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Short Communication

Reversed-phase high-performance liquid chromatographic determination of taxol in mouse plasma

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Abstract

A simple and selective high-performance liquid chromatographic technique was developed for the determination of taxol in mouse plasma. This technique employs a single extraction step followed by isocratic chromatography on a C_{18} analytical column and ultraviolet detection at 227 nm, and is suitable for the analysis of microsamples (50 μl plasma). A series of aromatic and aliphatic benzamides were synthesized as internal standards; N-octylbenzamide was evaluated most extensively. The calibration curve is linear within the range 0.15–10 nmol/ml. The intra-day and inter-day coefficients of variation were below 7%. The specificity of the assay was confirmed by comparing the chromatographic properties of samples with authentic taxol, using a diode-array detector. The assay was used to conduct pharmacokinetic studies of taxol in mice after administration in different formulations.

1. Introduction

Taxol [NSC-125973; tax-11-en-9-one,5β,20-epoxy-1,2α,4,7β,10β,13α-hexahydroxy-4,x10-di-acetate-2-benzoate-13-(α-phenylhippurate)] (Fig. 1), a diterpenoid derived principally from the bark [1] of the Western Yew, *Taxus brevifolia*, is under investigation as an anti-cancer agent [2–4] in Phase I, II, and III human clinical trials; target tumors include a variety of human cancers, including ovarian, breast, colon, non-small cell lung, head and neck cancer. Recently, the U.S. Food and Drug Administration (FDA) approved taxol for use against ovarian cancer. One problem associated with the administration of taxol is its low solubility in most pharmaceutically-acceptable solvents; the formulation used clinically contains Cremophor EL (polyethoxylated castor oil) and ethanol as excipients, which cause serious adverse effects. To eliminate this vehicle, we have formulated taxol in liposomes of various compositions. The taxol–liposome formulations showed a greater therapeutic effect [5] against a murine tumor model than did the taxol–Cremophor EL formulation.

The objective achieved in the present study was the development of a simple method for the determination of taxol in different formulations and in mouse plasma after administration of taxol formulations. Although several high-performance liquid chromatographic (HPLC) assays for analyzing taxol in human plasma have been

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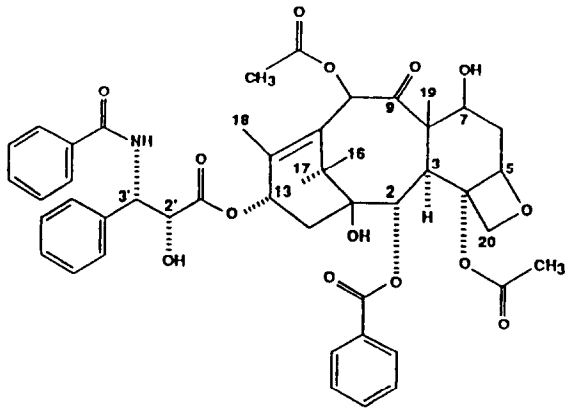

Fig. 1. Structure of taxol.

reported [6–10], no HPLC assay has been reported for small animals such as the mouse. Nor have previously reported assays provided information on the internal standard(s) that can be used.

2. Experimental

2.1. Apparatus

The Hitachi (Danbury, CT, USA) HPLC system used in this method consisted of a Model L6200A pump, a Model AS-2000 automated injector equipped with a 100-μl loop, and a Model L-4250 UV-Vis absorbance detector. Chromatographic separation was achieved with a Waters (Milford, MA, USA) μBondapak C_{18} column (300 × 3.9 mm I.D., 10 μm particle size) equipped with μBondapak C_{18} guard column (6.6 × 3.0 mm I.D., 10 μm particle size). Peak heights were recorded on a Hitachi Model D-2500 Chromato-Integrator.

2.2. Chemical and reagents

tert.-Butyl methyl ether and methanol were of HPLC grade and obtained from Aldrich (Milwaukee, WI, USA) and Fisher Scientific (Rochester, NY, USA) respectively. Reagents for the synthesis of the internal standards were obtained from Aldrich. Taxol was obtained from the National Cancer Institute (Bethesda, MD, USA).

Taxol and N-octylbenzamide stock solutions were prepared separately in methanol at a concentration of 1 μmole/ml and 2 mg/ml, respectively, and stored at −20°C. Both stock solutions were stable for at least 3 months. Standard solutions for calibration curves were prepared through serial dilution of the stock solution with drug-free mouse plasma.

2.3. Internal standard

Several aromatic and aliphatic benzamides were synthesized for evaluation as internal standards for the HPLC procedure. The Schotten–Baumann method [11,12], using benzoyl chloride and an alkyl amine, aromatic amine or hydrochloride salt, was employed as described below.

