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A HPLC-UV method for the determination of puerarin in rat plasma after intravenous administration of PEGylated puerarin conjugate

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ABSTRACT

A sensitive and reproducible HPLC method for quantitative determination of puerarin (PUE) in rat plasma was developed and validated using 4-hydroxybenzaldehyde as an internal standard. The separation of PUE was performed on a CAPCELL PAK C18 column by gradient elution with 0.2% aqueous phosphoric acid and acetonitrile as the mobile phase. The method was validated and found to be linear in the range of 80–12,000 ng/mL. The limit of quantification was 80 ng/mL based on 100 μL of plasma. The variations for intra- and inter-day precision were less than 8.3%, and the accuracy values were between 98% and 105.2%. The extraction recoveries were more than 85%. The method was successfully applied in the comparative study of pharmacokinetics of PEGylated puerarin (PEG-PUE) versus PUE in rats. Compared with PUE, PEG-PUE showed a 5.2-fold increase in half-life of PUE and a 4.7-fold increase in mean residence time. In addition, this method was also successfully applied to determine the low plasma concentration of PUE regenerated from PEG-PUE *in vitro*.

1. Introduction

Puerarin (PUE), a naturally occurring isoflavone *C*-glycoside isolated from the root of *Pueraria lobata* (Willd.) Ohwi. Research on the clinical application of PUE injection in recent years shows that PUE, being a vasodilator, is found effective to treat a variety of cardiovascular diseases, such as coronary heart disease, cardiac infarction, arteriosclerosis and arrhythmia [1]. However, PUE barely dissolves in aqueous solution. 50% propanediol are often used for solubilizing PUE in the preparing process of PUE injection [2]. Moreover, it was also reported that the elimination half-life of PUE in rat was 20 ± 8 min, and the cumulative excretion rate of unchanged PUE in urine and feces was 45.33% in 24 h [3,4]. Because of the unfavorable properties of PUE, it is very necessary to develop an effective strategy to improve its water solubility and extend biologic half-life of drug. One good approach to overcome these problems is the attachment of PUE to water-soluble polymers, namely PEGylated puerarin (PEG-PUE).

PEG-PUE was designed as one of new highly water-soluble polymeric prodrugs. Recent reports in the scientific literature have indicated that these newly developed polymeric prodrugs exhibited promise for controlled delivery of prototype drugs, and they could also protect drugs from metabolic deactivation and preserve their pharmacological activities during circulation [5,6]. Thus, the biological half-life of prototype drugs was greatly prolonged by PEG modification. Moreover, PEG is not only water-soluble and biodegradable, but also nontoxic. Based on the theory mentioned above, all these desirable characteristics of PEGylation may overcome the poor aqueous solubility and short half-life of PUE by PEG conjugation.

To study the pharmacokinetics of PEG-PUE, two groups of prototype drugs should be considered: PUE which remains attached to the polymeric carrier and free PUE which has been released from the conjugate. Methods for investigating the pharmacokinetics of PEGylated prodrugs in previous research were usually adopted to determine the total remaining prototype drugs via hydrolysis of PEGylated prodrugs during sample preparation [7–9]. In general, only free prototype drugs are thought to be pharmacologically active, and prototype drugs remaining in the PEGylated prodrugs could not exert medicinal functions until they were released *in vivo*. Thus, these methods have misinterpreted the actual metabolic processes of active prototype drugs regenerated from their prodrugs. We therefore have developed a non-disruptive and simple determination method for revealing the regeneration kinetics of PUE after intravenous administration of PEG-PUE. Moreover, the method has also been successfully applied to the determination of PUE regenerated from PEG-PUE *in vitro* stability of the conjugate in rat plasma. In addition, the test method has more simple preliminary treat-

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ment to the plasma sample than published assay [10] and could also provide an economic alternative to published LC–MS method [11] for LC–MS instrument is not available in some laboratories due to the costs associated with acquisition and maintenance of this expensive equipment.

