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Sensitive determination of docetaxel in human plasma by liquid–liquid extraction and reversed-phase high-performance liquid chromatography

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Abstract

A sensitive reversed-phase high-performance liquid chromatographic method has been developed and validated for the quantitative determination of docetaxel (**I**) in human plasma. The concentrations in plasma, for validation procedures spiked with known amounts of **I**, are read from calibration curves in the range of 10–20 000 ng/ml. The sample preparation involved a liquid–liquid extraction of 1000 μl of sample with a mixture of acetonitrile–*n*-butylchloride (1:4, v/v). The related compound paclitaxel (**II**) was used as internal standard. Chromatographic separations were performed an Inertsil ODS-80A column, with UV detection performed at 230 nm. The overall extraction recoveries were 84.3 and 90.0% for **I** and **II**, respectively. The lower limit of quantitation was 10 ng/ml, and the accuracy, within-run and between-run precisions at three tested concentrations fell within the generally accepted criteria for bioanalytical assays.

Keywords: Docetaxel

1. Introduction

Docetaxel (**I**; Taxotere; NSC 628503; 4-acetoxy-2α-benzoyloxy-5β,20-epoxy-1-7β,10β-trihydroxy-9-oxotax-11-ene-11α - (2*R*,3*S*)-3-*tert.*-butoxycarbonyl-amino-2-hydroxy-3-phenylpropionate) (Fig. 1) is a semisynthetic compound that belongs to a novel class of antineoplastic agents, viz. the taxanes. The compound is prepared by chemical manipulation of 10-deacetyl baccatin-III, an inactive precursor isolated from the needles of the European Yew tree, *Taxus baccata* L. [1]. Docetaxel is a potent inhibitor of cell replication, blocking cells in the late G_2-M

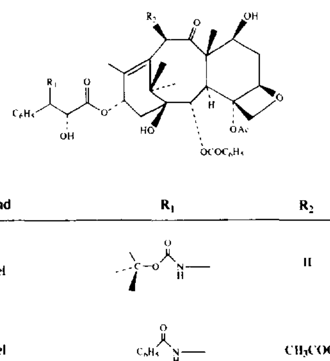

Fig. 1. Chemical structures of docetaxel and paclitaxel.

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phase of the cell cycle, by stabilization of the microtubule cytoskeleton [2]. In Phase II clinical studies, **I** has shown considerable activity against a variety of human neoplastic disorders, including breast, non-small cell lung, head and neck and ovarian carcinomas (reviewed in [3]).

The bioanalysis of taxane drugs, like **I** and its naturally occurring congener paclitaxel (**II**; Taxol) (Fig. 1), has been performed using high-performance liquid chromatography (HPLC) with ultraviolet (UV) [4–7] or mass spectrometric (MS) detection [8,9], or by enzyme-linked immunosorbent assays [10,11]. The lowest reported limits of quantitation of these techniques were 10, 0.2 and 0.3 ng/ml, respectively. Since chromatographic methods are, in general, more selective and may provide information on drug metabolism, HPLC is usually preferred to immunoassays. Although MS detection is by far superior to UV detection, this technique is at the disposal of few laboratories only, due to the high costs of the required equipment. For these reasons, analysis of taxanes by HPLC with UV detection is generally considered as first choice for pharmacokinetic studies.

In Phase I/II trials with **I** describing clinical pharmacological data [11–15], plasma concentrations of the drug were measured by HPLC similar to the method reported by Vergniol et al. [4], and include a time-consuming and expensive solid-phase extraction step. In view of the future usage of **I** in clinical practice with plasma drug-level monitoring, it was the aim of the present study to develop a simplified, less time-consuming analytical method for **I**. The procedure is based on a single solvent extraction prior to reversed-phase HPLC with UV detection, and has been subjected to a rigorous validation procedure according to the conference report on "Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies", as described previously [16].

