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Journal of Chromatography B, 664 (1995) 373–382

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

High-performance liquid chromatographic procedures for the quantitative determination of paclitaxel (Taxol) in human urine

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First received 7 July 1994; revised manuscript received 12 October 1994

Abstract

A reversed-phase high-performance liquid chromatographic (RP-HPLC) method has been developed and validated for the quantitative determination of paclitaxel in human urine. A comparison is made between solid-phase extraction (SPE) and liquid–liquid extraction (LLE) as sample pretreatment. The HPLC system consists of an APEX octyl analytical column and acetonitrile–methanol–0.2 μM ammonium acetate buffer pH 5 (4:1:5, v/v) as the mobile phase. Detection is performed by UV absorbance measurement at 227 nm. The SPE procedure involves extraction on Cyano Bond Elut columns. *n*-Butylchloride is the organic extraction fluid used for the LLE. The recoveries of paclitaxel in human urine are 79 and 75% for SPE and LLE, respectively. The accuracy for the LLE and SPE sample pretreatment procedures is 100.4 and 104.9%, respectively, at a 5 μg/ml drug concentration. The lower limit of quantitation is 0.01 μg/ml for SPE and 0.25 μg/ml for LLE. Stability data of paclitaxel in human urine are also presented.

1. Introduction

Paclitaxel (Taxol), tax-11-en-9-one, 5β,20 - epoxy - 1,2α,4,7β,10β,13α - hexahydroxy - 4,10 - diacetate - 2 - benzoate - 13 - (α-phenylhippurate), is a novel anticancer drug originating from the bark of the *Taxus brevifolia* (Fig. 1) [1]. This drug belongs to the group of tubulin promoting agents. Direct binding to tubulin causes massive stable microtubules, and thus interferes with mitosis, cell shape preservation, ciliary and flagellar motility and intracellular transport [2].

In preclinical experiments paclitaxel has demonstrated a broad spectrum of anti-tumour activity [3,4]. In clinical studies it has shown promising activity against ovarian, breast, head and neck, small cell and non-small cell lung cancer [2].

In pharmacokinetic studies, as part of the phase I evaluation, several high-performance liquid chromatographic (HPLC) procedures for the determination of paclitaxel in human plasma and urine have been employed [5–8]. The isola-

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0378-4347/95/$09.50
SSDI 0378-4347(94)00477-3

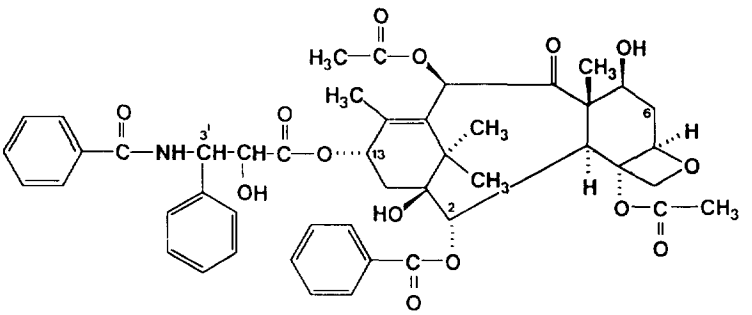

Fig. 1. Structure of paclitaxel.

tion of paclitaxel from human urine or plasma was based on either dilution (1:10) of the sample with the mobile phase (acetonitrile–1 m*M* perchloric acid, 58:42, v/v) followed by direct injection onto the HPLC system [7], a protein precipitation step with ice-cold acetonitrile [6] or a liquid–liquid extraction (LLE) with ethyl acetate [5] or a LLE procedure with *tert.*-butyl methyl ether followed by a solid-phase extraction (SPE) with C_{18} Sep-Pak columns [8]. Recently, a highly sensitive HPLC procedure with SPE as sample pretreatment procedure was developed for the bio-analysis of paclitaxel in human plasma [9,10]. The advantages of this assay are the low limit of quantitation (LLQ, 0.010 μg/ml), the short run time, the absence of interferences with endogenous material or common concomitant medications. We used this HPLC assay for the analysis and quantitation of paclitaxel in human plasma in a pharmacokinetic study which compared four different treatment schedules as a part of a European–Canadian randomized trial [10]. Putative metabolites (6α-hydroxy-, 3′-*p*-hydroxy and 6α,3′-*p*-dihydroxypaclitaxel) could also be detected in human plasma with this assay. No interference of these metabolites with the chromatographic profile of paclitaxel was found. Pharmacokinetic data of urinary excretion of paclitaxel and metabolites are very limited which may be due to the absence of appropriate and validated assays in this biological matrix. Although several HPLC techniques for paclitaxel analysis in human urine have been described these are not sensitive enough nor have been validated according to current requirements. Furthermore, stability data of paclitaxel in human urine are lacking.