2. Experimental

2.1. Materials and reagents

Methoxy PEG carboxymethyl with an average molecular weight of 4700 was obtained from Beijing Kaizheng Biotech Development Co., Ltd. (Beijing, China). PEG-PUE with a drug load of 4.1% was synthesized in our laboratory. The route and experiments for synthesis of PEG-PUE were reported in our previous research [12]. HPLC grade acetonitrile and methanol were purchased from Fisher (Fair lawn, NJ, USA). PUE injection was purchased from Shandong Fangming Pharmaceutical Stock Co., Ltd. (Dongming, China). 4-Hydroxybenzaldehyde (Internal standard, IS) was obtained from Sinopharm Chemical Reagent Beijing Co., Ltd. (Beijing, China). All other reagents were analytical grade and obtained through commercial sources. Super-pure water was obtained following distillation in glass and passage through a Milli-Q® system (Millpore, USA) and was used to prepare all solutions.

2.2. Liquid chromatographic conditions

The chromatographic separation was performed on a CAPCELL PAK C18 column (250 mm × 4.6 mm, 5.0 μm, Japan) at a column temperature of 40 °C. The mobile phase consisted of (A) 0.2% aqueous phosphoric acid and (B) acetonitrile using a gradient elution of 12.8% B at 0–6 min, 12.8–40% B at 6–7 min, 40–60% B at 7–12 min, 60–50% B at 12–13 min, 50–12.8% B at 13–14 min, and the re-equilibration time of gradient elution was 3 min. The flow rate was 1.0 mL/min. Detection was performed at 250 nm.

2.3. Standard and working solutions

Individual stock solutions of PUE (1.2 mg/mL) and IS (7.5 μg/mL) were prepared by accurately weighing the required amounts into separate volumetric flasks and dissolving in methanol. The calibration standard for PUE was prepared by spiking 4.95 mL of blank rat plasma with 50 μL of PUE stock solution. The resulting plasma standard had a concentration of 12,000 ng/mL. Further dilutions were made from this stock with blank plasma to afford plasma standards in the range of 80–12,000 ng/mL.

2.4. Sample preparation

To 100 μL of plasma sample was added 40 μL of IS solution. Following the addition of 1.0 mL of methanol, the sample was vortex-mixed for 5 min and then centrifuged at 15,000 rpm for 5 min. The resultant supernatant was evaporated to dryness in a water bath at 40 °C under the protection of nitrogen. The dried residue was reconstituted in 80 μL of the mixture of PG-acetate buffer (1 M) (50:50, v/v) (pH 4.8) and vortex-mixed for 1 min. After centrifugation at 5500 rpm for 5 min, the supernatant was transferred to an autosampler vial, and a 60 μL aliquot of the sample was injected into HPLC system.

2.5. Method validation

2.5.1. Selectivity

The selectivity of the method was tested by comparing the chromatograms of blank plasma controls from six rats with that of plasma spiked with PUE and IS.

2.5.2. Linearity

The linearity of the method was evaluated by calibration curves in the range of 80–12,000 ng/mL. Calibration curves were constructed by plotting peak area ratios of PUE to IS against concentration with a weight of $1/\chi^2$.

2.5.3. Accuracy, precision and extraction recovery

The intra- and inter-day accuracy and precision were investigated by determining QC samples at three different concentrations (80, 800, and 8000 ng/mL) over three consecutive days. QC samples of three different concentrations were tested in six replicates and calculated with calibration curves obtained daily. Accuracy was expressed as percentage value (% accuracy = [detected 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 LOQ, where accuracy should be between 80% and 120% and RSD should not exceed 20%.

The extraction recovery of PUE were determined at three concentrations levels by comparing the analyte peak areas of six extracted QC samples with those obtained from direct injection of standard solutions prepared at the same concentrations of six replicates. The recovery of IS was determined similarly.

2.5.4. Stability

The stability of QC samples was also investigated at three concentration levels, including (a) stability of the extracted samples at room temperature for 24 h, (b) stability after three freeze–thaw cycles with the frozen temperature of −20 °C and thawing temperature of 25 °C and (c) stability of plasma samples at −20 °C for 20 days. Thereafter, samples were analyzed and the resulted values for these samples were then compared with those of the respective freshly prepared QC samples.

