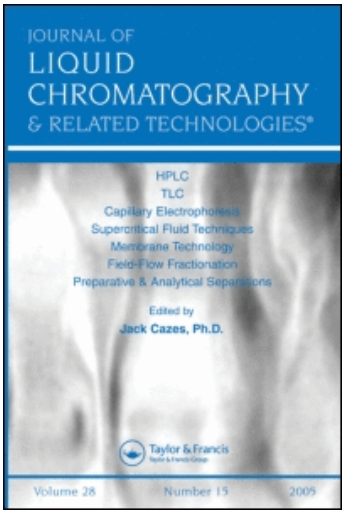

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF A NOVEL POLYMER-BOUND PACLITAXEL DERIVATIVE AND FREE PACLITAXEL IN HUMAN PLASMA

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ABSTRACT

A high-performance liquid chromatographic (HPLC) assay has been designed for the quantitative determination of polymer-bound paclitaxel (after hydrolytic release of paclitaxel from the polymer) and free paclitaxel in human plasma after intravenous administration of the antitumor polymer-drug conjugate PNU166945.

Chemical stabilization with 0.2 M ammonium acetate and solid-phase extraction (SPE) were required as sample pretreatment prior to the HPLC analysis. Separation was performed on an APEX Octyl analytical column and a mobile phase of acetonitrile-methanol-0.02 M ammonium acetate buffer pH 5.0 (4:1:5, v/v/v) and paclitaxel was detected at 227 nm. Total paclitaxel (polymer-bound pus free) levels were determined after chemical hydrolysis of the clinical samples with a mixture of 0.1 M KH_2PO_4 and methanol (1:1 v/v, pH 7.5) during 48 hours at room temperature. Concentrations of polymer-bound paclitaxel were calculated by subtraction of free from total drug levels.

Plasma samples containing paclitaxel were stable for at least 10 months and hydrolyzed plasma samples were stable for at least 3 months at -30°C. Within-run and between-run precisions were less than 10.9% and the accuracy of the assay ranged from 94-102%. The limit of quantification for paclitaxel in plasma was established at 10 ng/mL using a 500 µL sample volume. The presented method was successfully applied in a clinical pharmacokinetic study in our Institute.

INTRODUCTION

Polymer-drug conjugates comprise a class of potential drug delivery systems which are increasingly investigated in order to modulate the pharmacokinetics and consequently, the pharmacodynamics of anti-cancer drugs. Important advantages of these delivery systems are improved aqueous drug solubility, selective tumor targeting, and in some cases reduced toxicity.[1,2] Soluble carriers thus may have the ability to increase the therapeutic index of conventional anti-cancer drugs. Paclitaxel (Taxol®) belongs to a group of antineoplastic agents, the taxanes, that exert their cytotoxic action by promoting tubulin polymerization, causing massive stable microtubuli.[3] Activity has been reported in a variety of human tumors and in most countries, paclitaxel has been registered as first-line treatment in combination with cisplatin for ovarian cancer and as second-line treatment for anthracycline-pretreated breast cancer.[4]

Intravenous administration of paclitaxel, however, is associated with multiple side-effects related to its pharmaceutical formulation.[5] As paclitaxel is poorly soluble in aqueous fluids, a mixture of Cremophor EL and ethanol (1:1 v/v) is used to dissolve the drug. It is now well established that this formulation contributes largely to the hypersensitivity reactions observed after paclitaxel administration.[6,7] A new water-soluble polymeric derivative of paclitaxel (PNU166945, Figure 1) was developed with the intention of reducing toxicity and potentially improving activity, and without the use of the Cremophor EL vehicle. When tested against a murine lung carcinoma and in a murine reticu-

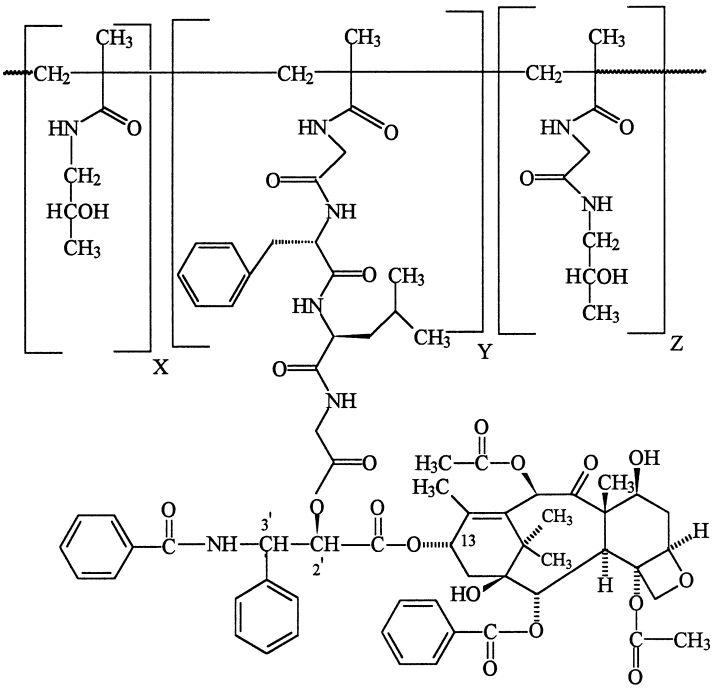


Figure 1. Structural formula of PNU166945.

losarcoma model, this compound showed increased antitumor activity in comparison with paclitaxel.[8] PNU166945 is a N-(2-hydroxypropyl) methacrylamide copolymer linked via a tetrapeptidyl amino-spacer to paclitaxel (Figure 1). After enzymatic cleavage of the esteric bond between the aminoacid chain and paclitaxel, free paclitaxel is released into the blood stream. To study the pharmacokinetics of PNU166945, polymer-bound drug as well as free drug should be considered.