2. Experimental

2.1. Chemicals and reagents

Docetaxel (**I**; Batch 14PROC92320; purity: 98.0% by reversed-phase HPLC) and the clinical docetaxel formulation in Tween 80 (Taxotere; 40 mg/ml) were supplied by Rhône-Poulenc Rorer (Vitry-sur-Seine Cedex, France). The internal standard, paclitaxel (**II**; Batch 96A10), was obtained from Bristol-Myers Squibb (Woerden, Netherlands), as a concentrated solution in 50% (v/v) of Cremophor EL in dehydrated ethanol USP (Taxol; 6 mg/ml). Methanol, acetonitrile (both HPLC grade), dimethylsulphoxide (DMSO) and *n*-butylchloride (both analytical grade) were purchased from Rathburn (Walkerburn, UK). Formic acid (98%, v/v in water; analytical grade), tetrahydrofuran (HPLC grade) and ammonium hydroxide (20%, w/v in water; analytical grade) were from Baker (Deventer, Netherlands). Water was filtered and deionized with a Milli-Q-UF system (Millipore, Milford, MA, USA) and was used throughout the study. Drug-free human plasma originated from the Central Laboratory of the Blood Transfusion Service (Amsterdam, Netherlands).

2.2. Stock solutions and standards

Stock solutions of **I** were made in triplicate by dissolving 18.4 mg of I in 18 032 μl (18 400×0.98 purity) of DMSO, resulting in a solution containing 1.0 mg/ml of **I**. A stock solution of **II** at 10 000 ng/ml was prepared by the addition of 83 μl of 6 mg/ml of Taxol to 50 ml of methanol–water (1:1, v/v). Working solutions of **I** were prepared in duplicate on each analysis day by serial dilutions in methanol–DMSO (1:1, v/v) from the primary stock solution.

Spiked plasma samples used as calibration standards were prepared daily by addition of 50 μl of the working solutions to 950 μl of drug-free human plasma, resulting in calibration standards of 10, 25, 200, 1000, 5000, 10 000 and 20 000 ng/ml of **I**. Three pools of quality control (QC) samples for **I** were prepared in human plasma in the concentrations of 50, 7500 and 15 000 ng/ml, as described above for the calibration standards.

2.3. Sample pretreatment

A volume of 100 μl of 10 000 ng/ml of **II** in methanol–water (1:1, v/v) and 5 ml of acetonitrile–*n*-butylchloride (1:4, v/v) were added to 1000 μl of human plasma in a glass tube with PTFE-lined screw

caps. The sample was mixed vigorously for 5 min, followed by centrifugation for 5 min at 4000 g at ambient temperature. The organic layer was collected in a glass tube and evaporated in 45 min at 60°C under a gentle stream of nitrogen. To the residue 125 μl of methanol–water (1:1, v/v) was added and, after vortex-mixing, the sample was centrifuged for 2 min at 4000 g. The clear supernatant was transferred to a low volume insert of glass, and a volume of 100 μl was injected into the HPLC system.

2.4. HPLC instrumentation and conditions

The HPLC equipment was composed of a constaMetric 3200 solvent delivery system (LDC Analytical, a subsidiary of Thermo Instruments Systems, Riviera Beach, USA), a Waters 717plus autosampling device (Milford, MA, USA) and a UV-2000 detector (Spectra Physics, Thermo Separation Products, Breda, Netherlands). Separations were achieved on a stainless-steel analytical column (150×4.6 mm I.D., 5 μm particle size) packed with Inertsil ODS-80A material (GL Science, Tokyo, Japan), protected by a Lichrospher 100 RP-18 endcapped-guard column (4.0×4.0 mm I.D., 5 μm particle size) obtained from Merck (Darmstadt, Germany). The mobile phase consisted of water–methanol–tetrahydrofuran–ammonium hydroxide (37.5:60:2.5:0.1, v/v), with the pH adjusted to 6.0 with formic acid. The mobile phase was degassed by ultrasonication and was delivered at a flow-rate of 1.00 ml/min. The column was maintained at 60°C, using a Model SpH99 column oven (Spark Holland, Meppel, Netherlands), and the eluent was monitored at a wavelength of 230 nm.

Peak recording and integration was performed with the Chrom-Card data analysis system (Fisons, Milan, Italy) connected to an ICW chromatographic workstation. Ratios of **I** to the internal standard **II** versus concentrations of the standard were used for quantitative computations. Calibration curves were fitted by weighted ($1/x^2$) linear regression analysis.

