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Short communication

Liquid chromatography–tandem mass spectrometry for the determination of paclitaxel in rat plasma after intravenous administration of poly(L-glutamic acid)-alanine-paclitaxel conjugate

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ABSTRACT

A specific and sensitive liquid chromatography–tandem mass spectrometric method for quantitative determination of paclitaxel in rat plasma was developed and validated using docetaxel as an internal standard. Liquid-liquid extraction using *tert*-butyl methyl ether was used to extract the drug and the internal standard from plasma. The separation of paclitaxel was performed on a C_{18} column with a mobile phase of acetonitrile:water:formic acid (65:35:0.1, v/v/v) over 5 min. The assay was based on the selected reaction monitoring transitions at *m/z* of the precursor-product ion transitions *m/z* 854.2 → 286.1 for paclitaxel and 808.3 → 527.2 for internal standard. The lower limit of quantification was 0.5 ng/mL based on 100 μL of plasma. Intra- and inter-day assay variations were less than 15%, and the accuracy values were between 95.4 and 105.4%. The extraction recoveries ranged from 96.7 to 103.7% across the calibration curve range. The method was successfully applied to measurement of low concentrations of paclitaxel or regenerated paclitaxel in plasma after intravenous administration of a single dose (10 mg/kg) of a poly(L-glutamic acid)-alanine-paclitaxel conjugate to rats.

1. Introduction

Paclitaxel, an anticancer agent isolated from the trunk bark of the Pacific Yew tree, *Taxus brevifolia*, shows a wide spectrum of anticancer activity for a variety of human cancers, including breast, ovarian, non-small-cell lung, prostate, head and neck, colon cancers and so on [1]. Paclitaxel is a taxane derivative that binds directly to tubulin, causing microtubular stabilization that arrests cell division in the G2 and M phase of the cell cycle. The use of paclitaxel is, however, limited by its low aqueous solubility and pharmaceutically used solvents. The clinical formulation of paclitaxel is supplied in 50% polyoxyethylated castor oil (Cremophor) and 50% dehydrated ethanol used for solubilizing paclitaxel. Several toxic effects have been attributed to Cremophor, including severe neurotoxicity and hypersensitivity reactions. Side effects of paclitaxel include nausea, vomiting, diarrhea, mucositis, myelosuppression, and cardiotoxicity [2,3]. Because of the unfavorable properties of paclitaxel, it is urgent to develop a more effective strategy to improve its water solubility and selectivity towards tumor tissues [4]. Among different approaches to overcome these problems, is the use of non-cytotoxic polymer–anticancer drug conjugates designed to locally deliver the antitumor agent by specific activation.

The concept of a polymer–drug conjugate, which was prepared by combining a suitable polymeric carrier, a biodegradable linker and a bioactive anticancer agent, is attractive and could form the basis of a new generation of anticancer agents. In an attempt to overcome the poor aqueous solubility and the side effects associated with the use of paclitaxel formulated with Cremophor/ethanol, poly(L-glutamic acid)-alanine-paclitaxel conjugate (PG-PTX, shown in Fig. 1A) is designed as a new highly water-soluble polymeric molecule containing the cytotoxic anticancer agent paclitaxel. The conjugate consists of a poly(L-glutamic acid) polymer bearing an amino acid (alanine) that is linked through an esteric bond at the 2′ position of paclitaxel. Cleavage of the esteric bond releases free paclitaxel into the blood stream. Compared with other synthetic polymers that have been tested in clinical studies, poly(L-glutamic acid) is unique because it is composed of natural L-glutamic acid linked together through amide bonds rather than the nondegradable C–C backbone. The free γ-carboxyl group in each repeating unit of L-glutamic acid is negatively charged under a neutral pH condition, which makes the polymer water-soluble. The carboxyl groups can also provide functionality for drug attachment. Poly(L-glutamic acid) is not only water-soluble and biodegradable, but also nontoxic. All these

