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Simple and sensitive high-performance liquid chromatography method for the determination of docetaxel in human plasma or urine

Madhu B. Garg, Stephen P. Ackland*

Department of Medical Oncology, Newcastle Mater Misericordiae Hospital, Waratah, NSW, Australia

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Abstract

Several methods for quantification of docetaxel have been described mainly using HPLC. We have developed a new isocratic HPLC method that is as sensitive and simpler than previous methods, and applicable to use in clinical pharmacokinetic analysis. Plasma samples are spiked with paclitaxel as internal standard and extracted manually on activated cyanopropyl end-capped solid-phase extraction columns followed by isocratic reversed-phase HPLC and UV detection at 227 nm. Using this system, the retention times for docetaxel and paclitaxel are 8.5 min and 10.5 min, respectively, with good resolution and without any interference from endogenous plasma constituents or docetaxel metabolites at these retention times. The total run time needed is only 13 min. The lower limit of quantification is 5 ng/ml using 1 ml of plasma. The validated quantitation range of the method is 5–1000 ng/ml with RSDs≤10%, but plasma concentrations up to 5000 ng/ml can be accurately measured using smaller aliquots. This method is also suitable for the determination of docetaxel in urine samples under the same conditions. The method has been used to assess the pharmacokinetics of docetaxel during a phase I/II study of docetaxel in combination with epirubicin and cyclophosphamide in patients with advanced cancer.

Keywords: Docetaxel

1. Introduction

Docetaxel (Taxotere®), [4-acetoxy-2a-benzoyloxy-5b,20 -epoxy -1,7b,10b-trihydroxy-9-oxotax-11-ene-13a-yl- (2*R*,3*S*) -3-*tert*.-butoxycarbonylamino-2-hydroxy-3-phenylpropionate], is a novel anticancer agent of the taxoid family. An analogue of paclitaxel, docetaxel (Fig. 1), was obtained by semisynthesis from 10-deacetyl baccatin III, extracted from the needles of the European yew tree *Taxus baccata* L. [1,2]. Docetaxel promotes tubulin assembly in to microtubules, stabilises microtubules and inhibits microtubule depolymerisation to free tubulin. This leads to disruption of the equilibrium within the microtubule system and ultimately to cell death [3,4]. Docetaxel has been demonstrated to be clinically effective against a variety of tumour types, including

*Corresponding author. Department of Medical Oncology, Newcastle Mater Misericordiae Hospital, Locked Bag 7, Hunter Region Mail Centre, NSW 2310, Australia. Tel.: +61-49-211-146; fax: +61-49-680-384.

E-mail address: mdspa@cc.newcastle.edu.au (S.P. Ackland).

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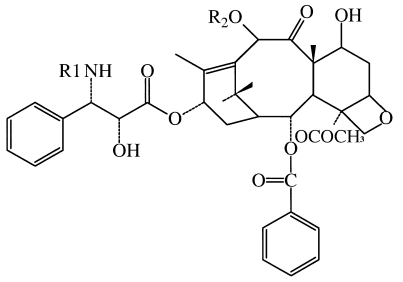

Docetaxel : R1= -COOC(CH3)$_3$; R2=H

Paclitaxel : R1= -COC$_6$H$_5$; R2= -COCH$_3$

Fig. 1. Structures of docetaxel and of the internal standard (paclitaxel).

breast, ovarian, head and neck and non-small-cell lung cancers [5–10].

Several methods for quantification of docetaxel have been described mainly using high-performance liquid chromatography (HPLC) [10–14]. Previous methods have lower limits of quantification of 9 ng/ml or higher. One recently described method has a quantitation limit of 5 ng/ml and a detection limit of 2.5 ng/ml using a column switching technique [14]. We have developed a simpler HPLC method, which uses a manual solid-phase extraction (SPE) procedure and only one HPLC column and still retains the sensitivity of the most sensitive method reported so far. It has a lower limit of quantification of 5 ng/ml, which allows greater precision and is applicable to use in clinical pharmacokinetic analysis of docetaxel at later timepoints after administration of the drug when the concentration of docetaxel in plasma is quite low, and in patients receiving low doses of treatment. This method gives excellent resolution between two taxanes (docetaxel and paclitaxel), does not have the problem of endogenous compounds causing late eluting peaks and is very reproducible and robust with a run time of only 13 min.

