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ORIGINAL RESEARCH

Quantitative analysis of docetaxel in human plasma using liquid chromatography coupled with tandem mass spectrometry

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ABSTRACT: An assay for the quantitative determination of docetaxel in human plasma is described. Docetaxel was extracted from the matrix using liquid–liquid extraction with *ter*-butylmethylether, followed by high-performance liquid chromatographic analysis using an alkaline eluent. Paclitaxel was used as internal standard. Positive ionization electrospray tandem mass spectrometry was performed for selective and sensitive detection. The method was validated according to the FDA guidelines on bioanalytical method validation. The validated range for docetaxel was from 0.25–1000 ng/mL using 200 µL plasma aliquots. The method requires only a limited volume (200 µL) of human plasma and the method can be applied in studies requiring a low lower limit of quantitation of 0.25 ng/mL. The assay was applied successfully in several clinical and pharmacological studies with docetaxel. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: docetaxel; LC-MS/MS; internal standard paclitaxel; liquid–liquid extraction

INTRODUCTION

Docetaxel (Taxotere^{R}) belongs to the taxanes and is a semisynthetic analogue of paclitaxel (see Fig. 1). Docetaxel inhibits mitosis through stabilization of the polymerization and de-polymerization of microtubules (Dumontet and Sikic, 1999). The drug is registered for the treatment of advanced breast cancer and advanced non-small-cell lung cancer (NSCLC). Furthermore it is used in combination with doxorubicin or in combination with capecitabine for the treatment of advanced breast cancer. Docetaxel is also registered in combination with cisplatin for metastatic NSCLC (www.emea.eu.int/humandocs/PDFs/EPAR/Taxotere/058895en1.pdf, 2004).

Several studies have shown that plasma concentrations of docetaxel are correlated with drug response rates and toxicity (Clarke and Rivory, 1999; Bruno and Sanderink, 1993; Bruno *et al.*, 2001; McLeod *et al.*, 1998). Various assays have been published describing the determination of docetaxel, using HPLC with UV detection (Vergniol *et al.*, 1992; Ardiet *et al.*, 1999; Rosing *et al.*, 1997; Sparreboom *et al.*, 1998; Ciccolini *et al.*, 2001) HPLC/MS (Parise *et al.*, 2003) and HPLC/MS/MS (Wang *et al.*, 2003; Baker *et al.*, 2004; Gustafson *et al.*, 2003; Sparreboom *et al.*, 2002). A number of

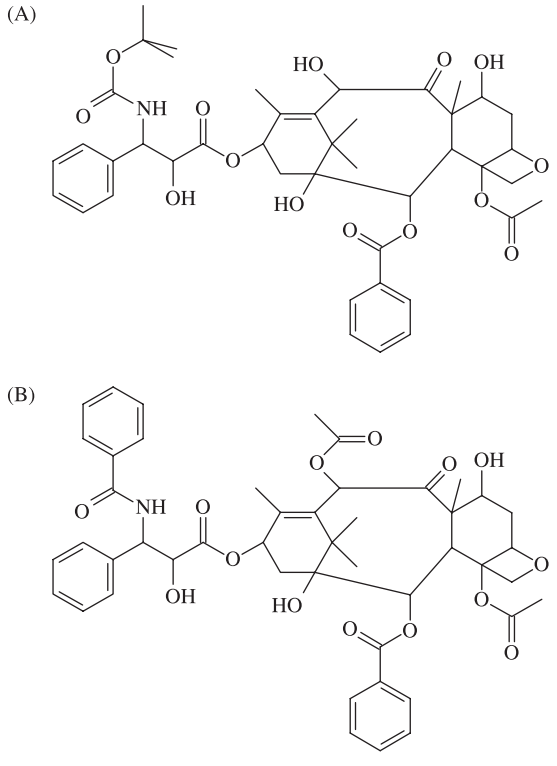

Figure 1. (A) Structure of docetaxel. (B) Structure of paclitaxel, used as internal standard.

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Abbreviations used: LLOQ, lower limit of quantitation; MRM, multiple reaction monitoring; NSCLC, non-small-cell lung cancer; ULOQ, upper limit of quantitation.