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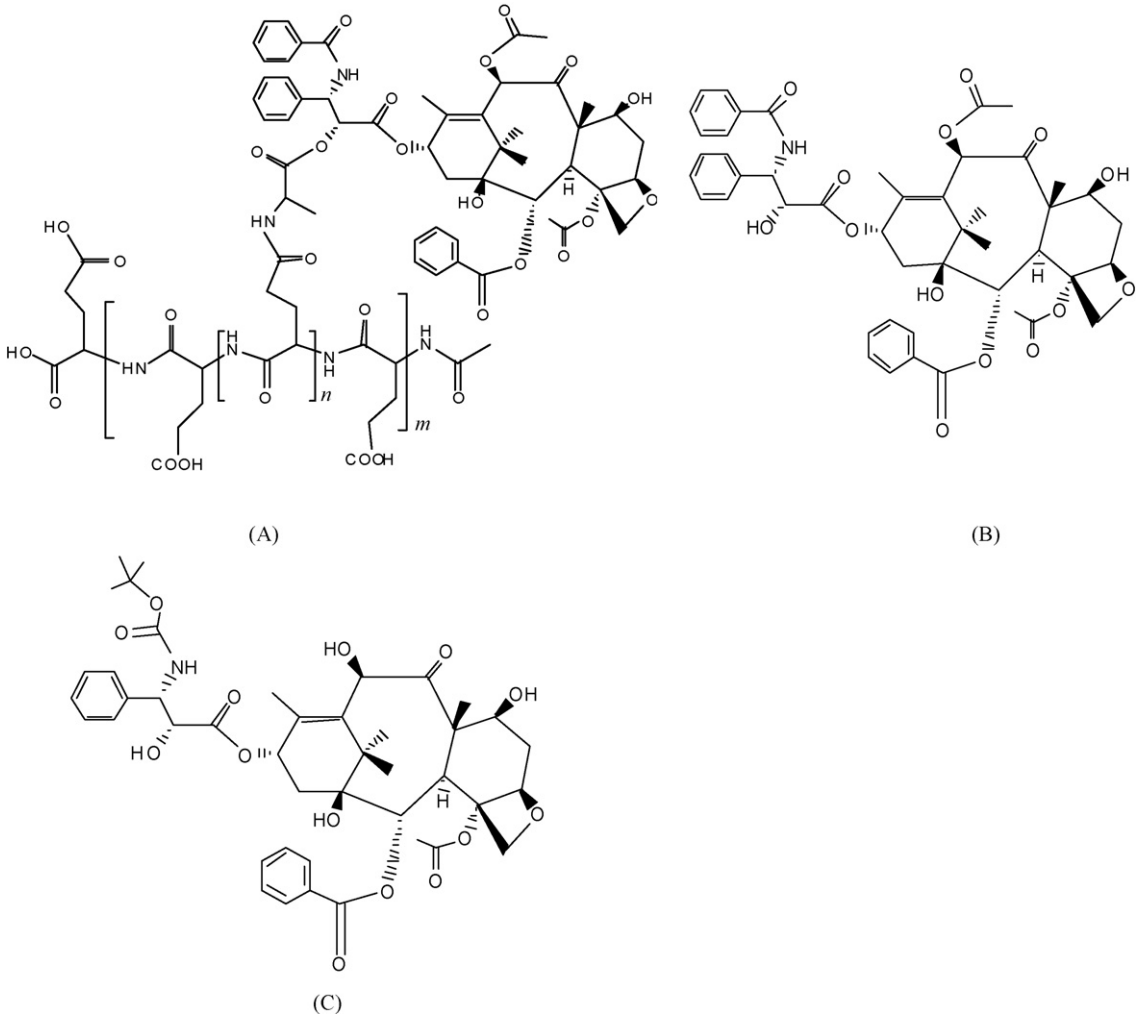

Fig. 1. Chemical structure of poly(L-glutamic acid)-alanine-paclitaxel conjugate (A), paclitaxel (B) and docetaxel (C).

characteristics make poly(L-glutamic acid) a unique candidate as the carrier of polymer–drug conjugates for selective delivery of chemotherapeutic agents, especially for paclitaxel [5].