Fraier et al developed a selective HPLC assay for the determination of polymer-bound paclitaxel, free paclitaxel and 7-epipaclitaxel in dog plasma and urine.[9] In this report, a selective HPLC method to quantify the levels of free and total (free plus polymer-bound) paclitaxel in human plasma is described, which is useful for clinical pharmacokinetic research with PNU166945.

Much attention has been paid to the stability of the analytes. The presented method has been applied successfully in a clinical phase I study with the drug in our hospital.

EXPERIMENTAL

Chemicals

PNU166945 (total paclitaxel 5.2%, batch no. 6001A335 and OF11361/68) was provided by Pharmacia & Upjohn (Milan, Italy). Paclitaxel (purity 98.1 %, Lot 124) and 2'-methylpaclitaxel (methylpaclitaxel) (purity 95%, Lot 002) were obtained from Bristol Myers Squibb Company (Syracuse, NY, USA). Methanol (ChromAR) was obtained from Promochem (Wesel, Germany). Acetonitrile (Gradient grade) was purchased from Biosolve (Barneveld, Netherlands). Merck (Darmstadt, Germany) provided glacial acetic acid (100% pure), *N*-hexane (extra pure), ammonium acetate, triethylamine, monobasic potassium phosphate (all analytical grade). Esterase (20,000 u/1060 mg, charge no. 64H7200) was obtained from Sigma Aldrich Chemie (Zwijndrecht, The Netherlands). Control human plasma was supplied by the Central Laboratory for Blood Transfusions (Amsterdam, the Netherlands).

Preparation of Stock Solutions and Reagents

Stock solutions of paclitaxel and methylpaclitaxel of 5.0 and 1.0 mg/mL, respectively, were prepared by dissolving accurately weighed amounts of each compound in methanol. A stock solution of PNU166945 (0.1 mg bound paclitaxel per mL) was prepared by dissolving 96.15 mg PNU166945 (5.2%, w/w bound paclitaxel) in 50.0 mL of human plasma. Stock solutions were stored at -30°C and fresh solutions were prepared every 6 months. A paclitaxel plasma standard of 100 μg/mL was obtained by mixing 100 μL of paclitaxel stock solution (5.0 mg/mL) with 4900 μL of control human plasma and a paclitaxel plasma standard of 10 μg/mL was prepared by mixing 200 μL of paclitaxel plasma standard 100 μg/mL with 1800 μL control human plasma. These solutions were freshly prepared prior to each calibration. A reconstitution solvent (AMW) was prepared by mixing 40 mL of acetonitrile with 10 mL of methanol and 50 mL of distilled water. The solution was stored at 4°C and a fresh solution was prepared every 3 months. A recovery standard of 250 μg/mL was obtained by mixing 100 μL of paclitaxel stock solution with 1900 μL of AMW. Recovery standards were freshly prepared for every calibration. An internal standard (IS) working solution (10 μg/mL) was prepared by dilution of the methylpaclitaxel stock solution (1.0 mg/mL) 100-fold in methanol. An IS working solution (200 μg/mL) was prepared by dilution of 5.00 mL methylpaclitaxel stock solution (1.0 mg/mL) in 25 mL methanol. The working solutions were stored at -30°C and freshly prepared every 6 months. An ammonium acetate solution 1.0 M was prepared by dissolving 19.27 g of ammonium acetate in 225 mL of distilled water and subsequently, the pH was adjusted to 5.0 with glacial acetic acid 100%.

Distilled water was further added to a final volume of 250 mL. Ammonium acetate solutions 0.2 M and 0.01 M were prepared by mixing 20

and 10 mL of ammonium acetate 1.0 M with 80 and 990 mL of distilled water, respectively. Storage was at 4°C and fresh solutions were prepared every 3 months. An 'hydrolysis mixture' was obtained by dissolving 1.36 g KH_2PO_4 in 100 mL of distilled water and with subsequently adding 100 mL methanol. The pH was adjusted to 7.5 with 4 N sodium hydroxide. The solution was stored at 4°C and freshly prepared every 3 months. Methanol-0.01 M ammonium acetate (2:8 v/v, pH 5) was prepared by mixing 400 mL of 0.01 M ammonium acetate pH 5 with 100 mL of methanol and acetonitrile-triethylamine (1000:1 v/v) was prepared by dilution of 1.0 mL of triethylamine in 1000 mL of acetonitrile. Both solutions were stored at 4°C and freshly prepared every 3 months.

Preparation of Calibration Standards, Quality Control Samples and Recovery Standards

Calibration standards of paclitaxel in plasma were prepared by spiking blank human plasma samples with aliquots of paclitaxel plasma standard (100 µg/mL) to final paclitaxel concentrations ranging from 10 to 10,000 ng/mL. Calibration standards of PNU166945 were prepared by diluting aliquots of PNU166945 stock solution (0.1 mg/mL) in plasma producing concentrations from 10 - 1,000 ng/mL and 1,000 - 100,000 ng/mL. Aliquots of 500 µL of the diluted plasma samples were stored at -30°C until analysis. Quality control samples of paclitaxel were prepared by spiking human plasma samples with paclitaxel plasma standard (100 µg/mL) to achieve nominal concentrations around 50, 500 and 5,000 ng/mL. Quality control samples of PNU166945 were prepared by spiking human plasma samples with PNU166945 stock solution (separately prepared, 0.1 mg bound paclitaxel per mL) to final concentrations of 50, 250, 750, 5000, 25,000, and 75,000 ng/mL.

For the preparation of recovery standards, the paclitaxel recovery standard (250 µg/mL) was further diluted to achieve nominal concentrations ranging from 25 - 25,000 ng paclitaxel/mL. The recovery of the internal standard methylpaclitaxel was determined by dilution of 50 µL of the internal standard working solution (10 µg/mL) with 350 µL of the paclitaxel recovery standard (250 µg/mL).