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these assays used solid-phase extraction (SPE) as a means of sample pretreatment (Parise *et al.*, 2003; Gustafson *et al.*, 2003). SPE can be a labour-intensive, time-consuming procedure if it is not automized. Others described liquid–liquid extraction, in which considerable amounts of plasma were required (Baker *et al.*, 2004; Sparreboom *et al.*, 2002). Only one article has been published thus far that used HPLC/MS/MS and a limited amount of 40 μL plasma (Hou *et al.*, 2004); however, a relatively high lower limit of quantitation (LLOQ) of 25 nM (22 ng/mL) was found. Recent progress in the field of oncology has raised the possibility of oral administration of docetaxel. This may be beneficial for patients as it offers them the possibility of treatment at home (Borner *et al.*, 2001), while at the same time sustained lower plasma concentration levels can be obtained compared with intravenous administration. To support clinical pharmacological studies with low dose oral docetaxel, evidently a sensitive assay with a low LLOQ is needed. It was also aimed to minimize the amount of blood taken from the patients. Therefore a sensitive assay was designed for the quantification of docetaxel in human plasma utilizing only 200 μL plasma volumes.

EXPERIMENTAL

Material. Docetaxel was obtained from Sequoia Research Products (Oxford, UK) and the paclitaxel internal standard originated from Hauser Laboratories (El Segundo, CA, USA). Methanol (Supra-Gradient grade) was purchased from Biosolve Ltd (Amsterdam, The Netherlands). Ammonia solution 25%, acetic acid and *tert*-butylmethylether (all analytical grade) were obtained from Merck (Darmstadt, Germany). Distilled water (B. Braun Medical, Emmenbrücke, Switzerland) was used throughout the analyses. Drug-free human heparinized plasma was obtained from Sanquin (Amsterdam, The Netherlands).

Instrumentation. Chromatographic separations were carried out using an HP1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) consisting of a binary pump, an autosampler, a mobile phase degasser and a column oven. A mobile phase of methanol–10 mM NH_4OH (7:3, v/v) was delivered at a flow rate of 0.2 mL/min through a Zorbax Extend C_{18} column (150 × 2.1 mm i.d., particle size 5 μm; Atlas Technologies) coupled to a reversed-phase guard column, 10 × 2 mm (Chrompack). Sample injections of 25 μL were carried out and the autosampler was thermostatted at 4°C. The run time was 7.5 min. The LC eluate was directed into an API-3000 triple quadropole MS equipped with a turbo-ionspray interface (PE Sciex, Toronto, Canada). The quadrupoles were operated with unit resolution in the positive ion mode. The resulting multiple reaction monitoring (MRM) chromatograms were used for quantification using Analyst software version 1.2 (PE Sciex). Mass transitions of m/z were optimized for docetaxel and paclitaxel, respectively, with dwell times of 150 ms. The nebulizer gas setting was set at 9.0 psi. Turbo gas flow was 7000 mL/min, while the curtain gas and collision gas (both N_2) were operated at 15 and 7.0 psi respectively. The ionspray voltage was kept at 5.5 kV, with a source temperature of 300°C.

Preparation of stock and working solutions. Two stock solutions of docetaxel with separate weighing were prepared in methanol at a concentration of 1.0 mg/mL. These solutions were further diluted with methanol to obtain working solutions. One set of working solutions was used to prepare calibration standards, the other to prepare quality control samples. A stock solution of paclitaxel was prepared in methanol at a concentration of 1.0 mg/mL. This solution was further diluted with methanol to obtain a working solution of 200 ng/mL. All solutions were stored at –20°C.

Preparation of calibration standards and quality control samples. Calibration standards were prepared in a range from 0.25 to 1000 ng/mL by diluting a fixed amount of working solution in control human plasma in volumetric flasks. Quality control (QC) samples were prepared in a similar way from the other set of working solutions in human plasma at concentrations of 0.75, 500 and 800 ng/mL. Furthermore, additional QC samples at the LLOQ level (0.25 ng/mL) and with a concentration higher than the upper limit of quantitation (ULOQ; 5000 ng/mL) were prepared. Sample aliquots of 200 μL were transferred to 2.0 mL polypropylene tubes (Eppendorf Merck) and stored at –20°C.

Sample processing. To 200 μL sample volumes 25 μL of internal standard working solution (200 ng/mL) were added. After mixing (10 s), *ter*-butylmethylether (1.0 mL) was added. The samples were mixed for 10 s and shaken automatically for 10 min at 1250 rpm (L46, Labinco, Breda, The Netherlands) and subsequently centrifuged for 10 min at 23,100 ***g*** (5403 Eppendorf, Netheler Hinz GmbH, Hamburg, Germany). The aqueous layer was frozen in an ethanol–dry ice mixture and the organic layer was decanted into a clean tube. The organic solvent was evaporated under a gentle stream of nitrogen gas at 40°C. The residue was reconstituted with 100 μL of acetonitrile–0.1 M NH_4Ac pH 5 (1:1, v/v) by vortex mixing for 30 s. After centrifuging for 10 min at 23,100 ***g*** the supernatant was transferred to a glass auto-sampler vial with insert and 25 μL were injected onto the analytical column.