To study the pharmacokinetics of PG-PTX, two groups of drugs should be considered: paclitaxel which remains attached to the polymeric carrier and free paclitaxel which has been released from the conjugate. It was therefore necessary to select a method which would be suitable for the quantification of both drugs within a sample (free and bound paclitaxel). The high hydrophilicity of PG-PTX makes its extraction from biological matrix difficult. In addition, PG-PTX is not a single chemical entity and its chromatography was not easy. Several assay methods have been published for the determination of paclitaxel in biological fluids, including capillary electrophoresis [6], immunoassays [7], high-performance liquid chromatography [8,9] and liquid chromatography–mass spectrometry (LC–MS) [10,11]. These reported methods had some different drawbacks limiting the ease of use, and only a few of them could support the pharmacokinetic study which often needs more sensitivity, accuracy, and specificity.

In the current research, we have developed a robust, fast and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) for the measurement of released paclitaxel from conjugate in biological samples. The method shows more sensitivity and/or shorter analytical time than published HPLC [8,9] and LC–MS [10,11] methods. Moreover, the method was employed to determine remained paclitaxel bound to the conjugate by chemical hydrolysis *in vitro*, which would be reported in subsequent investigation. This method has also been successfully applied to the determination of paclitaxel regenerated from PG-PTX in rat plasma after intravenous administration of the conjugate, as well as investigation of *in vitro* stability of the conjugate in rat plasma.

2. Materials and methods

2.1. Materials

Poly(L-glutamic acid) sodium salt with an average molecular weight of 15,000–50,000, internal standard (IS) docetaxel and Cremophor EL were obtained from Sigma (St. Louis, MO, USA). PG-PTX with a drug load of 35.9% was synthesized by Beijing Institute of Pharmacology and Toxicology. HPLC grade acetonitrile and *tert*-butyl methyl ether (TBME) was purchased from Fisher (Fair Lawn, NJ, USA). All other reagents were analytical grade and obtained through commercial sources.

2.2. LC–MS/MS instrumentation

A Thermo Finnigan TSQ LC–MS/MS system consisted of a Finnigan Surveyor Autosampler, a Finnigan Surveyor LC-Pump and a Quantum Discovery MAX triple quadrupole mass spectrometer (Thermo Electron Co., San Jose, CA, USA). Data acquisition was performed with Xcalibur software version 1.2 (Thermo Finnigan). The

data processing was carried out using the Thermo Finnigan LCQuan 2.0 data analysis program.

2.3. Chromatographic conditions

A BetaBasic C_{18} column (150 mm × 2.1 mm, 5 μm particle size, Thermo Fisher Inc., Waltham, MA, USA) was used for the analysis at ambient temperature with a mobile phase composed of acetonitrile:water:formic acid (65:35:0.1, v/v/v) at a flow rate of 0.2 mL/min. The injection volume was 20 μL, the analysis time was 5 min per sample, and the injection port was washed in between runs with 10 loop volumes of 50% methanol.

2.4. Mass spectrometric conditions

The ESI-MS spectrometer was operated in the positive ionization mode. Mass spectrometry conditions were optimized using a syringe pump infusion (10 μL/min). The optimal instrument conditions were as follows: capillary temperature of 300 °C, spray voltage of 4600 V, sheath gas of 25 arb units, auxiliary gas of 6 arb units. Nitrogen was used as sheath gas and auxiliary gas, and argon was employed as a collision gas at a pressure of 1.5 mTorr. Selected reaction monitoring (SRM) of the precursor-product ion transitions m/z 854.2 → 286.1 for paclitaxel and 808.3 → 527.2 for IS was used for quantification. The collision energy was 12 and 15 eV for paclitaxel and IS, respectively. Scan time was 0.5 s per transition.

2.5. Standard and working solutions

Individual stock solutions of paclitaxel (5 mg/mL) and IS (5 μg/mL) were prepared by accurately weighing the required amounts into separate volumetric flasks and dissolving in acetonitrile.

The calibration standard for paclitaxel was prepared by spiking 4.95 mL of blank rat plasma with 50 μL of paclitaxel stock solution. The resulting plasma standard had a concentration of 50,000 ng/mL. Further dilutions were made from this stock with blank plasma to afford plasma standards in the range of 0.5–5000 ng/mL. Quality control (QC) samples (2, 800 and 4000 ng/mL) in blank rat plasma were prepared in a similar manner.

