic acid (internal standard) were extracted from plasma by one-step extraction with *tert*.-butyl methyl ether. Peak purity was determined over a UV wavelength range of 200 to 400 nm. Paclitaxel and the internal standard were eluted at 3.4 min and 5.4 min, respectively, at a mobile phase flow-rate of 1.3 ml/min. No interfering peaks were observed and the total run time was 10 min. The standard curve was linear ($r=0.9999$) over the concentration range of 0.010–500 μg/ml. The extraction recovery was >90% for both paclitaxel and *n*-hexyl *p*-hydroxybenzoic acid. The intra- and inter-day assay variabilities of paclitaxel ranged from 0.4 to 2.2% and 0.6 to 7.8%, respectively. The LOD and LOQ were 5 and 10 ng/ml, respectively, for paclitaxel using a plasma sample volume of 100 μl. This highly sensitive and simple assay method was successfully applied to a pharmacokinetic study after i.v. administration of paclitaxel 20 mg/kg to mice.

Keywords: Paclitaxel

1. Introduction

Paclitaxel, tax-11-en-9-one,5β,20-epoxy-1,2α,4,7β,10β,13α-hexahydroxy-4,10-diacetate-2-benzoate-13-(α-phenylhippurate), is a novel antimicrotubule agent isolated from the bark of the Pacific yew tree, *Taxus brevifolia*. The antineoplastic activity of paclitaxel is known to be mediated by binding to tubulin, stabilizing microtubles and blocking the transit of cell cycling from the G_2-phase to the M-phase [1,2]. Currently, paclitaxel is indicated in the treatment of breast cancer after failure of combination chemotherapy, ovarian cancers refractory to cisplatin-based regimens, non-small cell lung cancers and melanoma [3–5].

A number of assay methods have been published for the determination of paclitaxel in biological fluids, including capillary electrophoresis [6], liquid chromatography–mass spectrometry (LC–MS) [7,8], immunoassays [9,10] and high-performance liquid chromatography (HPLC) [11–27]. Of these, the

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HPLC methods have been most frequently used in the pharmacokinetic studies of paclitaxel because of their simplicity, sensitivity and selectivity. These HPLC methods utilize either solid-phase extraction (SPE) [16,18–22], protein precipitation and SPE [23], liquid–liquid extraction (LLE) [12–14,17,24], solvent extraction followed by column-switching [13], or solvent extraction and SPE [11,15,25–27]. Most of these HPLC methods require relatively large (a maximum of 0.5–1.0 ml) biological sample volumes [11–14,16–19,24] and multiple extraction steps [25–27]. In cases where micro-sample volumes are required, the HPLC methods with single LLE are generally not considered sensitive enough due to the presence of endogenous interferences [26]. Although several HPLC methods are available that utilize LLE and small biological sample volumes (e.g., 100–200 μl), they require multiple extraction steps or precipitation [15,23,25,27].

In the present study, a simple and sensitive HPLC method with single LLE was developed for the micro-sample analysis (100 μl) of paclitaxel in plasma. The utility of the developed assay method was demonstrated in the pharmacokinetic disposition study after intravenous (i.v.) administration of paclitaxel in mice.

2. Experimental

2.1. Chemicals and reagents

Paclitaxel (purity 99.74%) and commercially available paclitaxel formulated in Cremophor EL–dehydrated ethanol USP (1:1, v/v, Taxol) were purchased from Hauser Chemical Research (Boulder, CO, USA) and Bristol-Myers Squibb (Princeton, NJ, USA), respectively. The internal standard, *n*-hexyl *p*-hydroxy benzoic acid, was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Acetonitrile and *tert*.-butyl methyl ether (all HPLC grades) were purchased from J.T. Baker (Phillipsburg, NJ, USA) and Sigma (St. Louis, MO, USA), respectively. Phosphoric acid was obtained from Aldrich (Milwaukee, WI, USA).

2.2. Instrumentation

The chromatographic system used in the study was a Hewlett-Packard 1100 series with a Model G1311A quaternary pump, Model G1313A autosampler, Model G1315A diode array detector, Model G1316A column compartment and Model G1322A degasser. Data were acquired and processed with HP Chem Station (Ver. 4.02) chromatography manager software from Hewlett-Packard (Santa Clara, CA, USA).