Validation procedures. For the docetaxel assay in human plasma a full validation program according to the FDA guidelines was executed (www.fda.gov/cder/guidance/4252fnl.htm, 2004).

Linearity. Eight non-zero calibration standards (0.25, 0.5, 1.5, 10, 100, 250, 500 and 1000 ng/mL) were prepared in human plasma and analysed in duplicate in three separate analytical runs. Calibration curves were calculated by least-squares linear regression using a weighting factor of $1/x^2$ (the reciprocal of the squared concentration). Concentrations were back-calculated from the constructed calibration curve and deviations from the nominal concentrations should be within ±20% for the LLOQ and within ±15% for other concentrations with coefficient of variation (CV) values less than

20 and 15%, respectively (www.fda.gov/cder/guidance/4252fnl.htm, 2004).

Accuracy and precision. Five replicates of the QC samples in plasma were analysed together with a calibration curve, independently prepared from the quality control samples, in at least three analytical runs. Quality control samples with concentrations higher than the ULOQ were diluted 10 times in human plasma prior to sample processing, in order to validate dilution of samples that were originally above the ULOQ. Five replicates were analysed in one analytical run. The accuracy was determined in percentage difference between the mean concentration and the nominal concentration. The coefficient of variation (CV) was used to report the precisions. The intra- and interassay accuracies should be within ±20% for the LLOQ concentration and within ±15% for other concentrations. The precisions should be less than 20% and less than 15%, respectively (www.fda.gov/cder/guidance/4252fnl.htm, 2004).

Specificity and selectivity. From six individual batches of control drug-free human plasma samples containing neither analyte nor internal standard (double blank), samples containing only internal standard (blank), and LLOQ samples were prepared. The samples were prepared to determine whether endogenous compounds interfere at the mass transitions chosen for docetaxel and the internal standard. Samples were processed according to the described procedures and analyzed. Peak areas of compounds co-eluting with the analyte or internal standard should not exceed 20% of the analyte peak at the LLOQ or 5% of the internal standard area. Deviations from the nominal concentrations should be within ±20% (www.fda.gov/cder/guidance/4252fnl.htm, 2004).

Ion suppression and recovery. Control drug-free human plasma was processed and dry extracts were dissolved with working solutions that represent 100% recovery containing the analyte and internal working standard in acetonitrile–0.1 M NH_4AC pH 5 (1:1, v/v). Ion-suppression was determined by comparing the analytical response of these samples to that of the working solutions. The loss of signal represents the ion suppression. Liquid–liquid extraction (LLE) recovery was determined by comparing the analytical response of processed quality control samples with the analytical response of blank samples reconstituted with working solutions as described earlier. Overall recovery corresponds to the net response after subtraction of the ion-suppression and the signal loss due to the extraction. Ion suppression and recovery experiments for the internal standard were performed in a similar way.

Stability. The stability of docetaxel was evaluated in human plasma at two concentration levels (0.75 and 800 ng/mL) after three freeze–thaw cycles by comparing quality control samples that have been frozen (–20°C) and thawed three times with freshly prepared quality control samples. Furthermore, stability in human plasma under processing conditions (ambient temperatures) was evaluated. In addition, stability in the dry extract at 2–8°C and in the final extract at ambient temperatures was determined for plasma. Re-injection reproducibility was tested after 24 h in the autosampler. The analytes are considered stable in the biological matrix or extracts thereof when 85–115% of the inital concentration was found (www.fda.gov/cder/guidance/4252fnl.htm, 2004). Long-term stability at –20°C has been repeated earlier (Rosing *et al.*, 1997). Docetaxel was found to be stable in human plasma for at least 6 months.

Implementation of the docetaxel assay. The analytical method described in this article has been used to support phase I studies in humans investigating the exposure to oral docetaxel 10 mg in plasma in combination with ritonavir 100 mg compared with i.v. docetaxel 100 mg. Blood samples were collected at several time points and, after centrifuging, plasma was removed and stored at –20°C until analysis.