2. Experimental

2.1. Chemicals and reagents

Docetaxel was supplied by Rhône-Poulenc Rorer (Sydney, Australia) as a concentrated sterile solution and internal standard paclitaxel was supplied by F.H. Faulding (Mulgrave, Australia). Stock solutions of docetaxel and of paclitaxel were prepared in ethanol and stored at −18°C. Standard solutions were prepared by dilution of the stock solutions in mobile phase. Acetonitrile (ACN), triethylamine (TEA), methanol (MeOH) and hexane were HPLC-grade Omnisolv solvents (Merck, Kilsyth, Australia). All other chemicals used were of best commercially available grades. The water used was purified by passage through a Nanopure II systemB (Sybron/Barnstead, Boston, MA, USA) and was further filtered through a 0.45-μm membrane filter (Millipore Australia, Lane Cove, Australia). Cyanopropyl (CN) end-capped SPE columns (Sep-Pak Vac, 1 ml containing 100 mg sorbent) were purchased from Waters Australia (Rydalmere, Australia).

2.2. Plasma samples

Drug-free pooled human plasma was kindly provided by Red Cross Blood Bank, Newcastle, Australia. Clinical samples were obtained from patients undergoing treatment for advanced cancer with docetaxel 60–75 mg/m^2 per day given intravenously over 1 h in combination with epirubicin and cyclophosphamide. Patient's blood was collected at various times before, during and after infusion in 10-ml polypropylene tubes containing lithium heparin and kept on ice until centrifuged at 2500 *g* for 10 min. Plasma was transferred to 5-ml polypropylene tubes and kept at −70°C until analysis.

Plasma standard solutions for calibration and control samples were prepared by spiking the pooled plasma with the standard solutions to achieve concentrations from 5 to 5000 ng/ml and stored together at −70°C with the patient's samples. Urine standard solutions were prepared using blank urine.

2.3. Sample preparation

Frozen clinical plasma samples were first thawed at 37°C in a water bath and then, after vortexing well, were centrifuged for 10 min at 1750 *g* (Beckman GPR centrifuge; Beckman Instruments, Gladesville, Australia) to remove fibrous materials and to avoid clogging of the SPE columns.

A 0.6–1.0-ml volume of thawed plasma samples were aliquoted into labelled 5-ml polypropylene

tubes and spiked with 30–50 μl of internal standard working solution (10 μg/ml in ethanol) and mixed well. Plasma standards and controls were treated the same way. An equal volume (0.6–1.0 ml) of 0.2 *M* ammonium acetate buffer, pH 5.0 (AAB) is added before vortex mixing and centrifugation at 750 *g* for 2–3 min.

2.4. Sample extraction (manual solid-phase extraction)

The extraction columns were first activated with 2 ml of methanol and 2 ml of 0.01 *M* AAB using a vacuum extraction manifold and vacuum pump. After loading with 1.0–1.8 ml of prepared samples, each column was washed with 2 ml of 0.01 *M* AAB followed by 2 ml of MeOH–0.01 *M* AAB (20:80). Then each column was further washed with 1 ml of hexane and dried under vacuum for about 1 min. Compounds of interest were eluted with 2 ml of ACN–TEA (1000:1) into borosilicate glass tubes, and the eluate was dried under a stream of nitrogen at 35°C. The residue was reconstituted with 200 μl mobile phase, vortex-mixed and centrifuged briefly at 750 *g*, and 160 μl was transferred to crimp-top polypropylene autosampler vials (Alltech Associates, Baulkham Hills, Australia). A 10–50-μl volume was injected onto the HPLC system, 10 μl for highly concentrated samples and larger volumes for less concentrated samples.

2.5. HPLC instrumentation and conditions

The chromatographic system consisted of a Shimadzu Model LC-10AD dual piston pump, a Shimadzu autosampler Model SIL-10A and a Shimadzu Model SPD-10A variable-wavelength UV detector governed by a microcomputer running Shimadzu LC10 version 1.2 software (Shimadzu Oceania, Rydalmere, Australia). Separation was accomplished by isocratic elution of the mobile phase (acetonitrile–0.02 *M* ammonium acetate buffer, pH 5, 43:57, v/v) at a flow-rate of 1 ml/min through a C_{18} radial compression column (10 cm×5 mm I.D., 4 μm particle size) (Waters Australia) preceded by Waters C_{18} Nova-Pak guard column (20×3.9 mm I.D.), at room temperature.