2.5. Validation

A validation run included a set of calibration samples assayed in duplicate and QC samples at three levels in quintuplicate, and was performed on four separate occasions. The accuracy or percentage deviation (%DEV) was calculated by the formula:

$$\%\text{DEV} = (\text{observed concentration}/\text{nominal concentration}) \times 100\% \quad (1)$$

The precision was calculated by one-way analysis of variance (ANOVA) for each test concentration, using the run-day as the classification variable. The between-group mean square (bgMS), the within-group mean square (wgMS) and the grand mean (GM) of the observed concentrations across run days were calculated using the software package Number Cruncher Statistical System (NCSS v5.0; J.L Hintze, East Kaysville, UT, USA, 1991) on an IBM compatible computer. The between-run precision (BRP) was calculated by:

$$\text{BRP} = \{[(\text{bgMS} - \text{wgMS})/n]^{0.5}/\text{GM}\} \times 100\% \quad (2)$$

where n is the number of replicates within each day. The within-run precision (WRP) was calculated by:

$$\text{WRP} = \{(\text{wgMS})^{0.5}/\text{GM}\} \times 100\% \quad (3)$$

To determine the lower limit of quantitation (LLQ), blank samples from five different individuals were spiked at 10 ng/ml of **I**, and analyzed on four separate occasions along with a standard curve in duplicate. The deviation from the nominal concentration and the WDP of each LLQ sample were calculated as described above [Eqs. (1) and (3), respectively].

The absolute recovery of **I** and **II** was calculated in four analytical runs, by comparing peak heights obtained by direct injection of the standard solutions containing 2000 ng/ml of **I** and 800 ng/ml of **II** in methanol–water (1:1, v/v), to those obtained in plasma samples subjected to the complete extraction procedure.

The stability of **I** in human plasma was established during three freeze–thawing cycles (15 min each) with samples spiked at concentrations of 50, 7500 and 15 000 ng/ml of docetaxel. The long-term storage stability of **I** at −80°C was tested at the same concentrations.

The following potentially co-administered drugs

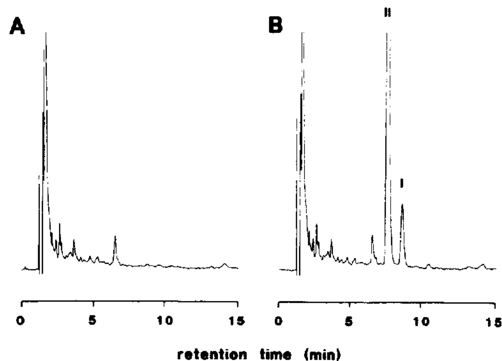

Fig. 2. Chromatograms of a blank plasma sample (A) and a plasma sample spiked with 200 ng/ml of I (B). The peaks labeled I and II correspond to docetaxel and paclitaxel, respectively.

were tested for interference with I or II: paracetamol (0.50 mg/ml), alizapride, codeine, domperidon, morphine and ranitidine (0.1 mg/ml each), dexamethasone (0.05 mg/ml) and metoclopramide (0.01 mg/ml).

To demonstrate the applicability of the analytical method, blood samples were taken from a patient participating in a Phase I clinical study in our institute, after the administration of 75 mg/m^2 of Taxotere by a 1-h intravenous infusion. Samples were obtained in heparinized tubes at 0, 0.5, 1, 1.5, 2, 3, 5, 7 and 24 h after drug dosing. After sampling, the blood was centrifuged for 5 min at 3000 *g* at ambient temperature, and the plasma supernatant was separated and stored immediately at −20°C. Within one day the plasma samples were stored at −80°C.

3. Results and discussion

Chromatograms of a blank and a spiked human plasma sample containing 200 ng/ml of I and 1000 ng/ml of II, used as internal standard, are shown in Fig. 2. The selectivity for the analytes is shown by the sharp and symmetrical resolution of the peaks, with no significant interfering peaks with the LLQ or quantitation of I or II in drug-free plasma samples, obtained from five different individuals. The composition of the mobile phase was selected in order to optimize the peak shape and separation factor for the analytes, resulting in retention times of 8.5 and 7.5 min for I and II, respectively. The overall chromatographic run time was established at 30 min.