For preparation of N-octylbenzamide, 1.9 ml (1.5 g) of N-octylamine was dispersed in 15 ml of cold 1 *M* NaOH in a 50-ml screw-cap test tube; a small amount of crushed ice was added to the tube, followed by 1.35 ml of benzoic chloride. The tube was vigorously shaken and then allow to stand for *ca.* 30 min in an ice bath with intermittent shaking. The product was removed by filtration, washed with water, dried in air (2.51 g, 93% yield) and recrystallized by dissolving in 3 ml of hot ethanol with subsequent addition of 1 ml water and cooling (2.11 g). For the synthesis of other amines, the general procedure employed 1.5 g of amine or amine hydrochloride dissolved in a 25% molar excess of 1 *M* NaOH, and one molar equivalent of benzoyl chloride with respect to the amine. If amine hydrochlorides were used, an additional molar equivalent of NaOH was added to compensate for the additional acid. The synthesized material eluted chromatographically as a single peak (under conditions described below) with a uniform spectrum, as determined with a diode-array detector. Yields of air-dried product ranged from 70 to 93% of the theoretical value.

The capacity factor (k') for each internal standard was determined as follows:

$$k' = \frac{t_R - t_H}{t_H}$$

where t_R is the retention time and t_H is the hold-up time.

2.4. Extraction procedure

Prior to extraction, 0.05–0.5 ml of mouse plasma was diluted to a total of 0.5 ml with double distilled water, and mixed with 1.0 μg internal standard in methanol (25 μl of methanolic solution containing 40 μg/ml N-octylbenzamide). Extraction of taxol was accomplished by adding 4.0 ml of *tert.*-butyl methyl ether [10] and vortex-mixing the sample for 30 s. The mixture was then centrifuged for 10 min at 500 *g* at 4°C, after which 3.0 ml of the organic layer was transferred to a clean tube and evaporated to dryness under a gentle stream of nitrogen (20°C).

2.5. Chromatography

Approximately 100 μl of methanol was used to reconstitute the residue, and a 70-μl aliquot was injected onto the column. The mobile phase was methanol–water (70:30, v/v) and was pumped at a flow-rate of 2.0 ml/min. HPLC was carried out at room temperature.

2.6. Taxol recovery

The recovery of taxol by the extraction procedure was assessed at three different concentrations by the following method. Standards were made in plasma and extracted with 4.0 ml of *tert.*-butyl methyl ether and centrifuged as described above. Two ml of the ether layer was removed and 1.0 μg of N-octylbenzamide was added to the ether as internal standard. After evaporation and reconstitution with methanol (100 μl), 70 μl was injected onto the column. The ratio of the peak height of taxol relative to N-octylbenzamide was calculated. Then, the same amounts of taxol were added to 1.0 μg N-octylbenzamide, reconstituted with methanol and injected directly. The peak-height ratio was calculated as before. The procedure was repeated 3 times ($n = 4$) and recovery was calculated using the following equation: recovery = {(mean peak-height ratio, extracted drug)/(mean peak-height ratio, direct injection)} · 100%

2.7. Animal studies

The assay was used to compare the pharmacokinetics of taxol after administration as a liposomal formulation or dissolved in the clinically-used Cremophor–ethanol vehicle. The studies were performed in healthy female Balb/c mice, aged 6–7 weeks, which had access to food and water *ad libitum*. All procedures involving animals were approved in advance by the Institutional Animal Care and Use Committee. The different taxol formulations were administered as intravenous bolus injections (20 mg/kg body weight) via the tail vein (average injection time, 5 s) and 3 mice were used per time point. Animals were anesthetized with diethyl ether, and blood samples were obtained by cardiac puncture and collected in heparin-containing tubes at $t = 5$ min and 1, 4 and 16 h. Plasma was separated immediately by centrifugation (5 min, 20 000 *g*) and stored at −20°C. Analysis was performed within 15 days.

3. Results

The UV-absorption of taxol results from the contribution of all three isolated phenyl groups in the structure (Fig. 1). One of these is a 3′-benzamide group in the C13 side chain of taxol. Therefore, a number of aromatic and aliphatic benzamides were synthesized as possible internal standards because they show chromatographic behaviour, lipophilicity and UV absorbance patterns similar to taxol. Capacity factors (k') were determined for these benzamides (Table 1). On the basis of its k' value, N-octylbenzamide was selected as the internal standard. The purity of N-octylbenzamide was determined by diode-array detection. The UV spectrum of N-octylbenzamide closely resembles that of taxol, with a peak absorbance around 227 nm (data not shown).