2.6. Sample preparation

To 100 μL of plasma sample was added 20 μL of IS solution. Following the addition of 1.5 mL of TBME, the sample was vortex-mixed for 5 min and then centrifuged at 10,000 rpm for 5 min. The resultant supernatant (1.2 mL) was transferred to another tube and dried under vacuum at 40 °C. The dried residue was reconstituted in 100 μL of mobile phase and vortex-mixed for 1 min. After centrifugation at 5000 rpm for 5 min, the supernatant was transferred to an autosampler vial, and a 20-μL aliquot of the sample was injected into LC–MS/MS.

2.7. Method validation

2.7.1. Selectivity and matrix effect

To serve as blanks, plasma samples were obtained from six different sources and analyzed to assure that they were free of interfering response values, and the results were compared with those obtained from an aqueous solution of paclitaxel at the lower limit of quantification (LLOQ). The blank plasma samples were also used to test the matrix effect. This was done by comparing the response values of the extracted blank samples that were spiked with the drug to those of standards injected directly.

2.7.1.1. Extraction recovery. The extraction recovery of paclitaxel from plasma was determined at three concentrations at the lower, middle, and higher parts of the calibration curves (2, 800, and 4000 ng/mL). Samples (n = 6) were then subjected to the extraction procedure, and the resultant paclitaxel peak areas were compared to those obtained from direct injection of standard solutions of equivalent concentrations. The recovery of IS was determined similarly.

2.7.2. Linearity

The linearity of the method was evaluated by calibration curves (n = 5) in the range of 0.5–5000 ng/mL. Calibration curves were constructed by plotting peak area ratios of paclitaxel to IS against concentration with a weight of $1/x$.

2.7.3. Accuracy and precision

The intra- and inter-day accuracy and precision were evaluated by analysis of LLOQ, low, middle, and high quality control (QC) samples with six determinations per concentration on the same day over 3 days. The concentration of each sample was determined using calibration standards prepared on the same day. Accuracy was expressed as percentage value (% accuracy = [measured concentration/nominal concentration] × 100%). The precision was estimated as percentage relative standard deviation (%RSD). For acceptable intra- and inter-day values, accuracy should be within 85–115% and RSD values should be ≤15% over the calibration range, except at the LLOQ, where accuracy should be between 80 and 120% and RSD should not exceed 20%.

2.7.4. Stability

The stability tests were conducted to cover the experimental conditions that real samples may experience. The stability of paclitaxel in plasma was assessed using 2 and 4000 ng/mL QC samples for short-term temperature and post-preparative stabilities. In short-term temperature stability, three aliquots of each of the low and high concentrations were thawed unassisted at ambient temperature and kept at this temperature for 6 h. The post-preparative stability of the prepared plasma samples was determined after keeping the samples at ambient temperature for 12 h. Thereafter, samples were analyzed and the resulted values for these samples were then compared to those of the respective freshly prepared QC samples. The analytes were considered stable in the different conditions when a deviation of less than ±15% from the actual value was obtained.

2.8. Application of the method

2.8.1. In vitro release of paclitaxel from PG-PTX in rat plasma

In order to evaluate the release rate and extent of paclitaxel from PG-PTX, an *in vitro* study in rat plasma at 37 °C was carried out. Rat plasma containing PG-PTX with a concentration of 100 μg/mL (paclitaxel equivalent) was incubated at 37 °C in a reciprocating water bath. At the designated time, 100 μL of plasma samples were taken and immediately added to 1.5 mL of cold TBME and subsequently 20 μL of the internal standard stocking solution (5 μg/mL) was added, and then followed the above mentioned extraction procedure.