Optimization of the Analytical Method

Sample Pretreatment for Free Paclitaxel Analysis

Sample pretreatment involved a solid-phase extraction of free paclitaxel from 500 µl of human plasma, stabilized with an equal volume of 0.2 M ammonium acetate pH 5. Preceding the solid phase extraction, 25 µL of IS 10,000 ng/mL (500 ng internal standard per mL plasma) was added to 1 mL samples. The samples were centrifuged for 3 minutes at approximately 9500 g. Cyano

Bond Elut columns 1 mL (Varian) were first conditioned with, consecutively, 2 mL of methanol and 2 mL of 0.01 M ammonium acetate pH 5 before use. The plasma/buffer mixture was then transferred to the columns. The columns were subsequently washed with 2 mL of 0.01 M ammonium acetate pH 5, 1 mL of methanol-0.01 M ammonium acetate pH 5 (2:8 v/v) and 1 mL of *N*-hexane. Thereafter, the columns were dried under maximum vacuum (15 mm Hg) for 1 min. Elution of the analytes was performed with 2 mL of acetonitrile-triethylamine (1000:1 v/v) and the elution solvent was evaporated to dryness under a gentle stream of nitrogen at 40°C.

Sample Pretreatment for Total Paclitaxel Analysis

Determination of total paclitaxel levels was performed after release of bound paclitaxel from the polymeric carrier by chemical hydrolysis with a mixture of 0.1 M KH_2PO_4 and methanol (1:1 v/v, pH 7.5) at room temperatures for 48 hours. After addition of the internal standard methylpaclitaxel, the samples were centrifuged for 3 minutes at approximately 9500 g. A solid phase extraction was performed according to the above described procedure. Concentrations of polymer-bound paclitaxel were calculated by subtraction of free from total paclitaxel concentrations.

Handling of Clinical Samples

Whole blood samples (10 mL) were collected from patients in heparinized tubes at 14 time points up to 48 hours after PNU166945 administration. The samples were chilled in an ice bath (0°C) immediately after withdrawal and subsequently centrifuged at 0-4°C for 10 minutes. The obtained plasma was divided in aliquots of 0.5 mL each. One aliquot (500 µL) was immediately frozen in a CO_2-ethanol bath (-70°) and another aliquot was first stabilized with 0.5 mL of 0.2 M ammonium acetate before it was frozen in the CO_2-ethanol bath. All samples were stored at -70°C until analysis.

Stability

The release of paclitaxel from its polymeric carrier in plasma was studied during 30 hours at room temperature without stabilization and with addition of the stabilizing buffer reagents ammonium acetate (0.2 M) and monobasic potassium phosphate (0.5 M). Plasma samples were spiked with 500 ng/mL paclitaxel attached to PNU166945 and were subsequently diluted 1:1 v/v with buffer. Samples were analyzed in duplicate at t=0 and after 6, 23, and 30 hours. Plasma samples spiked with 500 ng/mL free paclitaxel were used as calibration standards.

To investigate the rate and extent of paclitaxel release from the polymeric carrier at 37°C, control human plasma was spiked with 500 ng/mL paclitaxel attached to the polymer. Aliquots of plasma for analysis of free paclitaxel were removed at t=0 and after 1, 2, 4, 6, and 24 hours. Control human plasma spiked

with 500 ng/mL paclitaxel was used as calibration standard for the quantification of paclitaxel and 7-epipaclitaxel.

The experiments were repeated at room temperature (22°C) with control human plasma. Plasma was diluted 1:1 v/v with the hydrolysis mixture (0.1 M KH_2PO_4/methanol, 1:1 v/v, pH 7.5) and kept at room temperature for 54 hours. Samples were taken at t=0, and after 3, 6, 22, 30, 46, and 54 hours.

The influence of esterase addition to plasma on the rate and extent of paclitaxel release at room temperature was evaluated after spiking control human plasma containing PNU166945 (500 ng bound paclitaxel/mL) and 10 units esterase per mL. Aliquots of plasma were removed, diluted 1:1 v/v with 0.2 M ammonium acetate pH 5 and subsequently analyzed. Samples were taken at t=0, and after 1, 2, 4, and 6 hours.

Comparison of Cyano Solid Phase Extraction Columns

Varian (Bond Elut, non-endcapped, 1210-2008, batch number 132217, Harbor City, CA, USA), IST (Isolute, endcapped, 421-0010-A, batch number 5247501 AA, Hengoed, UK), and Baker (Bakerbond, 7021-01, batch number F15501, J. T. Baker, Philipsburg, NJ, USA) cyano SPE-columns (100 mg) were tested to study the influence on the recovery of paclitaxel extracted from plasma at a concentration of 500 ng/mL.

High Performance Liquid Chromatography

The chromatographic system comprised a Model 510 pump (Waters Ass., Milford, MA, USA) and an automatic sample injection device Model SP 8880 (Thermo Separation Products, Fremont, CA, USA) with an injection volume of 50 µL. An APEX Octyl analytical column (150 mm x 4.6 I.D., particle size 5 µm) (Jones Chromatography, Littleton, CO, USA) was used, which was protected by a guard column (3 x 10 mm) (Chrompack, Middelburg, The Netherlands) packed with reversed-phase material. Separation of paclitaxel was obtained with a mobile phase composed of acetonitrile-methanol-0.02 M ammonium acetate buffer pH 5.0 (4:1:5, v/v/v).

The mixture was degassed with helium during 15 minutes before use. The flow-rate was 1.0 mL/min and the detection was performed with a Spectra 200 UV-detector (Thermo Separation Products), which operated at a wavelength of 227 nm. Retention times and peak areas were measured with a Datajet integrator coupled to a WINner data system (Thermo Separation Products).