Stability tests of paclitaxel in urine performed in our laboratory showed that samples stored at −20°C for prolonged periods (>2 weeks) are not stable. For population kinetic studies prolonged storage of human urine is desirable.

This report describes two analytical procedures based on SPE and LLE as sample pretreatment for the determination, and quantification of paclitaxel in human urine. The anticipated concentrations in urine vary between 5 and 15 μg/ml depending on the dose. A comparison between the SPE and LLE procedures is presented and the pros and cons of both techniques are discussed.

2. Experimental

2.1. Chemicals and reagents

Paclitaxel (Lot. FB 10110; purity 98.1%) reference for HPLC analysis and the pharmaceutical formulation [Taxol; paclitaxel in Cremophor EL–ethanol (1:1) mixture at a concentration of 6 mg/ml] were obtained from Bristol Myers Squibb Company (Syracuse, NY, USA). Acetonitrile (HPLC-s gradient grade) was obtained from Biosolve (Barneveld, Netherlands). Methanol (ChromAR) was obtained from Promochem (Wesel, Germany). Ethanol, ammonium acetate, glacial acetic acid, *n*-hexane, triethylamine (all analytical grade) and *n*-butylchloride (Lichrosolv) were obtained from

Merck (Darmstadt, Germany). Cremophor EL originated from Sigma (Prague, Czech Republic). Control human urine was collected from healthy volunteers.

2.2. Preparation of stock solutions

Paclitaxel stock solutions were made by dissolving 10 mg of paclitaxel reference material in 2 ml of ethanol. From these stock solutions several dilutions were made with a mixture of acetonitrile–methanol–distilled water (4:5:1, v/v) (AMW) to concentrations ranging from 0.025 to 250 μg/ml. The paclitaxel stock solution was stored at −30°C. Every 3 months a fresh stock solution was made.

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

Standard samples for HPLC analysis and calibration were made by spiking control blank urine with paclitaxel stock solutions in AMW to achieve analyte concentrations ranging from 0.01 to 100 μg/ml. Quality control samples were prepared from a second fresh stock solution at concentrations of 0.05, 0.50 and 5 μg/ml.

2.4. Preparation of standards and quality control samples for liquid–liquid extraction

A 19.0-ml volume of blank human urine was first stabilized with a 1.0-ml mixture of Cremophor EL–ethanol (1:1, w/v). Standard samples were prepared by spiking blank stabilized urine with paclitaxel stock solution in AMW to obtain urine concentrations ranging form 0.25 to 100 μg/ml. Quality control samples were similarly prepared independently at concentrations 5, 50, and 90 μg/ml.

2.5. Sample processing

Standard, quality control, and study samples were processed as a batch. All stabilized and non-stabilized samples (0.5 ml) were buffered with 0.5 ml of 0.2 *M* ammonium acetate buffer (pH 5.0) and vortex-mixed for 20 s before subjection to LLE and SPE, respectively.

Solid-phase extraction

Cyano Bond Elut columns (1 ml, Betron Scientific, Rotterdam, Netherlands) were first conditioned with consecutive washings with 2.0 ml methanol and 0.01 *M* ammonium acetate buffer pH 5.0. Next, 1.0 ml of diluted urine/buffer mixture was applied to the columns. The columns were then washed with 2 ml of 0.01 *M* ammonium acetate pH 5.0, 1 ml of methanol–0.01 *M* ammonium acetate pH 5.0 (2:8, v/v) and 1 ml of hexane. The columns were dried under maximum vacuum for 1 min (15 mmHg). 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.