2.8.2. Pharmacokinetic study

All procedures involving animals were conducted in accordance with the Principles of Laboratory Animal Care (NIH publication #85-23, revised in 1985), and approved by our Institutional Animal Care and Use Committee. Adult male Sprague–Dawley rats, weighing 260 ± 15 g, were obtained from Academy of Military Medical Sciences Animals Center (Beijing, China). The rats were housed in

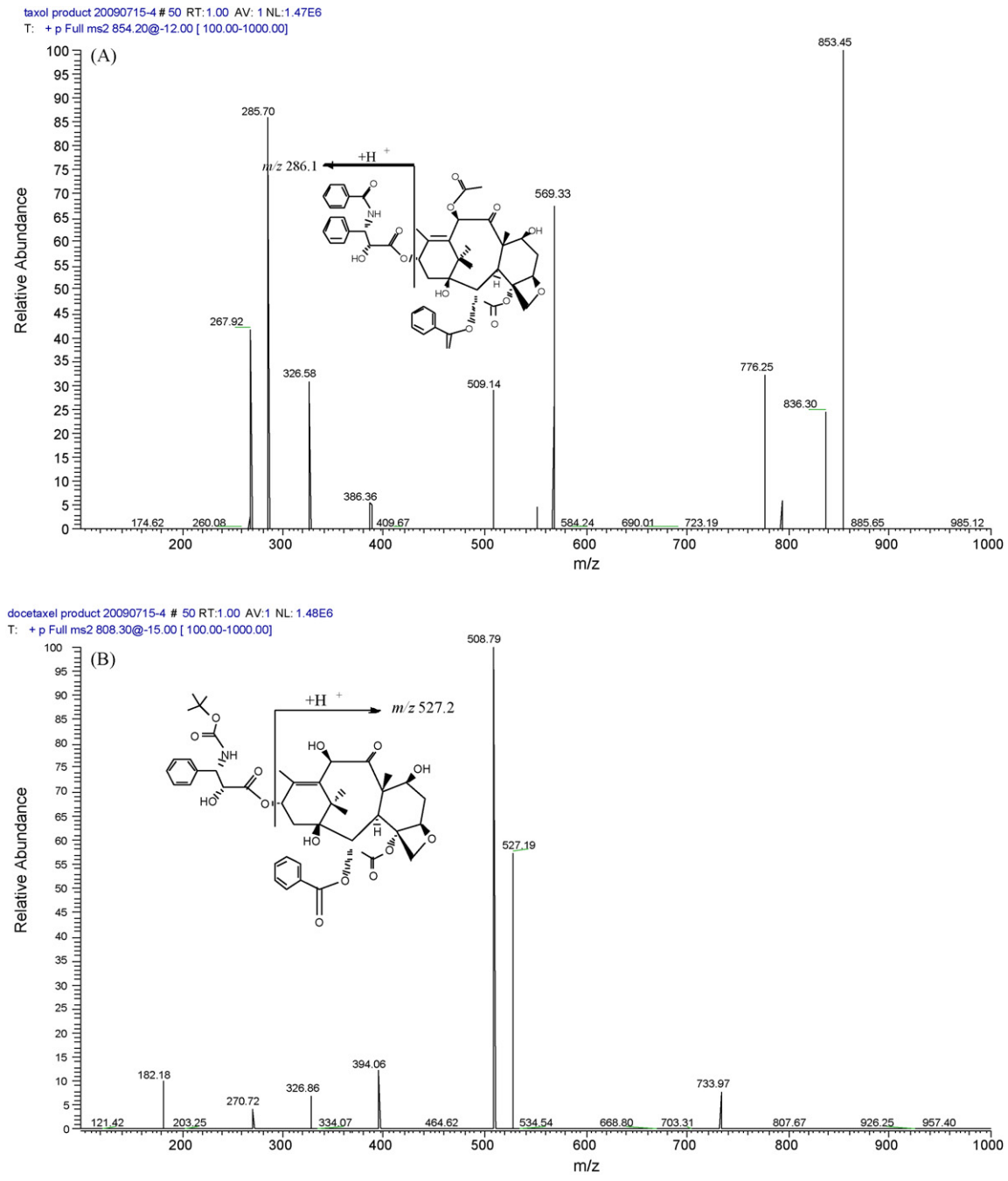

Fig. 2. Mass spectra of product ions of paclitaxel (A) and docetaxel (B) in positive electrospray ionization mode.

standard cages and allowed free movement and access to food and water during the whole experiment.