2.3. Chromatographic conditions

Chromatographic separations were achieved using a Capcell-pak C_{18} UG120 column (150×4.6 mm I.D., particle size 3 μm) and a Capcell-pak C_{18} UG120 guard column (10×4.0 mm I.D., particle size 5 μm) supplied by Shiseido (Tokyo, Japan). The mobile phase consisting of acetonitrile–0.1% phosphoric acid in deionized water (55:45, v/v) (Milli-Q Plus System, Millipore, Milford, MA, USA) was passed through a 0.22-μm membrane filter and degassed by ultra sonication under vacuum before use. The flow-rate of the mobile phase was maintained at 1.3 ml/min. Chromatography was performed at ambient temperature (20±2°C). The UV detection wavelength was 227 nm and the peak purity analyses were performed over a wavelength range of 200 to 400 nm.

2.4. Stock solution

Stock solutions of paclitaxel and *n*-hexyl *p*-hydroxy benzoic acid were prepared by dissolving 10 mg of paclitaxel in 10 ml of acetonitrile and 25 mg of *n*-hexyl *p*-hydroxy benzoic acid in 10 ml of acetonitrile, respectively. All solutions were stored in a freezer (−20°C) for a period of four weeks without degradation.

2.5. Preparation of analytical standard solution

Standard solutions were prepared by diluting the stock solution with 60% acetonitrile in deionized water immediately prior to use. All preparations were made in borosilicate glass tubes (Corning Glass Works, NY, USA). For the calibration samples, serial dilutions were made with 60% acetonitrile to provide drug concentrations of 0.01, 0.025, 0.05, 0.1, 1, 10, 100 and 500 μg/ml.

2.6. Extraction procedure

To 100 μl of mouse plasma in borosilicate glass tubes was added 10 μl of the internal standard solution (25 μg/ml) and the mixture was extracted with 2 ml of *tert*.-butyl methyl ether on a vortex mixer for 60 s. Upon centrifugation at 4000 *g* for 10 min, the organic layer was transferred to a clean test tube and was evaporated under nitrogen at 40°C. The residue was then reconstituted with 200 μl of 60% acetonitrile in deionized water and was mixed on a vortex mixer for 90 s. A portion (50 μl) of the reconstituted sample was injected onto the chromatograph. Drug concentrations were determined from the peak area ratios with respect to the internal standard.

2.7. Validation parameters

2.7.1. Linearity

Calibration samples were prepared by spiking 0.1 ml of blank mouse plasma at final paclitaxel concentrations of 0.01, 0.025, 0.05, 0.1, 1, 10, 100 and 500 μg/ml. Calibration curves were constructed in the daily series of analysis using a weighted regression method ($1/y$).

2.7.2. Recovery

To determine the extraction efficiency, aliquots of the prepared stock solutions of paclitaxel and *n*-hexyl *p*-hydroxy benzoic acid were added separately to 0.1 ml of plasma to yield concentrations of 1, 10 and 100 μg/ml. The samples were subjected to extraction as described above. Peak area ratios of the extracted and non-extracted samples were compared. Extraction recovery was determined as the mean (±SD) of five samples. Intra- and inter-day assay variabilities were determined at 0.01, 0.025, 0.05, 0.1, 1, 10, 100 and 500 μg/ml concentrations ($n=5$ each).

2.7.3. Determination of LOD and LOQ

The limit of detection (LOD) was defined as the plasma paclitaxel concentration resulting in a peak height greater than three-times the baseline noise levels ($S/N > 3$). The limit of quantitation (LOQ) was determined by spiking five aliquots of blank plasma with paclitaxel at the concentration of the lowest plasma calibrator and assaying with a set of calibration curve samples. This determination was repeated on five different days. The mean relative standard deviation (RSD) was calculated and the accuracy of the calculated drug concentrations relative to the theoretical concentrations was determined.

2.8. Animal study

SPF male ICR mice (six weeks of age, body mass 31–33 g) were supplied by Charles River Japan (Kanagawa, Japan). Animals were handled and housed according to the institutional guidelines in a protected environment in conventional plastic cages with free access to food and water. The animals were maintained at a temperature of 22–24°C. Paclitaxel formulated in Cremophor EL–ethanol (1:1, v/v) was diluted with isotonic saline to a final concentration of 2.0 mg/ml. Paclitaxel was administered to the animals fasted overnight at 20 mg/kg doses by a bolus tail vein injection. Blood samples were collected from the vena cava into heparinized syringes at 5, 15 and 30 min, 1, 2, 3, 4, 5, 6, 7, 9 and 12 h after drug administration ($n=4$ at each sampling time). Plasma samples were harvested by centrifugation at 1500 *g* for 10 min and were stored at −20°C until analysis.