Validation of the Method

A three run validation was completed for the determination of paclitaxel in human plasma consisting of the following experiments: linearity; within-run

and between-run precision; bias; separation from endogenous plasma constituents. Calibration standards ranging from 10 - 10,000 ng/mL for paclitaxel and ranging from 10 - 1,000 and 1,000 - 100,000 for PNU166945 were analyzed in duplicate. Weighted linear regression analysis (reciprocal of the concentration) was used to calculate the calibration curves. The *F* test for lack of fit (α=0.05) was used to evaluate the linearity of the calibration curve.

Precision and accuracy of the method were evaluated in three separate validation runs, using three quality control samples of paclitaxel and two sets of quality control samples of PNU166945 in the lower, mid and higher part of each concentration range. An estimate of the precision was obtained by one-way analysis of variance (ANOVA). The bias was calculated as the percentage deviation of the nominal concentration.

Six batches of control human plasma were processed and analyzed to determine whether endogenous plasma constituents co-eluted with paclitaxel. Potential interference of the co-medication with the analytical method was investigated by addition of the tested drugs at therapeutic relevant levels to control human plasma. The tested drugs were: paracetamol (20 µg/mL), metoclopramide (0.2 µg/mL), tramadol (1 µg/mL), magnesium oxide (20 µg/mL), temazepam (0.8 µg/mL), oxazepam (0.2 µg/mL), codeine (0.2 µg/mL), and digoxin (2.5 ng/mL). The overall paclitaxel extraction efficiency was determined by comparing the slope of the processed human calibration curve to a standard curve prepared in reconstitution solvent.

Stability

A total of six experiments were performed to evaluate the stability of paclitaxel during handling and storage of the samples.

The stability of the paclitaxel stock solution in methanol at -30°C was studied at concentration of 5.0 mg/mL during 6 months. The samples were analyzed before storage and after a storage period of 6 months.

The stability of paclitaxel in plasma was evaluated at concentrations of 560 ng/mL and 980 ng/mL at -30° during 10 months. The samples were analyzed in duplicate.

The stability of paclitaxel in reconstitution solvent after plasma extraction at room temperature was studied at a concentration of 750 ng/mL during 68 hours. Four samples were analyzed at t=0 and after 6, 12, 24, 48, and 68 hours.

The release of paclitaxel from the polymer in plasma samples during handling of blood samples in the clinic was evaluated by mimicking conditions after blood collection. A 50 µL volume of stock solution of PNU166945

(1 mg/mL) was added to a 5.0 mL sample of fresh control human blood of 37°C, to achieve a concentration of 10,000 ng/mL paclitaxel attached to the polymer. Subsequently, the blood sample was chilled on ice-water for 10 minutes and then centrifuged at 2500 g (4°C) during 10 minutes. After separation, plasma was diluted (1:1 v/v) with 0.2 M ammonium acetate pH 5 and the samples were immediately frozen in a CO_2-ethanol bath. The samples were thawed and the internal standard was added prior to the solid phase extraction. The samples were processed (see *sample pretreatment* section) and subsequently analyzed together with calibration standards. To determine the amount of free paclitaxel in a stock solution of PNU166945 (separately prepared, 1 mg polymer-bound paclitaxel/mL in water), 50 µL of stock solution was injected onto the HPLC column together with recovery standards. The following formula was used to calculate the release of paclitaxel from PNU166945 after blood collection and stabilization:

$$\frac{\text{free paclitaxel concentration in plasma}}{(\text{bound paclitaxel concentration in blood})\,(\text{hematocrit ratio})} \times 100\%$$

Paclitaxel release from PNU166945 in plasma samples during storage was studied at concentrations of 10,000, 50,000, and 100,000 ng/mL PNU166945 during 5.5 months at -30°C and -70°C. The stability of paclitaxel in a mixture of plasma and 0.1 M KH_2PO_4/methanol (1:1 v/v) after hydrolysis from PNU166945 was studied at -30°C. Control human plasma was diluted 1:1 v/v with the hydrolysis mixture (0.1 M KH_2PO_4/methanol, 1:1 v/v, pH 7.5) to release bound paclitaxel (500 ng/mL) and the sample was allowed to react at room temperature for 48 hours. Aliquots of 1 mL were immediately analyzed, while the remaining plasma mixture was stored at -30°C and analyzed after 3 weeks.

Case Study

The presented method was used to support a phase I and pharmacokinetic study of PNU166945. Patients were treated with a 1-hour intravenous infusion of PNU166945 at a starting dose of 80 mg/m^2 every three weeks and doses were escalated in subsequent patient cohorts until dose limiting toxicities were observed. Whole blood samples of a patient treated with a 1-hour infusion of PNU166945 at a dose of 196 mg/m^2 were withdrawn at 11 time points up to 24 hours after administration: before start of the infusion, at 30, 60, 75, 90, and 105 minutes, and 2, 3, 5, 8, 12, and 24 hours after start of the infusion. The samples were processed as described under *Handling of Clinical Samples* and subsequently analyzed according to the presented method. Non-compartmental methods were applied to calculate the pharmacokinetic parameters of total and free paclitaxel. The Area Under the plasma concentration-time Curve (AUC) was determined using the linear trapezoidal method without extrapolation to infinity.

The maximum plasma concentration (C_{max}) was derived from the experimental data. The elimination rate constant (k) was calculated by linear regression analysis of the logarithmic plasma concentration-time curve. The total body clearance (Cl), the elimination half-life (t½) and the volume of distribution at steady state (Vss) were calculated using standard equations.[10]

RESULTS AND DISCUSSION

Optimization of the Analytical Method

For the determination of free paclitaxel levels, plasma samples were diluted with 0.2 M ammonium acetate pH 0.5 as a stabilizing agent prior to storage, to ensure a minimum of in vitro release.