2.6. Application of the method

2.6.1. Inhibition of the degradation of PEG-PUE in vitro

In order to accurately evaluate the release of PUE from PEG-PUE in rat plasma, an analytical problem associated with the hydrolysis of PEG-PUE *in vitro* should be minimized as little as possible, for a small degradation of the conjugate *in vitro* could significantly overestimate the actual PUE concentrations regenerated from the conjugate *in vivo*. A previous work and some pertinent literature on the *in vitro* stability of PEG-PUE showed that the conjugate was more stable when acetic acid was added to the samples [6]. Therefore, the *in vitro* stability of PEG-PUE in the plasma was studied in the presence and absence of acetic acid. Blank plasma was spiked with 12% acetic acid solution (5:1, v/v) or water (control). A PEG-PUE aqueous solution was then added to plasma to produce a final concentration of 8.2 μg/mL (PUE equivalent).

To determine the stability of the samples during sample preparation and analysis, a set of spiked samples were prepared at room temperature (25 °C) and analyzed for their content of released PUE without any storage. Further, to determine the stability of the samples during storage, additional sets of samples were stored at −80 °C, and aliquots were analyzed at 7, 14 and 21 days after storage. All determinations were conducted in triplicates.

2.6.2. In vitro release of PUE from PEG-PUE in rat plasma

For hydrolysis in plasma, an *in vitro* study in rat plasma at 37 °C was carried out. Rat plasma containing PEG-PUE with a concentration of 8.2 μg/mL (PUE equivalent) was incubated at 37 °C in a reciprocating water bath. At scheduled times, plasma samples (100 μL) were transferred to a silanized microcentrifuge tube containing 20 μL of 12% acetic acid solution to prevent PEG-PUE

hydrolysis *in vitro*, and then followed the above mentioned sample preparation procedure.

2.6.3. Pharmacokinetic study

The animal experiment was approved by the Animal Ethics Committee at the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences. Male Sprague–Dawley rats (230–250 g) were used and supplied by Beijing Laboratory Animal Center (Beijing, China). The rats were housed in standard cages and allowed free movement and access to food and water during the whole experiment.

The dosing solution of the conjugate PEG-PUE was dissolved in physiological saline to produce a concentration of 5 mg/mL (PUE equivalent). The dosing solution of prototype drug PUE (5 mg/mL) was prepared by dilution of PUE injection with proper physiological saline. A single 20 mg/kg (PUE equivalent) dose of either PUE or PEG-PUE was administered into the tail veins of rats under mild ether anesthesia. At designated time points after dosing, blood samples (0.4 mL) were collected from the retro-orbital plexus of rats under light ether anesthesia into microcentrifuge tubes containing heparin as anti-coagulant at 0, 5, 15, 30, 45, 60, 90, 120, 180, 240, 300 min post-dosing. Plasma samples (100 μL) were spiked with 20 μL of 12% acetic acid and processed as described above. All the samples were stored at −80 °C until analysis within a week.

3. Results and discussion

3.1. Method development

The method for eliminating interference from endogenous substance is a prerequisite for a successful HPLC-UV analysis of analytes in biological fluids, especially in the case of rat plasma samples obtained by protein precipitate method. In this study, we optimized the HPLC condition for the separation of PUE and IS by using a gradient solvent system consisted of (12.8–60%) acetonitrile/0.2% phosphoric acid (87.2–40%). In spite of this, chromatographic behaviors of PUE and IS under this gradient elution were not very satisfactory. Such problems as split peak for PUE and leading peak for IS emerged. Feasibility of various mixture of solvents such as acetonitrile and water as a solution to dissolve the plasma samples were tested to improve peak shape, however, PUE still appeared split peak resulting from solvent effect. The peak shape yielded greatly improvement for both PUE and IS by replacing acetonitrile with propylene glycol. Ultimately the mixture of PG-acetate buffer (1 M) (50:50, v/v) (pH 4.8) was selected as a solution to dissolve the plasma samples, resulting in improved peaks for both PUE and IS, which may be relevant to reducing the solvent effect of acetonitrile with strong eluting power. Moreover, the mixture of PG-acetate buffer (1 M) (50:50, v/v) (pH 4.8) could also be used as a quenching solution to inhibit the degradation of PEG-PUE in our previous research [6].

