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Quantification of paclitaxel metabolites in human plasma by high-performance liquid chromatography

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Abstract

A reversed-phase high-performance liquid chromatographic (HPLC) method has been validated for the quantitative determination of the three major paclitaxel metabolites (6α-hydroxypaclitaxel, 3′-*p*-hydroxypaclitaxel, 6α,3′-*p*-dihydroxypaclitaxel) in human plasma. The HPLC system consists of an APEX-octyl analytical column and acetonitrile–methanol–0.02 *M* ammonium acetate buffer pH 5 (AMW; 4:1:5, v/v/v) as the mobile phase. Detection is performed by UV absorbance measurement at 227 nm. The sample pretreatment of the plasma samples involves solid-phase extraction (SPE) on Cyano Bond Elut columns.

The concentrations of the metabolic products could be determined by using the paclitaxel standard curve with a correction factor of 1.14 for 6α,3′-*p*-dihydroxypaclitaxel. The recoveries of paclitaxel and the metabolites 6α,3′-*p*-dihydroxypaclitaxel, 3′-*p*-hydroxypaclitaxel and 6α-hydroxypaclitaxel in human plasma were 89, 78, 91 and 89%, respectively. The accuracy of the assay for the determination of paclitaxel and its metabolites varied between 95 and 97%, at a 50 ng/ml analyte concentration. The lower limit of quantitation was 10 ng/ml for both the parent drug and its metabolites.

1. Introduction

Paclitaxel (Taxol), tax-11-en-9-one,5ß,20-epoxy-1,2α,4,7ß,10ß,13α-hexahydroxy-4,10-diacetate-2-benzoate-13-(α-phenylhippurate), is a novel anticancer drug originating from the bark of the Pacific yew, *Taxus brevifolia* (Fig. 1) [1]. The drug belongs to a new group of tubulin promoting and microtubules stabilizing agents [2].

In clinical studies paclitaxel showed activity against several tumor types including platinum-resistant ovary cancer [3].

Both from in vitro experiments and analysis of bile of treated rats it became clear that paclitaxel is extensively metabolised by cytochrome P450

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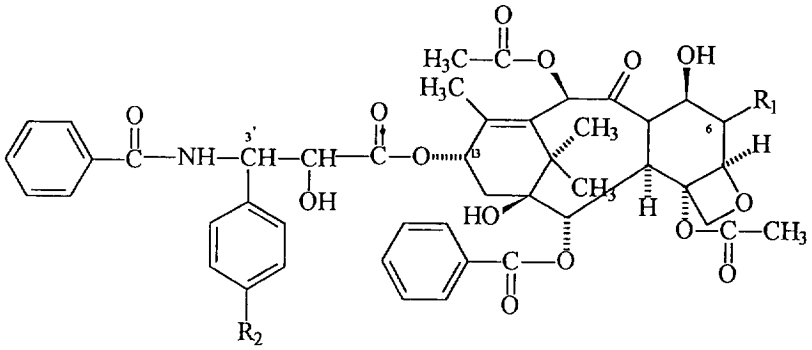

Fig. 1. Chemical structures of paclitaxel (R_1, R_2 = H), 6α-hydroxypaclitaxel (R_1 = OH, R_2 = H), 3′-*p*-hydroxypaclitaxel (R_1 = H, R_2 = OH) and 6α,3′-*p*-dihydroxypaclitaxel (R_1, R_2 = OH).

enzymes; however, no metabolic products could be found in plasma of patients in early pharmacokinetic studies [2,4,5]. This was probably due to the prolonged paclitaxel infusion schemes yielding only low levels of the drug and the use of relatively insensitive high-performance liquid chromatography (HPLC) assays.

In the first validated assay for the analysis of paclitaxel in human plasma one extra peak in the chromatograms of a plasma and urine sample of a treated patient was found [6]. No structural identification or quantitation was performed. In the phase I pharmacokinetic studies of paclitaxel no metabolites could be detected in plasma of treated patients [7–9]. So far, HPLC has been the methodology of first choice for paclitaxel analysis in biological fluids. Protein precipitation, in combination with solid-phase extraction (SPE), liquid–liquid extraction also followed by SPE and SPE alone have been utilized as sample pretreatment procedures [10–12]. The limits of quantitation of the various reversed-phase HPLC assays differ substantially, ranging from 6 [11] to 85 ng/ml [6], as does their capability to detect metabolites in plasma.