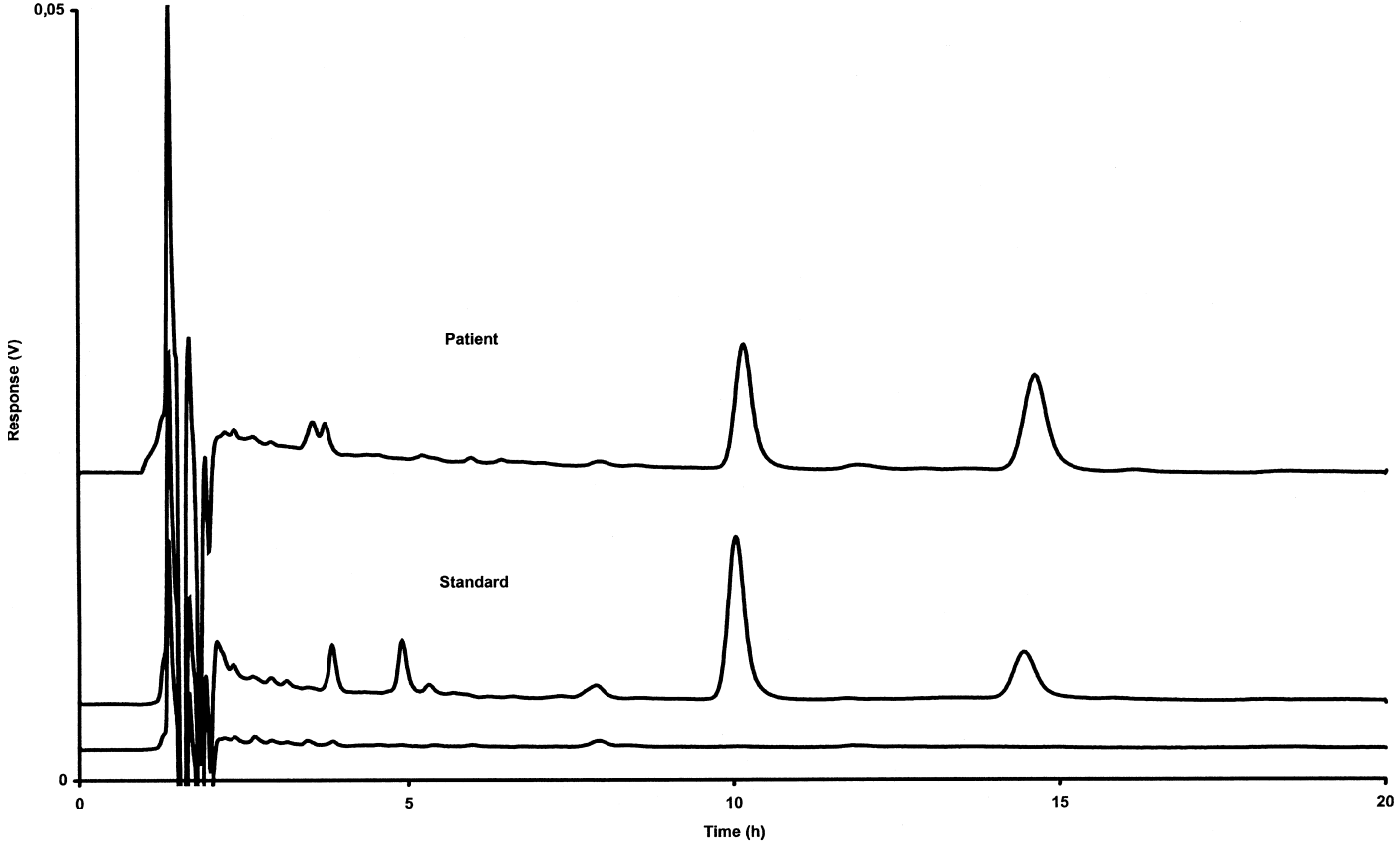
Determination of free and polymer-bound paclitaxel was best accomplished by reversed phase HPLC preceded by a solid-phase extraction using CN SPE columns from Varian, since the chromatograms of blank human plasma samples of the other tested columns contained interfering peaks. Representative chromatograms obtained for free and total paclitaxel from spiked human plasma and from a patient treated with PNU166945 at a dose of 196 mg/m^2 are shown in Figure 2 and 3, respectively.