3. Results and discussion

3.1. Assay specificity

Representative chromatograms of the extracted blank plasma, plasma spiked with paclitaxel and *n*-hexyl *p*-hydroxy benzoic acid, and a plasma sample obtained 7 h after i.v. injection of paclitaxel (20 mg/kg) are shown in Fig. 1. Paclitaxel and the internal standard were eluted at 3.4 min and 5.4 min, respectively, with a total run time being 10 min without carry-over. The capacity factors (k') were calculated to be 2.18 for paclitaxel and 3.99 for the internal standard. No endogenous or extraneous peaks were observed interfering with the assay. Peak purity was further confirmed by photodiode array detection over UV wavelengths from 200 to 400 nm.

In humans, paclitaxel is extensively metabolized, with 6α-hydroxypaclitaxel being the major metabolite identified to date [28]. In mice, both 6α-hydroxytaxol and 3′-*p*-hydroxypaclitaxel have been deter-

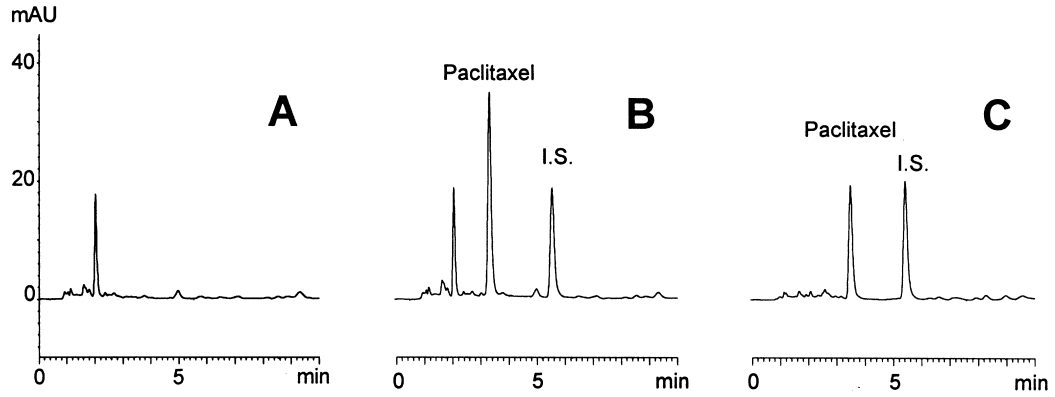

Fig. 1. Representative chromatograms of (A) extracted blank mouse plasma, (B) plasma spiked with paclitaxel (t_R=3.4 min, 10 μg/ml) and *n*-hexyl *p*-hydroxy benzoic acid (internal standard, t_R=5.4 min) and (C) plasma (5.6 μg/ml) obtained 7 h after a 20 mg/kg i.v. bolus dose.

mined in the liver, bile, feces and the small intestine as the major metabolites after 2–20 mg/kg i.v. doses but these metabolites were not detectable in the plasma [25]. Therefore, it is unlikely that these metabolites interfered with the present paclitaxel assay.

3.2. Assay validation characteristics

Spiked drug concentrations were linearly related to the peak area ratios of the drug vs. internal standard over the concentration range from 10 ng/ml to 500 μg/ml. A typical equation obtained by the weighted regression was $y=0.1972x+0.0001$, with a correlation coefficient of 0.9999. The extraction recovery with *tert*.-butyl methyl ether was >97% for paclitaxel and >90% for the internal standard determined at 1, 10 and 100 μg/ml (Table 1). Paclitaxel recovery obtained in this assay was found to be greater than in several previously published methods that have utilized various extraction methods [13–18]. Sample extraction with methylene chloride, chloroform and hexane resulted in a low recovery of paclitaxel (<40%), while extraction with diethyl ether or precipitation with acetonitrile resulted in noisy baselines.