By using weighted ($1/x^2$) linear regression analysis, deviations of the interpolated concentrations of >98% of all standards in the daily calibration curves of I in human plasma were within the acceptable 85–115% range (for the LLQ: 80–120% range). The calibration curves were linear in the range of 10 to 20 000 ng/ml of I, with regression correlation coefficients ≥0.997.

All the tested potentially co-administered drugs did not give significant interfering peaks for I and II in the assay. The LLQ for I was determined to be 10 ng/ml, and the range of the accuracy for I at the concentration of the LLQ was 88.5% to 108.6%. The within-run precision of the LLQ samples of I was 12.4%. The extraction recovery of I was determined at concentrations of 200, 1000, 5000, 10 000 and 20 000 ng/ml, and was found to be 84.3±6.8% (n=40). The overall extraction recovery of the internal standard (II) in all calibration samples used for validation was 90.0±5.0% (n=56). The accuracy of the QC samples showed values ranging within 95.5–105.9% of the nominal values (Table 1). The within-run and between-run precision for I at the three tested concentrations varied up to 3.34% and 1.94%, respectively (Table 1).

Compound I was found to be stable during three freeze–thaw cycles and for at least 6 months, when stored at −80°C. Processed human plasma samples containing I and II in methanol–water (1:1, v/v)

Table 1
The mean accuracy, the average within-run precision and the between-run precision of the QC samples in human plasma of I

QC sample (ng/ml)	Mean accuracy (%)	Precision (%)	
		Mean within-run	Between-run
500	105.9	3.34	1.94
7500	97.4	0.64	0.36
15 000	95.5	0.68	0.33

were also stable at room temperature, allowing the pretreatment of a large number of samples in each analytical run, and the use of an autosampler for injection (data not shown).

The described method was applied in our institute to a phase **I** and pharmacokinetic study of **I** in combination with capecitabine in patients with advanced solid cancer. Plasma collected from patients prior to the drug administration did not reveal the presence of interfering endogenous peaks. Concentrations of **I** could be readily estimated from protein-free extracts following liquid–liquid extraction and HPLC with UV detection. The plasma concentration–time profile of **I** from a 72-year old female treated with a 1-h intravenous infusion of **I** at a dose level of 75 mg/m^2 is given in Fig. 3. After the end of the infusion, the plasma levels declined tri-exponentially, which is in agreement with data obtained from other patients [14].

In conclusion, a thoroughly validated assay for the quantitative determination of docetaxel in human plasma has been described, which meets the current requirements as to validation of bioanalytical methodologies [17]. Previous experiments have shown that Cremophor EL concentrations in plasma exceeding 0.2% (v/v) can result in reduction in extraction efficiencies of upto 37% for paclitaxel using solid-phase extraction (SPE) [18]. In order to avoid these dramatic recovery problems, a simple and selective liquid–liquid extraction was developed for sample pretreatment. This enabled us also to improve the clinical applicability of the assay, while maintaining a high degree of sensitivity, with an LLQ of 10 ng/ml of **I**.

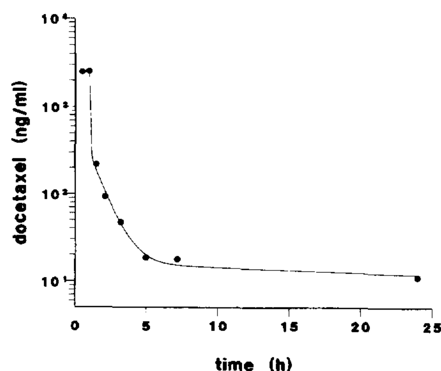

Fig. 3. Plasma concentration–time profile of docetaxel after administration of 75 mg/m^2 of **I** by a 1-h intravenous infusion to a female patient. The curve was fitted to a three-compartment open model using the MW\Pharm software package (MediWare, Groningen, Netherlands [19]).

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