The recently developed HPLC assay [10], utilizing a SPE as sample pretreatment procedure, has been used in a large pharmacokinetic study whereby several metabolic products could be detected in plasma of patients treated with both short and prolonged infusions of paclitaxel [10,11].

Several metabolic products of paclitaxel have also been detected in bile of rats and humans, isolated and structurally identified, by the use of chromatographic methods, mass spectrometry and nuclear magnetic resonance spectroscopy [13–16]. So far, the three major metabolic products that have been identified in humans are 6α-hydroxypaclitaxel, 3′-*p*-hydroxypaclitaxel and 6α,3′-*p*-dihydroxypaclitaxel (previously reported as metabolites I, II, and V [11]) (Fig. 1).

The importance of these metabolites in the pharmacology and toxicology of paclitaxel is largely unknown. Growth inhibition tests with two human derived cell lines MOLT-4 and U-937 showed approximately 30-fold less cytotoxicity of the principal metabolite 6α-hydroxypaclitaxel in comparison with paclitaxel [14]. 3′-*p*-Hydroxypaclitaxel retained some activity in vitro against the human A2780 cell line [15].

It is obvious that for investigating pharmacokinetic–pharmacodynamic relationships of paclitaxel the quantification of the major metabolites (6α-hydroxypaclitaxel, 3′-*p*-hydroxypaclitaxel and 6α,3′-*p*-dihydroxypaclitaxel) is an indispensable part. So far methods for the quantification of these compounds in human plasma have not been described, which may be due to the lack of reference compounds which are a prerequisite for the development and validation of such an assay. We have isolated and purified the metabolites in sufficient amounts from human faeces for this purpose [15]. Here

we describe the development, validation and use of a bioanalytical method for the quantification of paclitaxel and its three major metabolites.

Furthermore it is described how to use paclitaxel calibration curves for the quantification of the metabolites in plasma in the absence of these compounds as reference.

2. Experimental

2.1. Chemicals and reagents

Paclitaxel (Lot.80617492D; purity of 98.3%) and 3′-*p*-hydroxypaclitaxel (Lot.33343-111-2, purity of 96.9%), references were obtained from Bristol Myers Squibb (Syracuse, NY, USA).

The metabolites 6α-hydroxypaclitaxel (purity 96.9%) and 6α,3′-*p*-dihydroxypaclitaxel (purity 96.8%) reference material for HPLC analysis were obtained after isolation and purification of faeces of patients treated with paclitaxel 135 mg/m^2 or 175 mg/m^2 as a 3- or 24-h infusion, as described in detail previously [15]. The identity was confirmed by their chromatographic properties, on-line photodiode array (PDA) detection and fast-atom bombardment mass spectrometry (FAB-MS).

Acetonitrile (HPLC gradient grade) was obtained from Biosolve (Barneveld, Netherlands). Methanol (ChromAR) was obtained from Promochem (Wesel, Germany). Ethanol, ammonium acetate, glacial acetic acid, *n*-hexane, and triethylamine (all analytical grade) were obtained from Merck (Darmstadt, Germany).

2.2. Preparation of stock solutions

Paclitaxel, 6α-hydroxypaclitaxel, 3′-*p*-hydroxypaclitaxel and 6α,3′-*p*-dihydroxypaclitaxel stock solutions were made by dissolving the reference materials in ethanol obtaining concentrations of 5.00, 3.47, 0.50 and 1.57 mg/ml, respectively. A mixture of paclitaxel, 6α-hydroxypaclitaxel, 3′-*p*-hydroxypaclitaxel, and 6α, 3′-*p*-dihydroxypaclitaxel was prepared by dilution of the standard solutions in AMW to a concentration of 25 000 ng/ml for each compound. From this stock solution several dilutions were made in AMW to concentrations ranging from 25 to 2500 ng/ml.

2.3. Preparation of standards and quality control samples for solid-phase extraction

A mixture of the four reference compounds was obtained by dilution of the standard solutions in blank human plasma, yielding a final concentration of 10 000 ng/ml. This stock solution was further diluted in blank human plasma to achieve analyte concentrations containing 10, 50, 100, 500 and 1000 ng/ml for paclitaxel, 6α-hydroxypaclitaxel and 3′-*p*-hydroxypaclitaxel and 10, 50, 100 and 500 ng/ml for 6α,3′-*p*-dihydroxypaclitaxel. Quality control samples were prepared from the same stock solution at concentrations of 10, 50 and 500 ng/ml.