Table 1
Extraction recovery (%) of paclitaxel and *n*-hexyl *p*-hydroxy benzoic acid (n=5)

Concentration (μg/ml)	Recovery (mean±SD) (%)	
	Paclitaxel	*n*-Hexyl *p*-hydroxy benzoic acid
0.1	93.3±1.3	89.6±1.5
1	97.2±1.1	90.3±0.7
10	97.7±1.6	90.1±1.6
100	98.6±1.4	90.5±1.5
Mean	96.7	90.1

The inter- and intra-assay accuracy and precision values are given in Table 2, in which the coefficients of variations (CVs) were less than 7.8% over the concentration range from 0.01–500 μg/ml. The LOD and LOQ was determined to be 5 ng/ml and 10 ng/ml, respectively, using a 100 μl plasma sample volume. Most HPLC methods published previously for the analysis of paclitaxel have utilized plasma or urine sample volumes of 0.2 ml [13,15], 0.5 ml [12,18,19], 0.8 ml [24] and 1 ml [11,14,16,17]. Sparreboom et al. [25] used a plasma volume of 100 μl but the extraction procedure involved a combined liquid–liquid and solid-phase extraction. The present assay utilized a single LLE and was found suitable for measuring the paclitaxel concentrations ≥10 ng/ml using a 100 μl of plasma sample volume.

Paclitaxel possesses a poor water solubility and is commercially available as formulated in Cremophor EL–dehydrated ethanol USP (1:1, v/v) (Taxol). Cremophor EL is a complex mixture of the hydrophilic components (15–18%) consisting of glycerin polyglycol ethers and polyglycols, and the hydrophobic components (82–85%) consisting of polyoxyethyleneglycerol triricinoleate, esters of ricinoleic and oleic acids and unreacted caster oil [29]. The chromatographic appearance of Cremophor EL

Table 2
Within-day and between-day accuracy and precision of paclitaxel assay in mouse plasma

Concentration (ng/ml)	Intra-day (n=5)			Inter-day (n=5)		
	Concentration found (mean±SD) (ng/ml)	Accuracy (%)	CV (%)	Concentration found (mean±SD) (ng/ml)	Accuracy (%)	CV (%)
10	9.8± 0.2	98.1	2.2	9.8±0.8	98.0	7.8
25	24.6±0.1	98.6	0.4	23.8±0.6	95.3	2.7
50	50.7±0.6	98.6	1.2	49.1±1.5	98.2	3.1
100	98.9±0.8	98.9	0.8	97.2±2.3	97.2	2.4
1000	1005.4±9.7	99.5	1.0	1024.7±35.8	97.5	3.5
10 000	10 204.1±135.5	98.0	1.3	10 340.7±478.4	96.6	4.6
100 000	101 008.0±1359.6	99.0	1.4	103 262.8±2550.6	96.7	2.5
500 000	498 795.1±2049.7	99.8	0.4	495 627.5±2821.0	99.1	0.6

(λ_{max}=230 nm) has been reported for biological samples obtained after Taxol administration [27]. In the plasma samples obtained in our animal study, Cremophor EL was also present, eluting at 48 min. In an attempt to eliminate Cremophor EL, SPE with consecutive water and various concentrations of acetonitrile was tried on C_{18} Sep-Pak cartridges. At low acetonitrile concentrations (<30%), the peak size of Cremophor EL was reduced and at the same time the paclitaxel recovery was reduced. LLE with *tert*.-butyl methyl ether also resulted in a Cremophor EL peak but the peak size was found to be decreasing as the acetonitrile content of the final reconstitution volume was decreasing (Fig. 2). The Cremophor EL peak became minimal when the acetonitrile content was adjusted to 60% and, hence, the sample reconstitution was thereafter made with 60% acetonitrile in deionized water. In case a carry-over problem is encountered due to the presence of Cremophor EL (e.g., clinical samples obtained after administration