The eluent was monitored at 227 nm. Integration of detector output was performed using Shimadzu LC10 software to determine peak areas.

2.6. Calculations

Blank plasma samples were spiked with the appropriate volumes of docetaxel stock solution to prepare three sets of standards and three sets of controls of concentrations in the range of 5 to 1000 ng/ml and subjected to the sample preparation procedure described above. Calibration curves were drawn by plotting the peak area ratio of docetaxel to that of the internal standard versus the plasma drug concentration by using the software. The line of best fit was determined using the unweighted linear least-squares method. Docetaxel concentrations of controls and unknown samples were calculated from the calibration curves using the software.

3. Results and discussion

3.1. Assay validation

Various procedures were performed to validate the assay.

3.1.1. Selectivity and specificity

The extraction and HPLC assay resulted in symmetrical peak shape and good baseline resolution of docetaxel and paclitaxel. Plasma matrix components did not interfere with the analysis. Using this system, the retention times for docetaxel and paclitaxel were 8.5 min and 10.5 min, respectively. Fig. 2 illustrates typical chromatograms of plasma extracts obtained from a cancer patient (A) prior to and (B) 15 min after a 1-h intravenous infusion of docetaxel (60 mg/m^2). The total analysis time for each run is 13 min.

No interfering endogenous peaks were detected. Peak shape and retention time was the same for injection of pure standards in mobile phase as for extracted plasma standards and samples. Furthermore, in measuring samples of 18 patients we did not find any extra peaks in chromatograms representing possible metabolites of docetaxel.

This method is also suitable for determination of docetaxel in urine samples under the same conditions. Fig. 3 illustrates HPLC chromatograms of

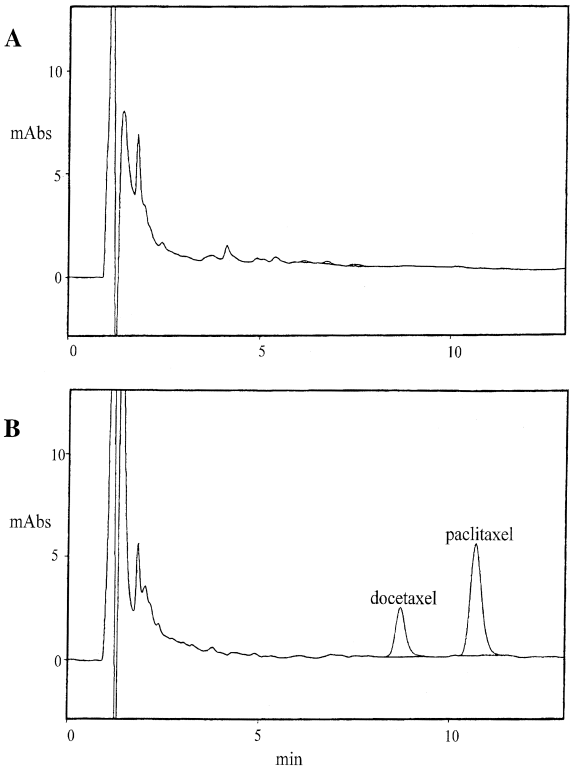

Fig. 2. HPLC chromatograms of extracts of (A) a blank plasma sample and (B) a patient plasma sample containing 210 ng/ml of docetaxel and 500 ng/ml of added paclitaxel (internal standard) taken 15 min after a 1-h intravenous infusion of docetaxel (60 mg/m^2).

extracts of a urine sample (injection volume 10 μl) from a cancer patient taken at the end of a 1-h infusion of docetaxel (75 mg/m^2).

In the course of development of this assay, different columns and mobile phases from other reported methods [10–12] were studied, with a manual transposition of the semiautomatic procedure. Some of the problems encountered with these methods were interference from endogenous plasma constituents, longer run times, low resolution between peaks, baseline instability and lack of sensitivity.