2.4. Sample processing

Cyano Bond Elut columns (1 ml, Varian, Harbor City, USA) were first conditioned with consecutive washings with 2.0 ml methanol and 0.01 *M* ammonium acetate buffer pH 5.0. Before use, 1 ml 0.2 *M* ammonium acetate was added to 1 ml spiked plasma containing the paclitaxel–metabolites mixture. Next, 1.0 ml diluted plasma–buffer mixture was applied to the columns. The columns were then washed with 2 ml 0.01 *M* ammonium acetate pH 5.0, 1 ml methanol–0.01 *M* ammonium acetate pH 5.0 (2:8, v/v) and 1 ml hexane. The columns were dried under maximum vacuum for 1 min (15 mm Hg, ca. $2 \cdot 10^3$ Pa). The analytes were eluted from the columns with 2 ml of a mixture of acetonitrile–triethylamine (1000:1, v/v), in 2.0-ml Eppendorf Safe-Lock tubes 3816 (Merck). The eluent was evaporated to dryness under a nitrogen stream at 30°C.

Samples were reconstituted with 200 μl AMW by vortex-mixing for 30 s. The reconstituted samples were transferred to autosampler vials containing limited-volume inserts and 50 μl was injected from each sample onto the HPLC column.

2.5. HPLC instrumentation and conditions

The HPLC system consisted of a Model 510 pump (Waters, Milford, MA, USA), and a SP 8880 autosampler (Thermo Separation Products (TSP), Santa Clara, CA, USA). An APEX-octyl analytical HPLC column (150 × 4.6 mm, particle size 5 μm) (Jones Chromatography, Lakewood, CO, USA) was used which was protected with a pre-column (4 × 4 mm, particle size 5 μm) packed by LiChrospher RP-8 material (Merck, Darmstadt, Germany).

The mobile phase consisted of acetonitrile–methanol–0.02 *M* ammonium acetate buffer pH 5.0 (4:1:5, v/v/v). Detection was performed with a 996 Photodiode Array Detector (Waters, Milford, MA, USA) coupled to a Millenium v 2.00 data station for obtaining UV spectra and purity analysis reports. A SP 4290 integrator coupled to a WINner data system (TSP) was used for data processing.

2.6. Molar absorptivity

The molar absorptivities (ϵ) in methanol for paclitaxel (10.0 μg/ml), 6α-hydroxypaclitaxel (11.6 μg/ml), 3′-*p*-hydroxypaclitaxel (10.0 μg/ml), 6α,3′-*p*-dihydroxypaclitaxel (10.5 μg/ml) were determined by using a UV–Vis spectrophotometer GBC 918 (GBC Scientific Equipment, Dandenong, Australia) operating at 227 nm. The molar absorptivity was calculated by $\epsilon = A/bc$, where A is the absorbance, b is the path length (in cm) of the radiation through the absorbing medium, and c the analyte concentration (in *M*).

2.7. Fast-atom bombardment mass spectrometry (FAB-MS)

FAB-MS spectra were obtained from the three metabolites and paclitaxel in a thioglycerol matrix using a JMS-SX/SX102A Tandem Mass Spectrometer (Jeol, Tokyo, Japan) with a 6-keV xenon atom beam and a 10-kV accelerating voltage.

2.8. Validation parameters

Calibration curves

A full validation for the analysis of paclitaxel and its three major metabolites in human plasma was completed. Calibration curves were calculated by weighted ($1/X$) linear regression analysis. For each standard curve five standard plasma samples were taken in duplicate at the concentrations 10, 50, 100, 500 and 1000 ng/ml.

Recovery

The extraction efficiencies of the analytes were determined by comparing the slopes of human plasma calibration curves to standard curves prepared in AMW.

Accuracy and precision

Accuracy and precision were determined with the use of quality control samples with concentrations 10, 50 and 500 ng/ml for all compounds.

Stability

The stability of the four compounds in AMW, at a concentration of 1000 ng/ml, was determined after storage at ambient temperature.