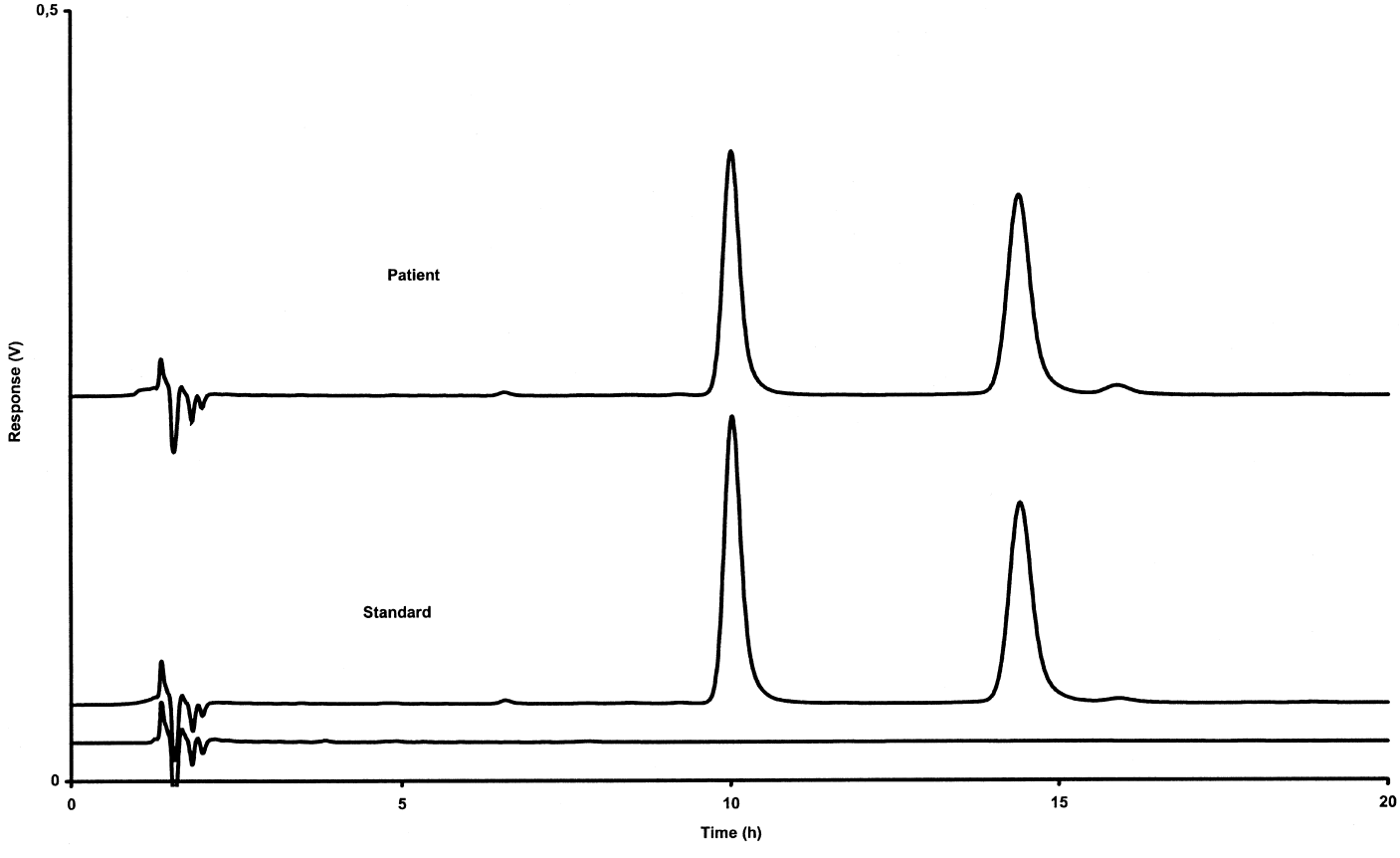
Stability

Concentrations of paclitaxel released from the polymer measured in plasma samples with and without stabilization are presented in Figure 4. After 30 hours at room temperature the free paclitaxel concentration in non-stabilized plasma samples was 53.90 ± 0.10% of the total paclitaxel concentration. After stabilization with 0.5 M monobasic potassium phosphate pH 4.2, 7.83 ± 0.46% paclitaxel was hydrolyzed from the polymer.

Using 0.2 M ammonium acetate pH 5 as stabilizing buffer, the release was 6.09 ± 0.03%. The *t* test for independent samples (α=0.05) indicated that 0.2 M ammonium acetate pH 5 should be used as stabilizing agent ($p < 0.05$).

Figure 2. HPLC chromatograms for the analysis of free paclitaxel. The lower limit of quantification was 10 ng/mL. The lower line represents a human control plasma sample, the upper line a plasma sample from a patient treated with PNU166945 196 mg/m^2 8 hours post infusion, 355 ng/mL, and the middle line a spiked plasma calibration sample, 1000 ng/mL.





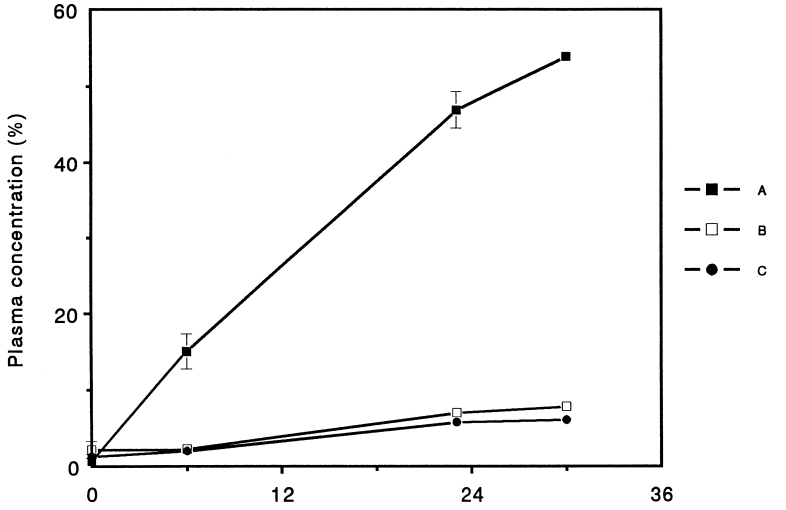


Figure 4. Release of paclitaxel from PNU166945 in plasma samples without stabilization (A), in plasma samples stabilized with 0.5 M monobasic potassium phosphate (B), and with 0.2 M ammonium acetate (C). Plasma concentration of free paclitaxel represented as % of the total paclitaxel plasma concentration.

Release of paclitaxel from PNU166945 in human plasma at 37°C has been evaluated in vitro and after 24 hours 71.15 ± 3.23% paclitaxel was released (Figure 5). Considerable amounts of 7-epipaclitaxel were found (25.29 ± 1.03%). After 48 hours at room temperature (22°C), release of paclitaxel in human plasma diluted 1:1 v/v with 0.1 M KH_2PO_4/methanol (1:1 v/v, pH 7.5) was approximately 92% (Figure 6). The amounts of 7-epipaclitaxel were less than 3% under these conditions. The latter procedure was therefore used to release bound paclitaxel from the polymeric carrier.

The addition of esterase did not influence the rate and extent of the paclitaxel release, which was 8.7 ± 0.4%, compared to 7.9 ± 0.1% in plasma containing esterase.

Figure 3. HPLC chromatograms for the analysis of total paclitaxel. The lower limit of quantification was 10 ng/mL. The lower line represents a human control plasma sample, the upper line a plasma sample from a patient treated with PNU166945 196 mg/m^2 12 hours post infusion, 8,884 ng/mL, and the middle line a spiked plasma calibration sample, 10,000 ng/mL.