Liquid–liquid extraction

A 5-ml volume of *n*-butylchloride was added to 1.0 ml of urine/buffer mixture in polypropylene tubes (Eppendorf-Netheler-Hinz, Hamburg, Germany). The tubes were capped and placed in a slow rotator to rotate for 10 min. Thereafter the tubes were centrifuged at 2500 *g* for 10 min. The tubes were then placed in a dry ice–acetone bath until the bottom layer was frozen. The organic layer was decanted into clean polypropylene tubes and weighted. The organic layer was evaporated to dryness under a gentle stream of nitrogen at 40°C.

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

2.6. HPLC instrumentation and conditions

The HPLC system consisted of a Model 510 pump (Waters Assoc., Milford, MA, USA), and a SP 8875 autosampler (Spectra Physics, Santa Clara, CA, USA) with an injection volume of 50

μl. An APEX octyl analytical HPLC column (150 × 4.6 mm I.D.; particle size 5 μm) (Jones Chromatography, Lakewood, CO, USA) was used which was protected with a pre-column (4 × 4 mm I.D.; particle size: 5 μm) packed with 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). Detection was performed with a SP 200 UV-detector (Spectra Physics) operating at 227 nm. A SP 4290 integrator coupled to a WINner data system (Spectra Physics) was used for data processing.

2.7. Validation parameters

Calibration curves

A full validation was completed for both the SPE and LLE in human urine. All urine samples were extracted and analyzed in duplicate. Calibration curves were calculated by weighted ($1/x$) linear regression analysis. For each standard curve seven standard urine samples were taken in duplicate at concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, and 10 μg/ml (0.012, 0.059, 0.12, 0.59, 1.2, 5.9 and 12 μ*M*) for the SPE and eight standard urine samples in duplo at concentrations of 0.25, 0.50, 1.0, 10.0, 40.0, 60.0, 80.0, and 100.0 μg/ml for the LLE.

Lower limit of quantitation

The lower limit of quantitation (LLQ) for both methods was investigated in urine samples from 6 different volunteers. For the concentration to be accepted as the LLQ, the percent deviation of the nominal concentration and the relative standard deviation (R.S.D.) are to be less than 20%.

Recovery

The paclitaxel extraction efficiency was determined by comparing the slopes of human urine calibration curves to standard curves prepared in AMW.

Accuracy and precision

Accuracy and precision were determined using five reference samples with concentrations of 0.05, 0.5 and 5 μg/ml for SPE, and 5, 50 and 90 μg/ml for LLE. The within-day and between-day precision and accuracy were determined in three separate validation runs. The mean observed concentrations and inter-assay precision were determined utilizing one-way ANOVA.

Stability

The stability of paclitaxel in human urine, at a concentration of 10 μg/ml, was determined after storage at −30°C, 8°C and ambient temperature. The dry extract of the processed urine samples after SPE was stored at −30°C. The stability of paclitaxel in Cremophor EL (a polyoxyethylated castor oil–dehydrated alcohol, 1:1, v/w) stabilised spiked urine was determined after storage at −30°C or at −20°C (depending on the Institute).

3. Results and discussion

Several analytical methods for the analysis of paclitaxel in urine have been published. These methods all made use of HPLC assays, though with different sample pretreatment procedures. These procedures include: direct injection of the sample onto the HPLC system [7], LLE [5,6], and LLE followed by SPE [8]. Validation characteristics of these analytical methods, needed to support pharmacokinetic studies, are lacking. The first validated assay for the analysis of paclitaxel in human plasma showed degradation of paclitaxel into 7-epipaclitaxel when the analyte was eluted from the solid-phase extraction cartridge with methanol–water (65:35, v/v) with evaporation of the solvent at room temperature [8]. Stability of the samples, however, was not investigated.

Instability of cytotoxic drugs in biological matrices is a frequently encountered problem. It is well known that paclitaxel undergoes epimerization in aqueous as well as non-aqueous solutions [11,12]. Leslie et al. [13] reported two major peaks, other than paclitaxel, in chromato-

grams of processed samples from mouse plasma, standing in the autosampler waiting for injection. An increased degradation rate was seen in alkaline solutions. The main factor in the degradation of paclitaxel seems to be base catalysis. At pH 9 two degradation products were identified as 7-epipaclitaxel and baccatin III [12]. Willey et al. [9] showed that paclitaxel is stable for at least 68 h in autosampler vials dissolved in the AMW mixture [acetonitrile–methanol–water (4:1:5, v/v)]. No significant degradation occurred in plasma exposed to room temperature (8 h) [9].