The dosing solution of the conjugate PG-PTX was dissolved in physiological saline to produce a concentration of 2.5 mg/mL (paclitaxel equivalent). The dosing solution of the parent drug paclitaxel (2.5 mg/mL) was dissolved in a mixture of Cremophor EL:ethanol:saline (1:1:8, v/v/v). A single 10 mg/kg (PTX equivalent) dose of either paclitaxel or PG-PTX was administered into the tail veins of rats that were under anesthesia. At designated time points after dosing, blood samples (0.3 mL) were taken and centrifuged in heparin-coated microcentrifuge tubes to obtain plasma. Plasma sample (100 μL) was transferred to a silanized microcentrifuge. All the samples were stored at −30 °C until analysis within a week.

3. Results and discussion

3.1. Liquid chromatography–mass spectrometry

Paclitaxel and docetaxel could be ionized under either positive ESI (ESI^+) or negative ESI (ESI^-) conditions because of the amide and hydroxyl groups in both of the structures. However, ESI^+ gave a better signal than ESI^-. Therefore, the ESI^+ mode was used for analyte quantification. Paclitaxel and docetaxel showed prominent protonated quasi-molecular ions $[M+H]^+$ at m/z 854.2 and 808.3, respectively. Therefore, these quasi-molecular ions were chosen as precursor ions for acquisition of product ion fragments. When H^+ adducts were subject to the collision induced dissociation (CID) condition, they resulted in strong product ions for paclitaxel and