2.9. Purity of metabolites

The peak purity was calculated using the Waters Millenium 2.00 PDA software.

The peak purity is based on the difference in the purity angle (PA) and the threshold angle (TA) expressed in degrees. Both the PA and TA were measured by the use of complex algorithms, based on the conversion of the peak spectra to vectors in a multi-dimensional space. For the determination of PA, the peak apex is used as the reference spectrum; all the other spectral data contained within that peak are compared to the spectra of the apex. The noise (TA) calculations are based on a segment of the baseline (20–40 s) within the chromatographic run. This segment does not contain any chromatographic peaks or chromophores beyond the mobile phase background.

3. Results and discussion

The pharmacokinetics of paclitaxel appears to be non-linear which may lead to dramatic differences in drug exposure, in terms of AUC, when dosages and or schedules are changed [11]. This non-linearity probably occurs at the level of saturable hepatic metabolism. The identification of the three major metabolites 6α-hydroxypaclitaxel, 3′-*p*-hydroxypaclitaxel and 6α,3′-*p*-dihydroxypaclitaxel of paclitaxel in human bile and in human plasma are supportive for extensive hepatic metabolism. More knowledge about the metabolic fate of paclitaxel as well as the pharmacokinetics of the metabolic products will be very helpful to elucidate the exact nature of the processes underlying the non-linear kinetic behaviour.

Although several HPLC methods including various sample pretreatment procedures have been published for the analysis of paclitaxel in biological matrices no metabolic products could be detected in human plasma. The recently developed HPLC assay with a solid-phase extraction procedure, however, makes it possible to detect systematically metabolic products in human plasma [10,11]. The quantification of these metabolites in human plasma has not yet been described, which may be due to the lack of reference compounds which are necessary for the development and validation of such an assay.

The principal metabolite 6α-hydroxypaclitaxel could also be isolated from human plasma for structural identification. For the identification of the other metabolic products as reference material for the performance of a validation procedure, we isolated sufficient amounts of 6α-hydroxypaclitaxel and 6α,3′-*p*-dihydroxypaclitaxel from human faeces of patients treated with paclitaxel [15].

The chromatographic behaviour, UV-PDA spectra, and mass spectrum of the principal metabolite (V, retention time 7.3 min) in human plasma indicated that this compound is 6α-hydroxypaclitaxel. FAB-MS of this metabolite and paclitaxel isolated from plasma of treated patients, showed protonated molecular ions $[M + Na]^+$ at *m/z* 892 and *m/z* 876, respectively. The increase of 16 mass units indicated the introduction of one oxygen atom. Prominent fragments of paclitaxel included *m/z* 591, *m/z* 531 and *m/z* 308 (Table 1). Fragment *m/z* 591 corresponds with the taxane nucleus after cleavage of the C13 side chain (R) $[M - R - H_2O + Na]^+$. The peak detected at *m/z* 531 corresponds with the taxane nucleus after release of the acetate group $[M - R - H_3CCOOH - H_2O + Na]^+$. The last fragment *m/z* 308 is the C13 side chain [C13 side chain + Na]$^+$. The mass spectrum of isolated 6α-hydroxypaclitaxel revealed fragment ion peaks at *m/z* 607, *m/z* 547 and *m/z* 308. This indicates that hydroxylation had taken place in the taxane nucleus itself.

The unidentified peaks I and II, previously reported [11], were identified as being 6α,3′-*p*-dihydroxypaxlitaxel and 3′-*p*-hydroxypaclitaxel by the chromatographic behaviour and the UV-PDA spectra [15].

Fig. 2 shows a representative chromatogram of plasma of a patient treated with paclitaxel 175

Table 1
FAB-MS fragments of paclitaxel and metabolite V (6α-hydroxypaclitaxel) isolated from human plasma and possible assignments

Fragments (*m/z*) found in		
Paclitaxel	Metabolite V	Possible identity
876	892	$[M + Na]^+$
591	607	$[M - R - H_2O + Na]^+$
531	547	$[M - R - H_3CCOOH - H_2O + Na]^+$
308	308	$[R + OH + Na]^+$