3.1.2. Precision and accuracy

We defined the limit of quantification as the minimum concentration that could be detected and quantified with ≤10% standard deviation from the

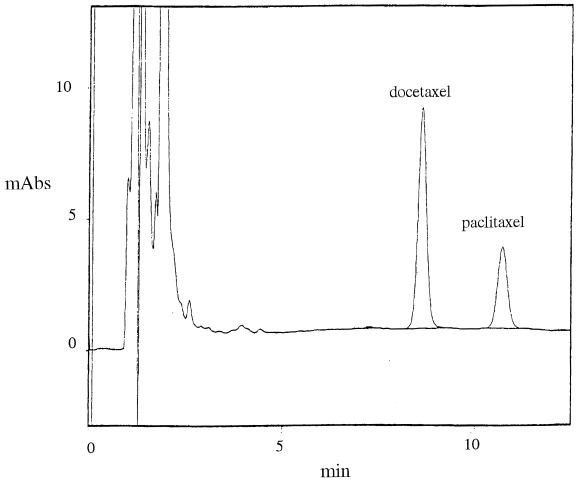

Fig. 3. HPLC chromatogram of extracts of a urine sample from a cancer patient containing 1560 ng/ml of docetaxel and 500 ng/ml of added paclitaxel (internal standard) taken at the end of a 1-h intravenous infusion of docetaxel (75 mg/m^2).

actual concentration. The limit of quantification that can be reliably and reproducibly measured is 5 ng/ml. Linearity of detector response was assessed for extracted plasma standards over the range 5–5000 ng/ml. Data were added serially from low to high concentrations. The limit of linearity was taken as the highest concentration included which still gave an r^2>0.998. For 0.6–1-ml aliquots of plasma standards linearity is satisfactory between 5 and 1000 ng/ml and plasma concentrations of 1000 to 5000 ng/ml can be accurately measured using smaller aliquots (0.5 ml or less) of plasma sample.

Assay precision and accuracy was satisfactory over the concentration range 5–1000 ng/ml. The within-day relative standard deviation (RSD) assessed at six concentrations varied between 0.4 and 5%, and between-day RSD assessed at two concentrations (500 ng/ml and 50 ng/ml) varied between 2.4 and 6.0% (Table 1). The between-day variation of the linear regression slope of six calibration curves was also satisfactory (slope= 0.0018±0.0001, correlation coefficient>0.999).

3.1.3. Extraction efficiency

Extraction efficiency of docetaxel and paclitaxel was determined by spiking 0.6–1-ml aliquots of plasma and mobile phase simultaneously with stan-

Table 1
Precision and accuracy of the method

Actual concentration (ng/ml)	Measured concentration (ng/ml)	Accuracy (%)	RSD (%)
Within-day (n=3)			
5	5.40±0.08	+8.00	1.42
10	10.39±0.47	+3.90	4.55
50	45.93±0.19	−8.14	0.43
100	100.96±1.27	+0.96	1.26
500	499.2±16.17	−0.16	3.2
1000	943.7±48.09	−5.63	5.1
Between-day (n=6)			
50	46.89±2.83	−6.22	6.03
500	507.52±12.12	+1.50	2.39

dard solutions, extracting the plasma samples as described above, analysing both sets and comparing the HPLC-derived peak areas. Extraction efficiency of docetaxel measured in triplicate at three concentrations (100, 500 and 1000 ng/ml) was 99±1.7%, and for paclitaxel, recovery was 100±2.0% (n=8) at a concentration of 500 ng/ml.

3.1.4. Stability of docetaxel

In frozen human plasma, docetaxel has been found to be stable for 6 months at −30°C [11,12]. Docetaxel is stable for at most 15 h in human plasma at ambient temperature [12]. We therefore assessed the stability of docetaxel under various other conditions to determine the optimum requirements for storage and processing during the analysis. We found that eluted samples from SPE columns should be dried on the same day and should not be left at 4°C to be dried next day as this creates baseline instability and some interfering peaks in HPLC chromatograms.