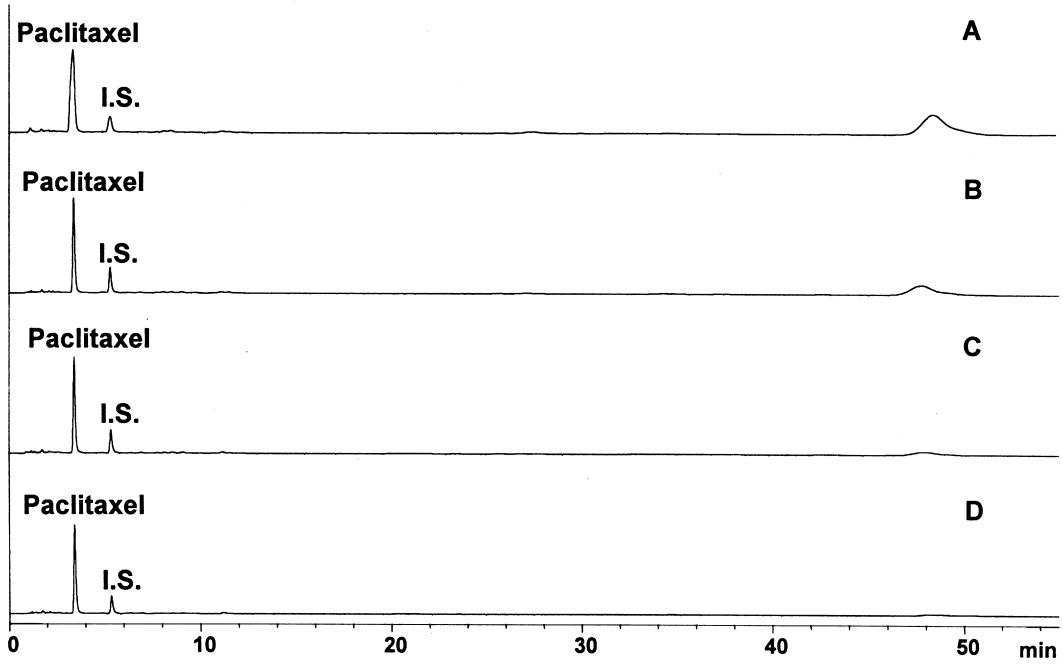

Fig. 2. Chromatographic disappearance of Cremophor EL peak (t_R=48 min) in extracted mouse plasma samples as a function of acetonitrile content in the reconstitution procedure: A, B, C and D represent 80%, 60%, 30% and 20% acetonitrile in deionized water, respectively.

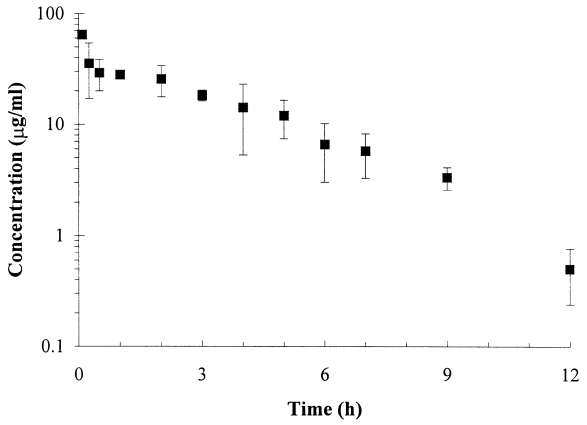

Fig. 3. Plasma paclitaxel concentration–time curve obtained after i.v. administration (20 mg/kg) in ICR mice. Each point represents the mean±SD (n=4).

of Taxol or other Cremophor EL-containing paclitaxel preparations), the content of the organic solvent may need to be adjusted in the sample reconstitution procedure.

3.3. Application of method

The applicability of the assay method was demonstrated in a pharmacokinetic disposition study after a bolus i.v. administration of 20 mg/kg paclitaxel in SPF male ICR mice. Paclitaxel disposition was best described by a bi-exponential equation as determined by the nonlinear least-squares regression program WinNonlin (Scientific Consultants, NC, USA) (Fig. 3). The best fit equation for the plasma paclitaxel concentration–time curve was determined to be: C=69.7 (μg/ml)$e^{-11.9586\ (h)\ t}$+37.3 (μg/ml) $e^{-0.0218\ (h)\ t}$. Table 3 shows the pharmacokinetic parameters obtained after i.v. injection of paclitaxel 20 mg/kg bolus doses in mice. These parameter values are comparable to those reported previously, including the apparent terminal elimination half-life (2.65 vs. 2.20 h), the systemic clearance (0.14 vs. 0.15 l/h/kg) and the volume of distribution (0.50 vs. 0.48 l/kg) [27].

4. Conclusions

A simple and sensitive HPLC method was developed for paclitaxel, utilizing single extraction with *tert*.-butyl methyl ether. This assay method provided excellent extraction recovery, sensitivity, accuracy and precision, with relatively short assay run time. This HPLC method was successfully applied to a paclitaxel pharmacokinetic study in mice where limited volumes were available.

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Table 3
Pharmacokinetic parameters of paclitaxel obtained after i.v. injection (20 mg/kg) in ICR mice (n=4)

$t_{1/2,\lambda1}$ (h)	$t_{1/2,\lambda2}$ (h)	Vss (l/kg)	Cl (l/h/kg)	MRT (h)	AUC (μg h/ml)	AUMC (μg h^2/ml)
0.06	2.65	0.50	0.14	3.68	148.40	546.18

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