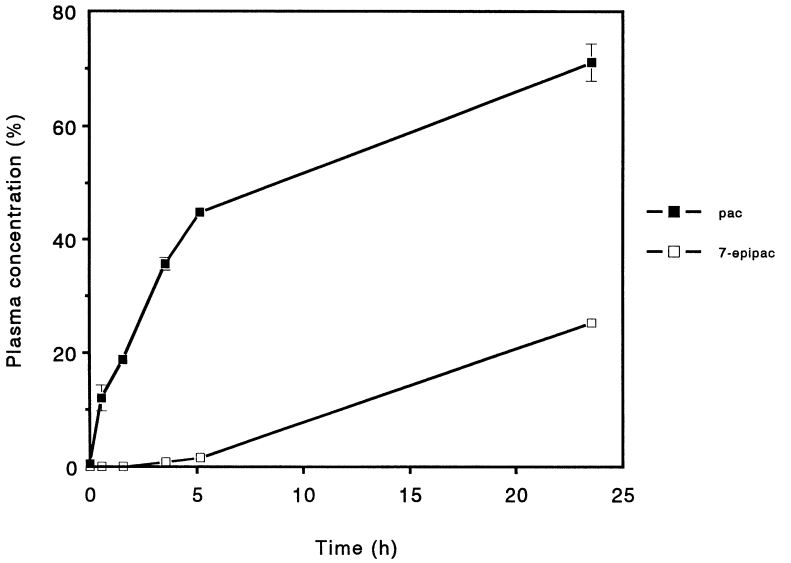


Figure 5. In vitro release of paclitaxel from PNU166945 (500 ng/mL) in human plasma at 37°C. Pac = paclitaxel, 7-epipac = 7-epipaclitaxel. Plasma concentration of free paclitaxel represented as % of the total paclitaxel plasma concentration.

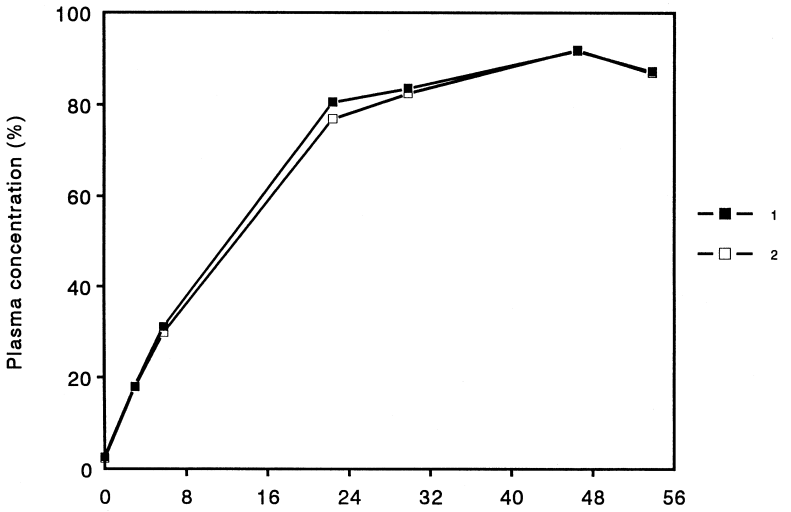


Figure 6. In vitro release of paclitaxel from PNU166945 (500 ng/mL) at room temperature (22°C) in plasma diluted with the hydrolysis mixture. 1 = control human plasma 1, 2 = control human plasma 2. Plasma concentration of free paclitaxel represented as % of the total paclitaxel plasma concentration.

Validation

Validation parameters in terms of linearity, accuracy and precision are outlined in Table 1. The assay for the determination of paclitaxel in human plasma was linear over a concentration range of 10 - 10,000 ng/mL as determined by the *F* test for lack of fit (α=0.05). For the determination of total paclitaxel, the assay was linear over the concentration ranges of 10 - 1,000 ng/mL and 1,000 - 10,000 ng/mL. Correlation coefficients (r) of 0.999 or better were obtained. For every calibration curve the calibration concentrations were back-calculated from the ratio of the peak areas from paclitaxel and methylpaclitaxel. The deviations of the nominal concentration for all concentrations were less than 15%.

The within-run precisians for the determination of free paclitaxel at 50, 500 and 5,000 ng/mL were 3.4, 1.7, and 1.1% respectively. The between-run precisions at 50, 500, and 5,000 ng/mL were less than 15% for all quality control samples. The within- and between-run precisions for the determination of total paclitaxel were less than 10% for all quality control samples. The accuracies were within ± 10%. The blinded evaluation samples originating from another laboratory were all measured within 95 and 105% of the nominal concentration.

For free paclitaxel, the mean (n=3) extraction efficiency of paclitaxel from human control plasma was 85.2 ± 3.4%, and 94.9 ± 3.0% for the internal stan-

Table 1

Accuracy and Precision of the Method for the Determination of Free and Total Paclitaxel

Nominal plasma conc. (ng/mL)	Number of replicates	Mean measured conc. ± SD (ng/mL)	Accuracy (%)	Between-run precision (%)	Within-run precision (%)
Free paclitaxel					
50	5	47 ± 4.6	94	10.9	3.4
500	7	501 ± 16.7	100	3.4	1.7
5000	5	5097 ± 70.00	102	1.0	1.1
Total paclitaxel					
50	6	47 ± 2.0	94	4.3	2.2
250	6	248 ± 17.2	99	7.9	3.4
750	6	759 ± 37.8	101	2.9	4.3
5,000	6	4,943 ± 194.4	99	2.5	3.3
25,000	6	24,485 ± 868.3	98	3.7	1.3
750,000	6	75,719 ± 2,438.0	101	1.9	2.8

dard methylpaclitaxel. After hydrolysis, the mean paclitaxel recovery (n=3) calculated at the concentration of 10,000 ng/mL was 87.9 ± 8.1%. The recovery values for plasma samples containing 1,000 ng/mL free paclitaxel in the presence of PNU166945 (10,000 ng/mL polymer-bound paclitaxel) were not significantly different from the recovery values for plasma samples containing paclitaxel without PNU166945 (p=0.210). Thus, the presence of PNU166945 in plasma samples did not influence the recovery of paclitaxel. The limit of quantification for paclitaxel was 10 ng/mL plasma, using a 500 µL sample volume. No interferences with endogenous constituents or with the tested drugs were observed in the chromatograms.