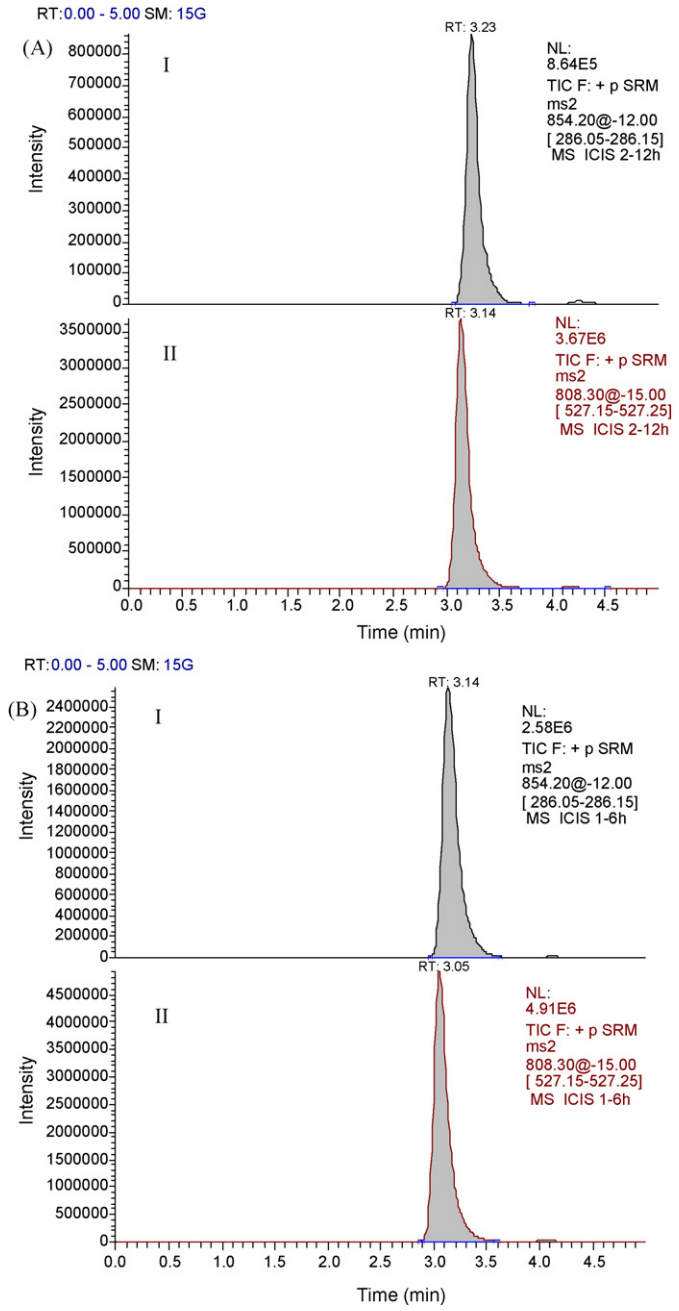

Fig. 3. Representative SRM chromatograms (I: m/z 854.2 → 286.1 for paclitaxel; II: m/z 808.3 → 527.2 for internal standard) for (A) a plasma sample obtained 12 h after intravenous administration of paclitaxel at a dose of 10 mg/kg; (B) a plasma sample obtained 6 h after intravenous administration of PG-PTX at a dose of 10 mg/kg (paclitaxel equivalent).

docetaxel at m/z 286.1 and 527.2, respectively. The product ion mass spectrum and the possible structures of these fragments are shown in Figs. 2 and 3.

3.2. Selectivity and extraction recovery

The retention times of paclitaxel and the internal standard were approximately 3.2 and 3.1 min, respectively. No endogenous or extraneous peaks were observed interfering with the separation and quantification of paclitaxel. Chromatographic conditions may cause co-elution of endogenous compounds that are undetected by the MS–MS but may affect the ionization efficiency. Therefore, the effect of matrix on the response of the analyte was also evaluated. However, no matrix effect was detected in our study.

Table 1
Intra- and inter-day accuracy and precision of the method for the determination of paclitaxel in rat plasma ($n = 6$).

Concentration (ng/mL)	Intra-day		Inter-day	
	Accuracy	RSD (%)	Accuracy	RSD (%)
0.5	105.4	6.7	103.2	9.1
2	96.1	7.2	95.4	8.9
800	104.0	4.4	98.2	7.0
4000	103.1	7.7	97.3	9.6

The mean extraction recoveries for MP in plasma were 103.7 ± 6.9% at 2 ng/mL, 96.7 ± 3.1% at 800 ng/mL, and 99.4 ± 1.9% at 4000 ng/mL. The mean recovery for IS from plasma was 86.9 ± 2.2%. These results suggested that there was no relevant difference in extraction recovery at different concentrations of MP in plasma samples.

3.3. Linearity and lower limit of quantification

The calibration model was selected based on the analysis of the data by linear regression with intercepts and $1/x$ weighting factor. Representative linear equation for paclitaxel in plasma was $y = 0.00126x + 0.00464$ in the range of 0.5–5000 ng/mL. Each standard point in every calibration curve was back calculated using its own equation. The non-zero standards showed less than 20% deviation at the 0.5 ng/mL concentration and less than 15% deviation at all other concentration levels. The coefficients of determination (r^2) for paclitaxel in plasma were ≥0.996. The value of LLOQ was 0.5 ng/mL for paclitaxel in rat plasma, based on 100 μL of plasma.

3.4. Accuracy and precision

The results of the accuracy and precision are shown in Table 1. The intra- and inter-day accuracy for paclitaxel at 0.5, 2, 800 and 4000 ng/mL levels in rat plasma fell in the ranges of 96.1–105.4% and 95.4–103.2%, and the intra- and inter-day precision (RSD) were in the ranges of 4.4–7.7% and 7.0–9.6%, respectively. These data indicate that the repeatability, intermediate precision, and bias values of the assay are within the acceptance limits of ±20% at LLOQ and ±15% at other concentration levels.

3.5. Stability

The stability was expressed as a percentage of the theoretical value. For short-term stability, the mean recoveries were 95.2 ± 9.7% and 96.5 ± 6.4% at 2 and 4000 ng/mL, respectively. The results showed that the plasma samples could be prepared and handled at ambient temperature during the period of a minimum of 6 h without any indication of degradation. The processed plasma samples of paclitaxel were stable for 12 h and the recoveries of the drug after 12 h were 103.2 ± 5.4% and 107.8 ± 5.1% at the two concentrations studied. These studies suggested that rat plasma samples containing paclitaxel can be handled under normal laboratory conditions without significant loss of compound.