The HPLC conditions described allow the analysis of taxol in plasma from taxol-treated mice. The chromatogram from the plasma of an untreated mouse shows no apparent interfering peaks (Fig. 2A). Complete resolution of taxol and the internal standard peaks was achieved in

Table 1
Capacity factors of aliphatic and aromatic benzamides

Compound	Capacity factor (k')[a]
N-Benzylbenzamide	0.61
N-Phenylbenzamide	0.67
N-Phenylethylbenzamide	0.84
N-Cyclohexylbenzamide	0.89
N-4-Methylphenylbenzamide	1.00
N-Hexylbenzamide	1.47
N-Heptylbenzamide	2.32
N-Octylbenzamide	3.65

[a]μBondapak C_{18} column; mobile phase, methanol–water (7:3, v/v)

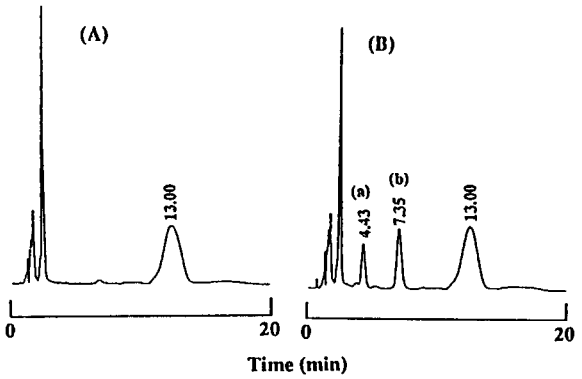

Fig. 2. Representative chromatograms of (A) extracted mouse plasma (no taxol), and (B) extracted mouse plasma spiked with 0.75 nmoles taxol. Peaks: a = taxol; b = internal standard.

15 min (Fig. 2B). The assay was linear over the tested concentration range of 0.15–10 nmol/ml taxol injected.

The intra-day and inter-day variation was determined at three concentrations (Table 2). The coefficients of variation ranged from 1.1 to 6.9.

The observed extraction recovery of taxol was 98–102%, and was independent of the concentration of taxol in plasma (Table 3). Because the calibration curves based on peak-height ratios for taxol and internal standard solutions in methanol were identical to those obtained using mouse plasma (data not shown), there was no need for preparing separate calibration curves for situations in which protein concentrations vary, such as in the measurement of drug–protein binding.

Fig. 3 depicts the plasma taxol concentration–time profile in mice after bolus i.v. injection of different taxol formulations. The plasma taxol concentrations were similar 5 min after taxol was given either in Cremophor EL–ethanol or in small unilamellar vesicles (SUV). The plasma taxol concentrations were significantly higher ($p < 0.01$) 1 h and 4 h after administration of taxol-containing SUV than after administration of taxol–Cremophor formulations. The pharma-

Table 3
Extraction recoveries of taxol

Concentration (nmol/ml)	Recovery (mean ± S.D.) (%)
1	9.87 ± 3.1
5	101.7 ± 3.8
10	99.0 ± 4.4
Mean	99.8

Table 2
Intra-day and inter-day assay variabilities

Actual amount (nmol)	Intra-day		Inter-day	
	Measured mean amount ($n = 6$) (nmol)	C.V.[a] (%)	Measured mean amount ($n = 6$) (nmol)	C.V.[a] (%)
0.5	0.51	5.0	0.52	6.9
1.5	1.51	3.7	1.48	5.5
3.0	3.12	1.6	3.08	1.2
9.0	9.37	1.1	9.48	2.3

[a]C.V. = (S.D./mean) · 100.

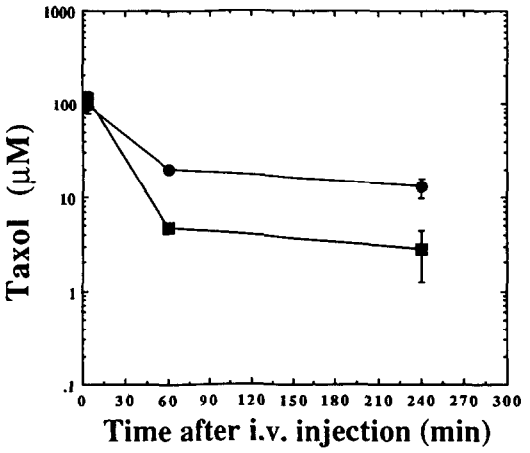

Fig. 3. Semilog plot of plasma taxol concentration *versus* time in mouse plasma after an intravenous dose of 20 mg/kg taxol administered in Cremophor EL–ethanol (■) or in liposomes of phosphatidylglycerol–phosphatidylcholine (1:9 mole ratio; ●). Bars indicate the standard deviation.

cokinetics of taxol–liposome formulations depends on a number of formulation parameters, including the size, charge, and fluidity of the liposome, as well as the dose of liposomes. A more complete description of the differences in the pharmacokinetics of free and liposomal taxol will be presented elsewhere.

4. Discussion

The number of studies published to date that deal with the determination of plasma taxol levels in animals is very limited. A number of HPLC assays have been developed to characterize plasma pharmacokinetics in human phase I studies [6–10]. With the exception of one study [7], the methods used did not employ internal standards. We synthesized several possible internal standards from which an appropriate one can be chosen depending on the requirement.

The limit of detection of the present assay (0.15 nmol applied to the column under the stated conditions) is adequate for pharmacokinetic studies in small animals, from which limited volumes (200–300 μl) of plasma are available. The absolute recovery of taxol from plasma was quantitative (98–102%), which allows the use of a methanolic standard for pharmacokinetic studies, rather than plasma standards. Sample preparation is rapid, and taxol and the internal standard eluted from the column within 15 min.

5. Acknowledgement

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6. References

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