Stability

In methanol no significant degradation of paclitaxel was measured. Paclitaxel was stable for at least 6 months stored at -30°C. Paclitaxel was found to be stable in plasma at -30°C for at least 10 months.

After plasma extraction, the paclitaxel concentration in reconstitution solvent after 3 days at room temperature was 100.8 ± 1.4% of the initial concentration.

The percentage of free paclitaxel to bound-paclitaxel in the stock solution was 0.12%. Assuming that the hematocrit ratio is 0.5 and the drug is not bound to the blood cells and remains completely in the plasma fraction, the calculated release was 0.32 ± 0.03%. Consequently, the fraction of paclitaxel released from the PNU166945 measured in clinical samples may be overestimated by approximately 0.4%. Assuming that free paclitaxel levels obtained in vivo were approximately 1% of the total paclitaxel levels (Figure 7), a great prudence should be used for any conclusion regarding pharmacokinetics, pharmacodynamics and toxicity of the free paclitaxel fraction.

After storage at -30°C and -70°C during 2.3 months paclitaxel release from PNU166945 was 2.30 ± 0.55% and 0.20 ± 0.45%, respectively. Although the overall release seems very small in percentage terms of bound paclitaxel, the influence of the additional release on the final figures may be considerable. As a result, plasma concentrations of free paclitaxel may be significantly overestimated. It is therefore of the utmost importance to prevent in vitro release where possible. Chemical stabilization and rapid solid phase extraction after sampling were necessary procedures to minimize these effects.

After 48 hours at room temperature, 81.4 ± 1.6% paclitaxel was released from the polymer in a plasma sample diluted 1:1 v/v with 0.1 M KH_2PO_4/methanol. Storage at -30°C for three weeks did not result in a significant change in the recovered amount of paclitaxel (85 ± 3.3%).

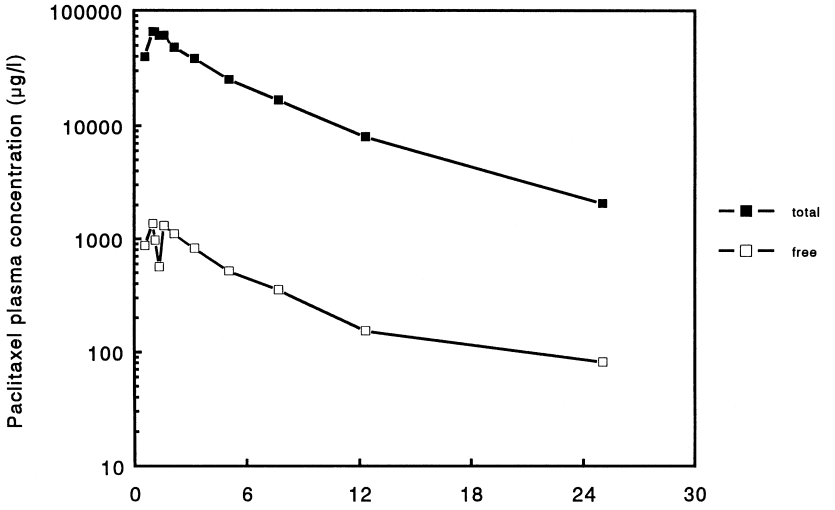


Figure 7. Plasma concentration-time curve of a patient treated with PNU166945 at a dose of 196 mg/m^2. The bound fraction is found by subtraction of free paclitaxel from total paclitaxel. The curve representing the bound fraction is invisible in this figure due to the relatively small deviation from the curve of total paclitaxel.

Clinical Pharmacokinetics

The presented method was successfully applied in a phase I and pharmacokinetic study performed in our Institute. Pharmacokinetics were determined in a patient treated with 196 mg/m^2 paclitaxel, administered as PNU166945. The plasma concentration-time curves of this patient are shown in Figure 7. The AUC of total paclitaxel in this patient was 385 h.mg/L, C_{max} was 65.9 mg/L, Cl was 745 mL/h, t½ was 5.9 h and Vss was 6.3 L. The calculated values for paclitaxel released from the polymer were: AUC = 8.99 h.mg/l, C_{max} = 1.37 mg/L and t½ = 7.8 h.

CONCLUSION

The results presented above demonstrate that a selective and sensitive HPLC assay has been developed for the determination of total paclitaxel levels quantitatively released from PNU166945 and free paclitaxel in human plasma. Polymer-bound paclitaxel concentrations are calculated as total levels minus free levels. For the determination of free paclitaxel, it is of major importance that the collected blood samples are chilled, centrifuged and stabilized appro-

priately and timely to avoid further ex vivo release of paclitaxel from its polymeric carrier.

Much attention has been paid to the stability of the analytes, during handling of the clinical samples as well as during storage. This is of great importance, since release of relatively small amounts of paclitaxel from the polymer may result in a significant overestimation of the free fraction present in the plasma compartment of patients. Plasma samples containing paclitaxel are stable for at least ten months and hydrolyzed plasma samples for at least 3 weeks when stored at -30°C.

The best results for the sample pretreatment are obtained by an accurately performed SPE procedure using CN SPE columns. The proposed assay is a suitable method to be applied in clinical studies to evaluate pharmacokinetics of PNU166945 in humans.

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