Our stability study showed that paclitaxel is stable in urine at ambient temperature and 8°C for at least 7 days (Table 1). However, significant disappearance (>30%) of paclitaxel at a concentration of 5 μg/ml was seen when stored at −20°C for more than 2 weeks. This decrease in recovery might be caused by precipitation (crystallization) of the poor water soluble drug during storage and/or chemical degradation, or possibly by adsorption onto the container wall. However, in the chromatograms no additional peaks were identified that would support the hypothesis of chemical degradation. We investigated the influence of paclitaxel adhesion to glass and polypropylene tubes. For this purpose quality control samples (50 μg/ml) for the LLE were prepared in both glass tubes ($n = 5$) and polypropylene tubes ($n = 3$). The mean paclitaxel concentrations found after sample processing were 53 μg/ml with a coefficient of variation (C.V.) of 4.4% and 54 μg/ml (C.V. 1.8%) for both glass and polypropylene tubes, respectively. This indicates that no substantial interaction between these two materials and paclitaxel occurs.

Table 2
Chemical stability of paclitaxel in stabilised human urine [5% Cremophor EL–dehydrated alcohol, (1:1, w/v)] stored for 17 months at −20°C ($n = 4$)

Initial concentration (μg/ml)	Mean measured concentration (μg/ml)	C.V. (%)	Deviation[a] (%)
1.96	2.07	1.9	−5.3
41.97	41.41	3.9	1.6
82.98	82.82	3.5	0.2

[a] % Deviation = [(nominal concentration − measured concentration)/(nominal concentration)] · 100.

Therefore alternative routes were investigated to prolong the physical stability of paclitaxel in urine and to prevent the potential formation of insoluble microcrystals. The first approach was to stabilise paclitaxel urine samples with Cremophor EL–dehydrated alcohol. Urine samples were then stable for more than 17 months (concentrations tested were between 2 and 80 μg/ml) (Table 2). The second approach was to process samples within 7 days after sampling by SPE and to obtain a dry extract (Table 3). This extract was stable for at least 4 months when stored at the temperature of −30°C.

We developed and validated two analytical methods based on SPE and LLE sample pretreatment procedures as stabilised urine could be

Table 1
Stability of paclitaxel in urine (initial concentration 10 μg/ml) stored at 8°C and at ambient temperature ($n = 5$)

Time (days)	Temperature			
	8°C		Ambient	
	Paclitaxel concentration (%)	C.V. (%)	Paclitaxel concentration (%)	C.V. (%)
0	100.0	2.2	100.0	2.2
2	98.0	4.3	97.0	4.1
4	102.0	3.0	98.0	3.0
7	100.0	2.7	102.0	5.2

Table 3
Stability of paclitaxel dry extract after solid-phase extraction of spiked urine samples (initial concentration 10 μg/ml) stored at −30°C ($n = 5$)

Time (weeks)	Paclitaxel concentration (%)	C.V. %
0	100.0	0.7
1	101.0	0.6
2	94.0	0.4
4	99.0	0.3
12	102.0	1.9

analyzed after LLE but not after SPE, which was, however, adequate for the analysis of non-stabilised urine samples.

LLE of paclitaxel from human urine stabilized with a mixture of Cremophor EL–dehydrated alcohol, has been tested using several organic fluids (*n*-butylchloride, diethyl ether, isopropylether, hexane, ethylacetate, ethylacetate–ether). Several problems were encountered with these organic solvents. After slow rotating of urine mixed with hexane an emulsion occurred, and no liquid layer could be decanted. Chromatographic analysis of both ether and isopropyl ether extracts showed late and broad eluting peaks (retention time ± 70 min), while multiple peaks interfered with paclitaxel after extraction with ethylacetate. High recoveries, however, were found for the extraction with *n*-butylchloride (HPLC grade), with only minor accompanying peaks seen in the chromatograms compared with the chromatograms obtained after LLE with the other organic solvents. *n*-Butylchloride (LiChrosolv) was, therefore, most suitable for LLE of paclitaxel from human urine. Fig. 2 shows typical HPLC chromatograms. Blank chromatograms after LLE sample pretreatment from 10 healthy volunteers showed late eluting peaks with retention times $t = 20$, $t = 32$ and $t = 38$ min. Therefore a relatively long run time (45 min) is needed after LLE as sample pretreatment. 2-Methyl taxol was available as internal standard for LLE, however impurities interfered with the paclitaxel peak. No other suitable internal standards were available at that time.