We found that docetaxel in solution is quite stable under a variety of conditions. Docetaxel standards in mobile phase, stored at 4°C are stable for periods up to 6 months and even solutions stored at room temperature are quite stable for at least 5 days. Thus samples can be left in the autosampler overnight or over weekends for long HPLC runs without any degradation. Also extracted plasma samples of docetaxel in mobile phase at 4°C are stable for 2–3 weeks. Thus, extracted plasma samples can be stored at 4°C along with extracted plasma standards and controls for subsequent HPLC analysis.

3.2. Application of the method

The method described has been used to assess the pharmacokinetics of docetaxel during a phase I/II study of docetaxel in combination with epirubicin and cyclophosphamide in patients with advanced cancer. In this study docetaxel is administered to cancer patients as a 1-h constant rate intravenous infusion. Fig. 4 shows a typical plasma concen-

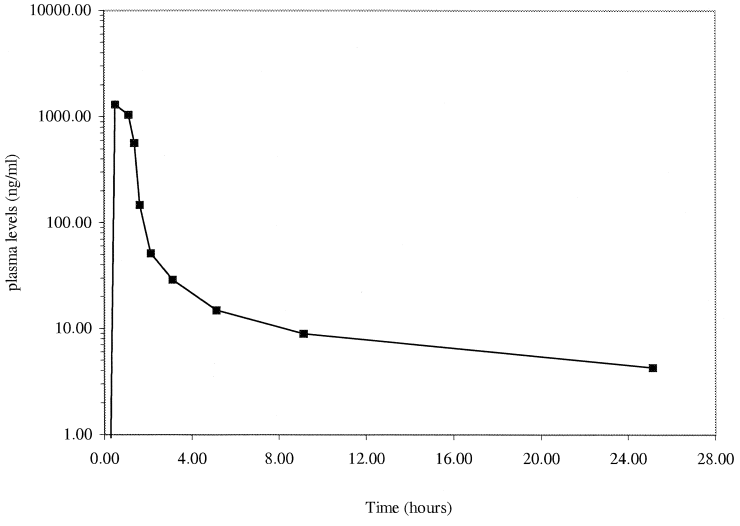

Fig. 4. Plasma concentration vs. time profile of docetaxel given a 1-h intravenous infusion (60 mg/m^2) to a cancer patient.

tration–time profile for a patient receiving a 60 mg/m^2 dose of docetaxel. After the end of infusion, the plasma concentrations declined tri-exponentially with a terminal half-life of 22 ± 11 h ($n=18$). The most likely explanation for the longer terminal half life in our hands compared to that previously reported (about 12 h, reviewed in Ref. [15]) is the increased sensitivity of the assay allowing accurate assessment of plasma concentrations at later timepoints (up to 72 h).

4. Conclusions

We have developed a simple and sensitive HPLC method to determine docetaxel concentrations in plasma or urine. The assay has a lower limit of quantification of 5 ng/ml, which is applicable to use in clinical pharmacokinetic analysis. Several methods for quantification of docetaxel have been described mainly using HPLC and SPE procedures [10–14]. Previous methods have lower limits of quantification of 9 ng/ml or higher. One recently described method has a limit of quantification of 5 ng/ml and a detection limit of 2.5 ng/ml but uses a column switching technique with two columns, one as a clean-up column and the other as an analytical column. The method described here is equally sensitive yet simpler, using a manual SPE procedure and only one HPLC column without the need for a clean-up column. This method also gives good resolution between docetaxel and paclitaxel, without the common problem of late eluting endogenous compounds.

In the course of this study we tried several previously published extraction and chromatographic procedures [10–12]. Some of the problems incurred with a manual transposition of these semiautomatic extraction procedures of these methods were interference from endogenous plasma constituents, longer run times, low resolution between peaks, baseline instability and lack of sensitivity. The improved sensitivity and precision of our assay is mainly attributed to the composition of our mobile phase, and the use of a C_{18} radial compression column (10 cm×5 mm I.D., 4 μm particle size) which eliminates the ''wall effect'' common to rigid columns. Because of the shorter length of this column, the run time of our assay is only 13 min, which makes it a very practical method to use.

Sensitive assay methods, such as described here, may allow better estimation of pharmacokinetic parameters for docetaxel during and after treatment especially if low doses are administered The ability to assay plasma up to 72 h after dosing facilitates a more reliable estimate of terminal half life.

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