RESULTS

Mass spectrometry

Docetaxel formed predominantly a protonated molecule ($[M+H]^+$) in the mobile phase containing methanol–10 mM NH_4OH (7:3, v/v) using the electrospray ion source. In Fig. 2(a) Q1 mass spectrum of docetaxel is depicted. Apart from the protonated species, ammonium, sodium and potassium adducts are also visible. The protonated molecular ion of docetaxel was introduced to fragment in the collision cell and the resulting product ion spectrum is presented in Fig. 3. A proposed fragmentation pattern for docetaxel is presented in Fig. 4. The main fragment ion corresponds to the cleavage of the side chain at the carboxyl group, resulting predominantly in the 527 amu product ion and a side chain (m/z 282). This latter smaller fragment, then may split in at least two different fragments with m/z 226 and m/z 182.

Chromatography

Several assays have described the quantitative analysis of docetaxel in biological fluids using LC-MS techniques (Parise *et al.*, 2003; Wang *et al.*, 2003; Baker *et al.*, 2004; Gustafson *et al.*, 2003; Sparreboom *et al.*, 2002; Hou *et al.*, 2004). In all assays the analytes are chromatographically separated from matrix components using acidic mobile phases containing mostly acetic or formic acid in a mixture of water and either methanol or acetonitrile. The presented assay is in concordance with earlier work described by Stokvis *et al.* (2004) for the analysis of paclitaxel and the sample pre-treatment was adapted from Alexander *et al.* (2003). In analogy, the alkaline eluent as mobile phase also favoured the high MS response that was observed in our study. Representative chromatograms of docetaxel and the internal standard paclitaxel at the LLOQ level of 0.25 ng/mL from human plasma are shown in Fig. 5.

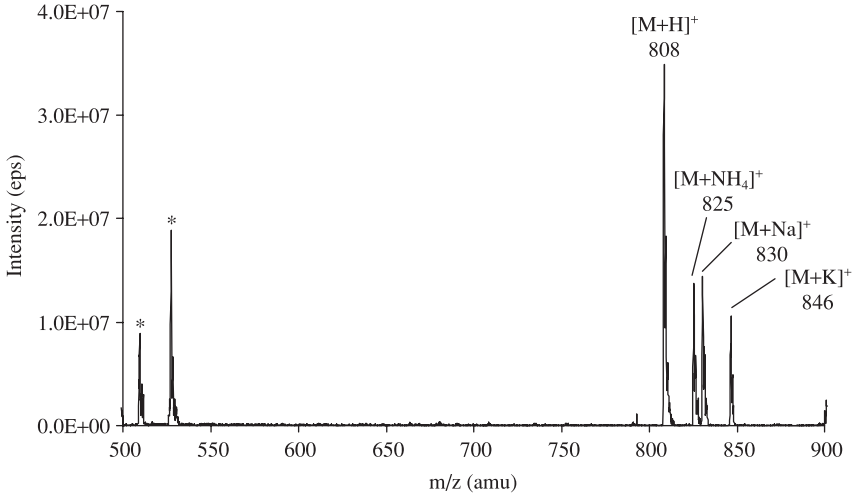

Figure 2. Q1 mass spectrum of docetaxel. Peaks labelled with an asterisk correspond to fragment ions.

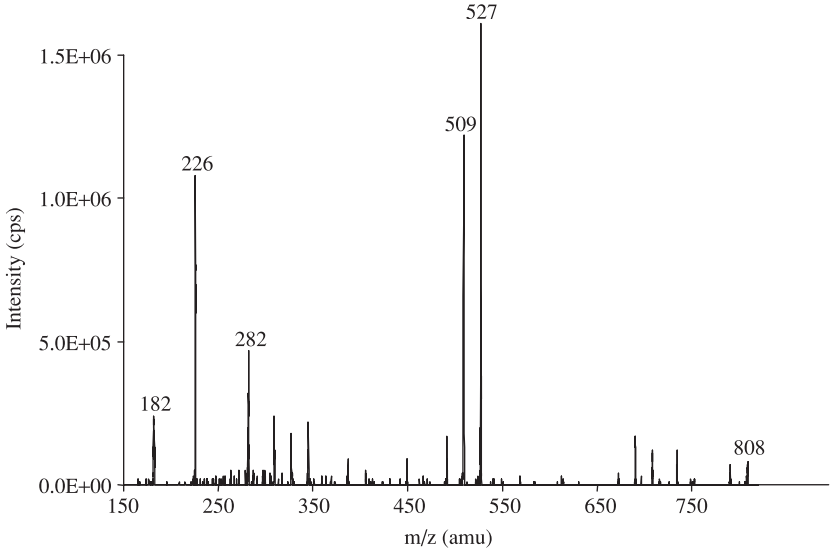

Figure 3. Product ion MS/MS spectrum of docetaxel from the protonated molecular ion at *m*/*z* 808.