The LLQ for the LLE was defined at a concentration of 0.25 μg/ml. Accuracy and within-day precision for the LLE were 100.0–100.4% and 3.8–7.3%, respectively (Table 4). Linearity for the LLE was observed over the range 0.25–100 μg/ml (Table 5).

Using this HPLC assay combined with SPE as sample pretreatment for the detection of paclitaxel in human plasma several metabolites could be detected [9,10]. Modifying this assay we also hoped to find and to identify metabolites in human urine, but these could, however, not be detected under the circumstances used.

The detection limit for the SPE was 0.008 μg/ml using a 500-μl urine sample with a 50-μl injection onto the HPLC column. The LLQ was 0.01 μg/ml also using a 500-μl sample with a 50-μl injection. The R.S.D and DEV for the SPE were 103–114% and 1.1–4.0%, respectively (Table 4). The assay was linear over the concentration range 0.0125–25 μg/ml (Table 5). The chromatograms showed no interference of endogenous urine substances with the SPE (Fig. 3).

However, several problems arose during the validation of this assay. (1) The reproducibility by different analysts was low. (2) Paclitaxel was not stable when stored at −30°C for prolonged periods. Procedures were, therefore, investigated to prolong the stability during storage of urine of patients treated with paclitaxel. Sample processing within 7 days after sampling to a dry extract which can be stored at −30°C and stabilisation of the samples with a mixture of Cremophor EL–dehydrated alcohol appeared adequate. The dry extract seems to be stable for more than 4 months provided it is prepared within 7 days after sampling. Stabilisation of urine with paclitaxel with the Cremophor El–dehydrated alcohol mixture was very effective for a period of at least 17 months. Stabilised urine samples could not be processed with SPE, and thus an LLE procedure was developed and validated. Correlations coefficients of 0.995 or better for both analytical procedures were obtained throughout the validation.

Results from the validation show that both methods are accurate and precise. Accuracy,