R = C_6H_5-CO-NH-CH(C_6H_5)-CHOH-CO-

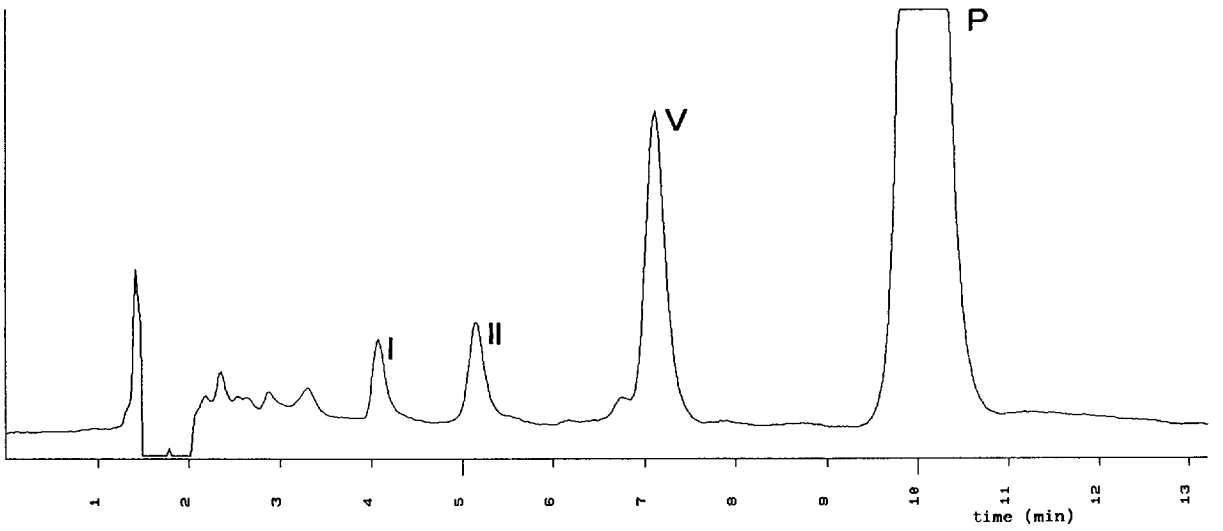

Fig. 2. HPLC chromatogram of plasma of a patient treated with paclitaxel at a concentration of 175 mg/m^2 during a 3-h infusion. Paclitaxel (P), 6α,3′-p-hydroxypaclitaxel (I), 3′-p-hydroxypaclitaxel (II), and 6α-hydroxypaclitaxel (V).

mg/m^2 during a 3-h infusion showing paclitaxel and its three major metabolites. The retention times for metabolites 6α,3′-p-dihydroxypaclitaxel, 3′-p-hydroxypaclitaxel and 6α-hydroxypaclitaxel (corresponding with metabolites I, II, and V of our previous clinical study [11]), and paclitaxel are 4.1, 5.2, 7.3 and 10.1 min, respectively. The peaks of paclitaxel and its three major metabolites possess adequate symmetry and are well separated. The UV-PDA detection of all compounds exhibit typical taxane spectra (absorbance maximum at 227 nm) (Fig. 3) [15].

PDA purity plots of the metabolites revealed no underlying interfering compounds.

The lower limit of quantification for all the three metabolites was 10 ng/ml. The calibration curves, peak area versus concentration, were linear over the concentration ranges tested, i.e. 10–1000 ng/ml for paclitaxel, 6α-hydroxypaclitaxel and 3′-p-hydroxypaclitaxel and over the range 10–500 ng/ml for 6α,3′-p-dihydroxypaclitaxel, with regression correlation coefficients $\geq$0.995. By using weighted ($1/X$) linear regression analysis, deviations of the interpolated concentrations of all standard samples were within the acceptable 85–115% range. Accuracy, and within-day precision data have been tabulated (Table 2). The within-day precision for paclitaxel