Sample pretreatment

LLE with *ter*-butylmethylether was suitable for obtaining good recoveries for docetaxel (92.4 ± 4.5%). The sample pre-treatment was similar to that as descibed by Alexander *et al.* (2003) for paclitaxel with the minor adaption that freezing of the aquous layer in a −60°C freezer was replaced by instant freezing in an ethanol–dry ice bath.

Validation procedures

The calibration concentrations were back-calculated from the responses. For all concentrations of docetaxel, the deviations of the nominal concentrations were between −8.92 and 9.84%. The CV values ranged from 1.78 to 12.0%.

Assay performance data for docetaxel are summarized in Table 1. For the assay of docetaxel, interassay accuracy and precisions were between −10.2 and 1.02% and less than 12.8%, respectively. MRM chromatograms of double blank and blank samples prepared in six individual batches of human plasma did not show peaks that co-eluted with docetaxel with areas exceeding 20% of the area at LLOQ level or show peaks that co-eluted with the internal standard with areas that exceeded 5% of the internal standard area. Deviations from the nominal concentrations at the LLOQ level in human plasma were between −19.5 and −14.2% for docetaxel.

The ion-suppression of docetaxel ranged from 13.4 to 20.5%. The ion-suppression of the internal standard was 27.2%. The total recovery of docetaxel (sample pretreatment recovery plus ion-suppression) was 76.1 ±

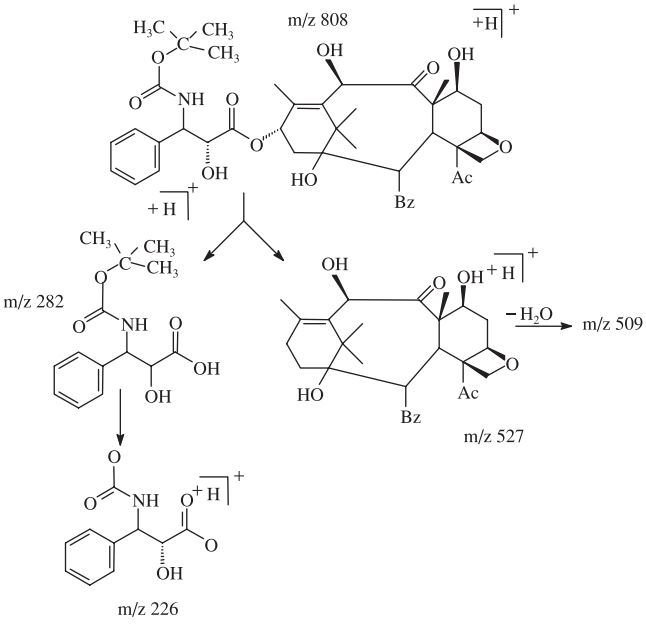

Figure 4. Proposed fragmentation scheme for docetaxel.

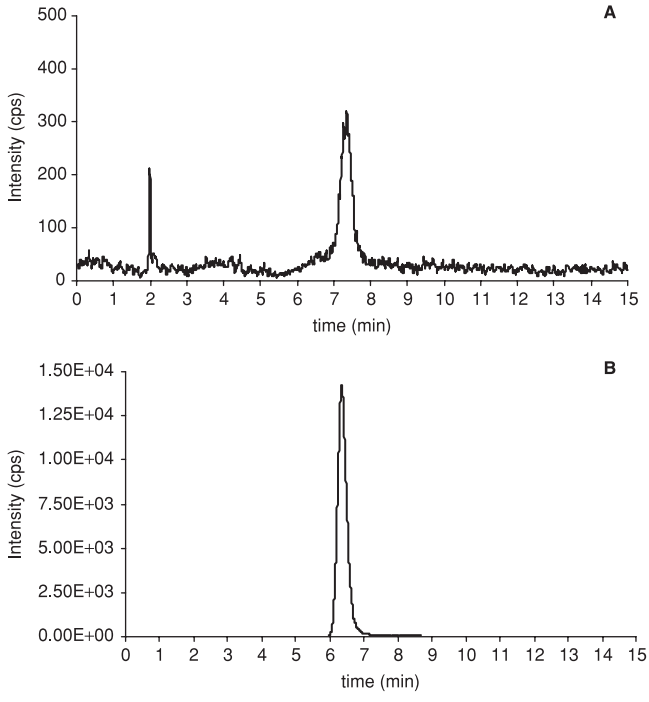

Figure 5. Representative chromatograms for docetaxel and paclitaxel at the LLOQ level (0.25 ng/mL) in human plasma.