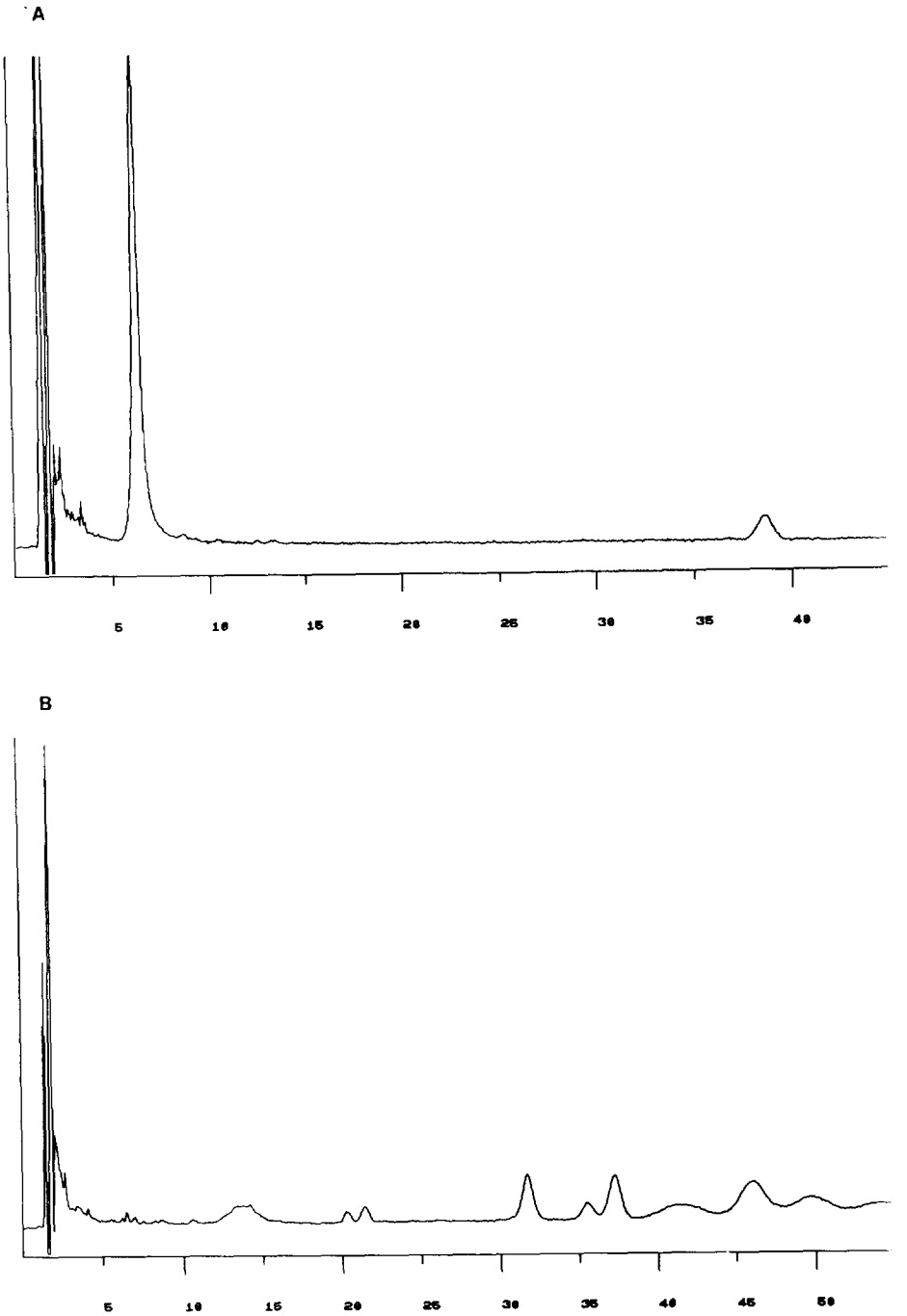

Fig. 2. (A) Chromatogram of a blank non-stabilised urine after SPE. (B) Chromatogram of a blank stabilised urine after LLE.

between-day and within-day precision for both extraction procedures are shown in Table 4. The within-day precision was 1.1–4.0% for the SPE and 3.8–7.3% for the LLE. The between-day variation (0.7–7.5%) gave similar results for both sample pretreatment procedures.

Table 4
HPLC validation characteristics ($n = 5$)

Nominal concentration (μg/ml)	Mean measured concentration (μg/ml)	Accuracy (%)	Precision (%)	
			Within-day	Between-day
Solid-phase extraction				
0.050	0.057	114.0	4.0	6.3
0.500	0.485	103.0	1.1	3.5
5.000	5.246	104.9	1.7	1.9
Liquid–liquid extraction				
5.00	5.02	100.4	7.3	1.0
50.00	49.98	100.0	5.0	7.5
90.00	89.68	100.4	3.8	0.7

With both analytical methods it was shown that the urinary excretion of paclitaxel in patients remains lower than 10% of the administered dose. In clinical samples no metabolic products could be determined in urine with both SPE and LLE.

4. Conclusions

Two methods for the analysis of paclitaxel in urine have been developed and validated. Both methods are accurate, precise and allow quantitation of paclitaxel concentrations of 0.01–50 μg/ml and 0.25–100 μg/ml for SPE and LLE, respectively.

Paclitaxel in human urine is not stable. A mixture of 5% Cremophor EL–dehydrated alcohol added to human urine containing paclitaxel is sufficient to stabilise the analyte.

Stabilised samples must be pretreated with LLE prior to HPLC analysis. Non-stabilised samples can be extracted by SPE but need to be processed within 7 days after sampling. The advantage of SPE is the high sensitivity of this sample pretreatment procedure. However, no SPE can be performed when human urine is stabilised with the Cremophor EL–dehydrated alcohol mixture.