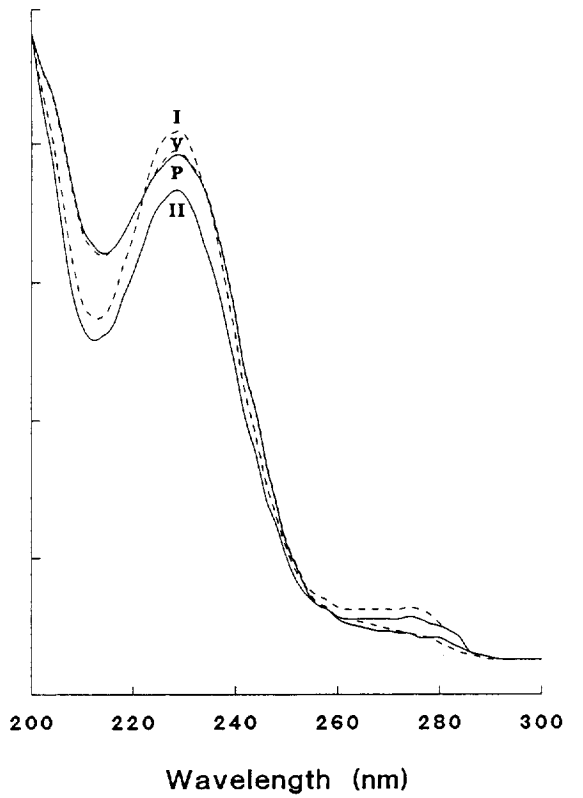

Fig. 3. UV-PDA spectra of paclitaxel (P) and its three major metabolites 6α,3′-p-hydroxypaclitaxel (I), 3′-p-hydroxypaclitaxel (II) and 6α-hydroxypaclitaxel (V).

Table 2
HPLC validation characteristics

Nominal concentration (ng/ml)	Mean measured concentration (ng/ml)	Accuracy (%)	Precision (%) within-day	*n*
Paclitaxel				
10	9.59	96	7.4	6
50	47.9	96	4.0	6
500	501	100	1.2	6
6α-Hydroxypaclitaxel				
10	10.9	109	8.5	6
50	47.6	95	4.0	6
500	493	99	2.9	6
3′-p-Hydroxypaclitaxel				
10	9.93	99	4.1	6
50	47.9	96	2.8	6
500	496	99	3.8	6
6α,3′-p-Dihydroxypaclitaxel				
10	11.3	113	6.3	3
50	48.7	97	3.3	3
500	508	102	2.2	3

n = number of replicates.

and metabolites varied between 1.2 and 8.5%. The extraction recoveries of 6α,3′-*p*-dihydroxypaclitaxel, 3′-*p*-hydroxypaclitaxel, 6α-hydroxypaclitaxel and paclitaxel were 78, 91, 89 and 89%, respectively.

Paclitaxel as well as metabolites 6α,3′-*p*-dihydroxypaclitaxel, 3′-*p*-hydroxypaclitaxel, 6α-hydroxypaclitaxel were found to be stable in AMW for at least three days at room temperature.

The molar absorptivities (in M^{-1} cm^{-1}) at 227 nm for paclitaxel, 6α-hydroxypaclitaxel, 3′-*p*-hydroxypaclitaxel and 6α,3′-*p*-dihydroxypaclitaxel were 33 000 (range 32 600–33 600), 32 000 (range 31 900–32 400), 34 000 (range 32 700–35 800) and 30 100 (range 30 000–30 150), respectively. There were no clear differences between the peak areas of 6α-hydroxypaclitaxel and paclitaxel (slope = 0.994; $r^2 = 0.997$) and for the 3′-*p*-hydroxypaclitaxel and paclitaxel (slope = 1.002; $r^2 = 0.999$). After correction for the recovery with a correction factor of 1.14 for the 6α,3′-*p*-dihydroxypaclitaxel there was also no clear difference between the peak areas compared to the peak areas of paclitaxel (slope = 0.983; $r^2 = 0.983$). The molar absorptivities, the extraction recoveries and the slopes of the calibration curves of 6α-hydroxypaclitaxel and 3′-*p*-hydroxypaclitaxel were all in the same range of that of paclitaxel. These two major metabolites can therefore be determined by the use of the paclitaxel calibration curves.

The quantification of the 6α,3′-*p*-dihydroxypaclitaxel using the paclitaxel calibration curve needs a correction factor of 1.14, as its extraction recovery is slightly lower than for paclitaxel.

We conclude that the previously described HPLC assay [10,11] is a useful tool not only for the determination of paclitaxel but also for its three major metabolites in human plasma. The concentrations of the metabolites (in ng/ml) can be quantified, in the absence of reference compounds, using the paclitaxel calibration curve and after correction for the molar mass differences. Only for the dihydroxy metabolite a correction factor of 1.14 is needed.

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