3.6. In vitro release of paclitaxel from PG-PTX in rat plasma

The method was applied to investigate the *in vitro* release extent and rate of paclitaxel from PG-PTX in rat plasma. The release percentage–time profile of paclitaxel in rat plasma was shown in Fig. 4. The results showed that approximately 8% and 20% paclitaxel were released from PG-PTX at 0.5 and 12 h, respectively. Interestingly, only about 16.6% paclitaxel was detected in rat plasma at

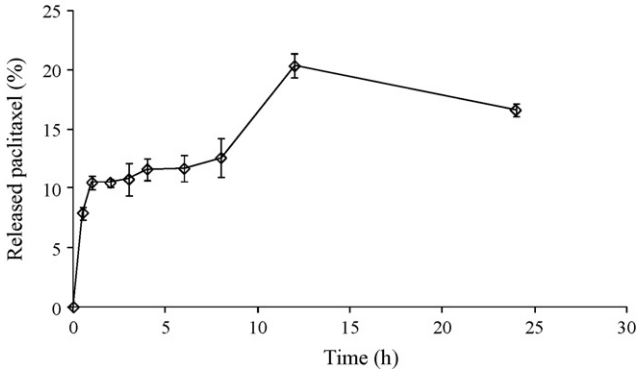

Fig. 4. *In vitro* release of paclitaxel from PG-PTX in rat plasma at 37 °C ($n = 3$).

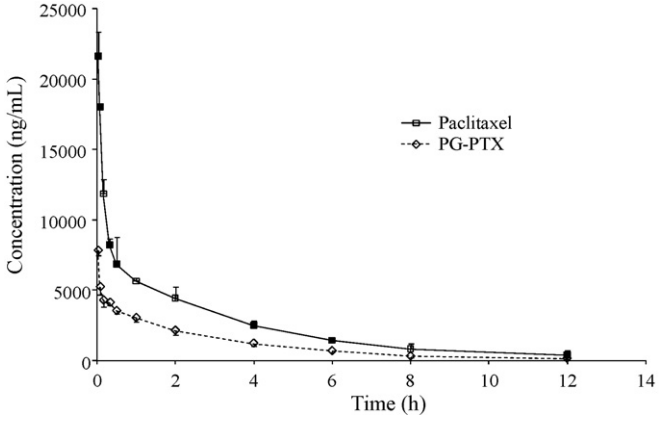

Fig. 5. The plasma concentration–time profiles of paclitaxel after single intravenous administration of paclitaxel (closed square) or PG-PTX (open diamond) to rats at a dose of 10 mg/kg (as paclitaxel equivalent) ($n = 3$).

24 h may be due to simultaneous degradation of released paclitaxel, which was consistent with our previous results [12,13].

3.7. Pharmacokinetic study

The developed assay method was applied to a pharmacokinetic study after intravenous administration of paclitaxel or PG-PTX to rats at a dose of 10 mg/kg and mean plasma concentration–time profiles are depicted in Fig. 5. Paclitaxel concentration was rapidly declined by roughly 80% within 2 h after administration, which was consistent with results of Tong et al. [14]. The LLOQ of the method was sufficient to characterize the pharmacokinetics of paclitaxel. For the administration of PG-PTX, pharmacokinetic parameter values of systemic clearance ($C1_S$), steady-state volume of distribution (V_{SS}) and mean residence time (MRT) were 0.72 ± 0.07 L/h kg, 2.23 ± 0.16 L/kg and 2.7 ± 0.1 h, respectively. Those values after paclitaxel injection were 0.32 ± 0.02 L/h kg, 1.20 ± 0.04 L/kg and 2.9 ± 0.5 h, respectively.

4. Conclusion

A sensitive, specific and reproducible liquid chromatography–tandem mass spectrometry method for quantitative determination of paclitaxel in rat plasma has been developed and validated, and found to be accurate and precise for the routine analysis of paclitaxel in plasma. The pretreatment of biological specimens involves only a rapid single liquid-liquid extraction method.

The method has been applied successfully, for the first time, to quantitatively analyze paclitaxel in rat plasma regenerated from poly(L-glutamic acid)-alanine-paclitaxel conjugate after intravenous administration.

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