The LLE is a suitable extraction procedure of paclitaxel in human urine when stabilised with Cremophor EL–dehydrated alcohol. The disad-

Table 5
Characteristics of paclitaxel urine standard curves

Validation	Slope	Intercept	Correlation coefficient
Solid-phase extraction			
SPE-1	230730	−437	1.000
SPE-2	226528	−3583	0.999
SPE-3	199110	−2058	0.998
Liquid–liquid extraction			
LLE-1	88308.7	−1854.89	0.998
LLE-2	88327.7	5081.92	0.997
LLE-3	89574.7	9152.89	0.996

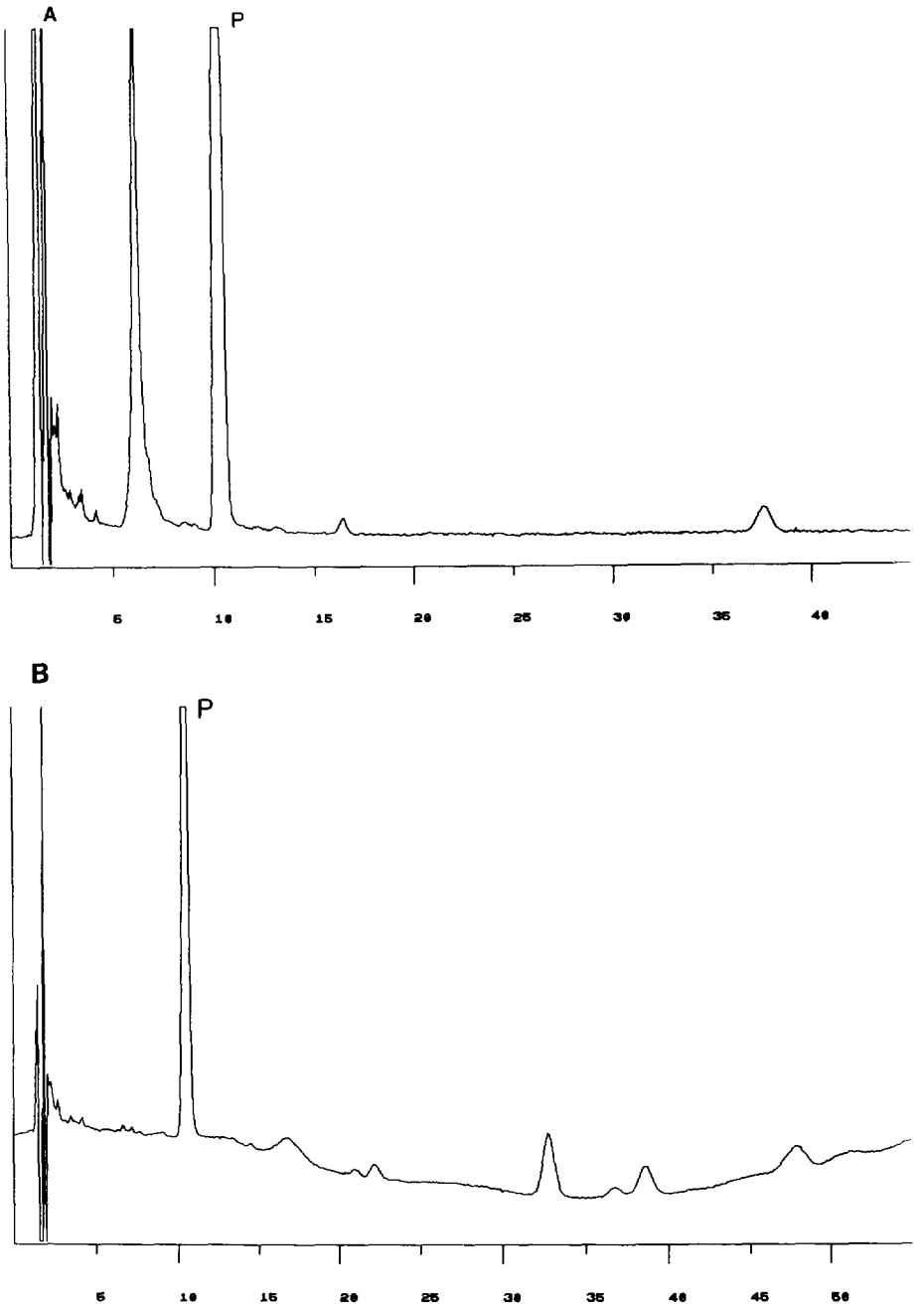

Fig. 3. (A) Chromatogram of a non-stabilised urine sample spiked with 10 μg/ml paclitaxel (P) after SPE. (B) Chromatogram of a stabilised urine sample spiked with 10 μg/ml paclitaxel (P) after LLE.

vantages of this method are the relatively long run time and the lower sensitivity.

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