3.3%. The total recovery of the internal standard was 66.4% at a concentration of 25 ng/mL plasma.

Stability

Docetaxel is stable in human plasma after at least three freeze–thaw cycles and it is stable in human plasma stored at ambient temperatures for at least 24 h. Furthermore, docetaxel is stable in the dry extract after LLE of human plasma stored at nominally 2–8°C for at least 8 weeks and it is stable in the final extract for at least 8 days, when kept at nominally 2–8°C. The deviation from time zero is less than 15% of the first result and indicates that docetaxel is stable in the reconstituted samples of human plasma when kept in the autosampler at nominally 2–8°C for 24 h. Long-term stability of docetaxel in control human plasma was investigated and published earlier (Rosing *et al.*, 1997). Previous stability data of docetaxel in human plasma stored under similar conditions found that docetaxel was stable at –30°C for at least 6 months. In addition re-injection reproducibility was established after 24 h at nominally 2–8°C.

Implementation of the docetaxel assay

The systemic exposure of docetaxel given per os as 10 mg in combination with ritonavir 100 mg was compared with docetaxel administered i.v. 100 mg. Typical results are presented in Fig. 6. Interestingly, we were able to measure low dose oral docetaxel plasma concentrations up to 48 h after dosing. The measured concentration range for our patients were between 0 and 33.0 ng/mL for the oral administration of docetaxel and between 0 and 2060 ng/mL for the i.v. administration.

Table 1. Assay performance data for docetaxel in human plasma

Nominal concentration (ng/mL)	Measured mean concentration (ng/mL)	Interassay		Number of replicates
		Accuracy (%)	Precision (%)	
0.261	0.234	–10.2	12.8	15
0.782	0.782	0.0640	10.0	15
521	514	–1.25	5.16	15
834	842	1.02	8.16	15
2610	2426	–6.87[a]	0.855[a]	5

[a] Intraassay accuracy and precision.

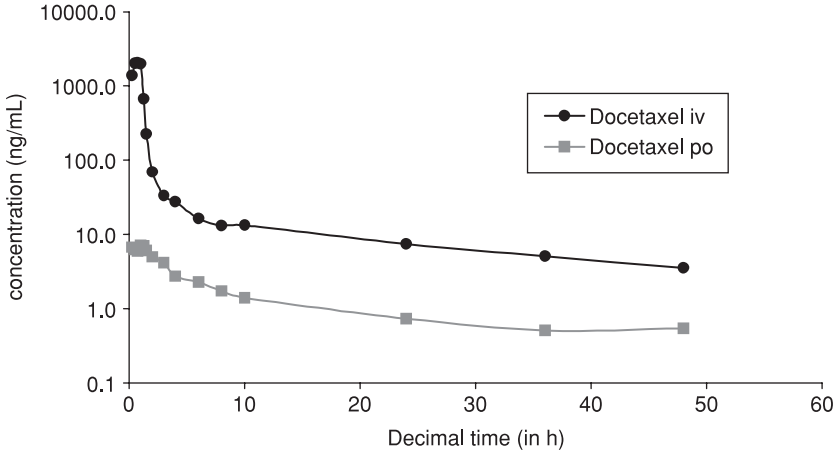

Figure 6. Concentration versus time curve of a patient receiving 100 mg docetaxel i.v. in 1 h and of a patient receiving 10 mg oral docetaxel in combination with 100 mg ritonavir per os.

CONCLUSIONS

The development and validation of an assay for the determination of docetaxel in human plasma is described. The validated range for docetaxel was from 0.25 to 1000 ng/mL using 200 μL human plasma sample aliquots. The obtained LLOQs for human plasma were comparable to the LLOQ obtained in an earlier study (Parise *et al.*, 2003), but with the advantage of LLE over SPE and with considerable less amount of plasma required. Compared with the assay of Hou *et al.* (2004) run time was reduced and the detection limit was 5-fold lower. This assay was validated according to FDA guidelines and has been successfully applied in clinical phase I studies with docetaxel.

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