An appropriate internal standard is needed for accurate quantitative analysis of analytes in biological fluids. In this study, we chose 4-hydroxybenzaldehyde as the internal standard. 4-Hydroxybenzaldehyde is a stable chemical raw material which is widely used and easily commercially available, and it displayed a similar solubility to solvent extraction and adsorbent function to cartridge, compared with those of PUE. Both compounds contain a *p*-hydroxybenzene ring system, and show maximum absorption at the wavelength of around 250 nm [10,13]. In addition, the retention times and extraction recoveries for PUE and 4-hydroxybenzaldehyde were very close, and the resolution between the peak of PUE and 4-hydroxybenzaldehyde was also good. Based on these observations, 4-hydroxybenzaldehyde was employed as the internal standard for quantitative analysis of PUE in rat plasma (Fig. 1).

3.2. Selectivity

There was no significant chromatographic interference around the retention times of PUE and IS in drug-free specimens. Furthermore, we found that this optimal HPLC system could also provide good chromatographic profiles of PEG-PUE and IS in rat plasma (Fig. 2). The retention times of PUE, IS and PEG-PUE were approximately 6.9, 8.8 and 12.8 min, respectively.

3.3. Linearity and limit of quantification

The calibration model was selected based on the analysis of the data by linear regression with intercepts and $(1/\chi^2)$ weighting factor. Representative linear equation for PUE in plasma was $y = 0.83512\chi + 0.02489$ in the range of 80–12,000 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 80 ng/mL concentration and less than 15% deviation at all other concentration levels. The coefficients of determination for PUE in plasma were ≥0.997. The value of LOQ was 80 ng/mL for PUE in rat plasma, based on 100 μL of plasma.

3.4. Accuracy, precision and extraction recovery

The results of the accuracy and precision are shown in Table 1. The intra- and inter-day accuracy for PUE at 80, 800 and 8000 ng/mL levels in rat plasma fell in the ranges of 101.0–105.2% and 98–103.8%, and the intra- and inter-day precision (RSD) were in the ranges of 2.9–6.6% and 5.1–8.3%, respectively. These data indicated that the repeatability, intermediate precision, and bias values of the assay were within the acceptance limits of ±20% at LOQ and ±15% at other concentration levels.

The mean extraction recoveries for PUE in plasma were 86.1 ± 1.7% at 80 ng/mL, 87.0 ± 3.4% at 800 ng/mL, and 88.9 ± 2.6% at 8000 ng/mL. The mean recovery for IS in plasma was 90.4 ± 3.5%.

3.5. Stability

PUE was found to be stable after three freeze–thaw cycles in plasma. It was also proved that PUE could be stable in the rat plasma at room temperature for at least 24 h and at −20 °C for 20 days. Table 2 lists the mean recoveries for PUE at each concentration, and ranged from 95.1 ± 3.5% to 99.3 ± 4.4%. Based on the above methodological study of PUE in rat plasma, it was expected that the present method would be applicable to pharmacokinetic study of PUE in rat plasma.

3.6. Application of the method

3.6.1. Inhibition of degradation of PEG-PUE in vitro

When acetic acid was added to spiked samples and samples were prepared at room temperature and analyzed without any storage. There were no significant degradation of PEG-PUE in the first 4 h, then it showed a slight ascendant trend in the next 5 h, ultimately 5.4 ± 0.3‰ PUE was released from PEG-PUE at 9 h. Interestingly, in the presence of acetic acid, only 8.5 ± 0.2‰ PUE was released from PEG-PUE stored at −80 °C during a three-decade study period. However, when acetic acid was not added to the samples, more than 75% of PEG-PUE was recovered as PUE after 9 h of incubation at room temperature. These data indicate that the addition of acetic acid not only prevents hydrolysis during the storage (Fig. 3), but is also necessary for the stability of PEG-PUE during sample preparation.

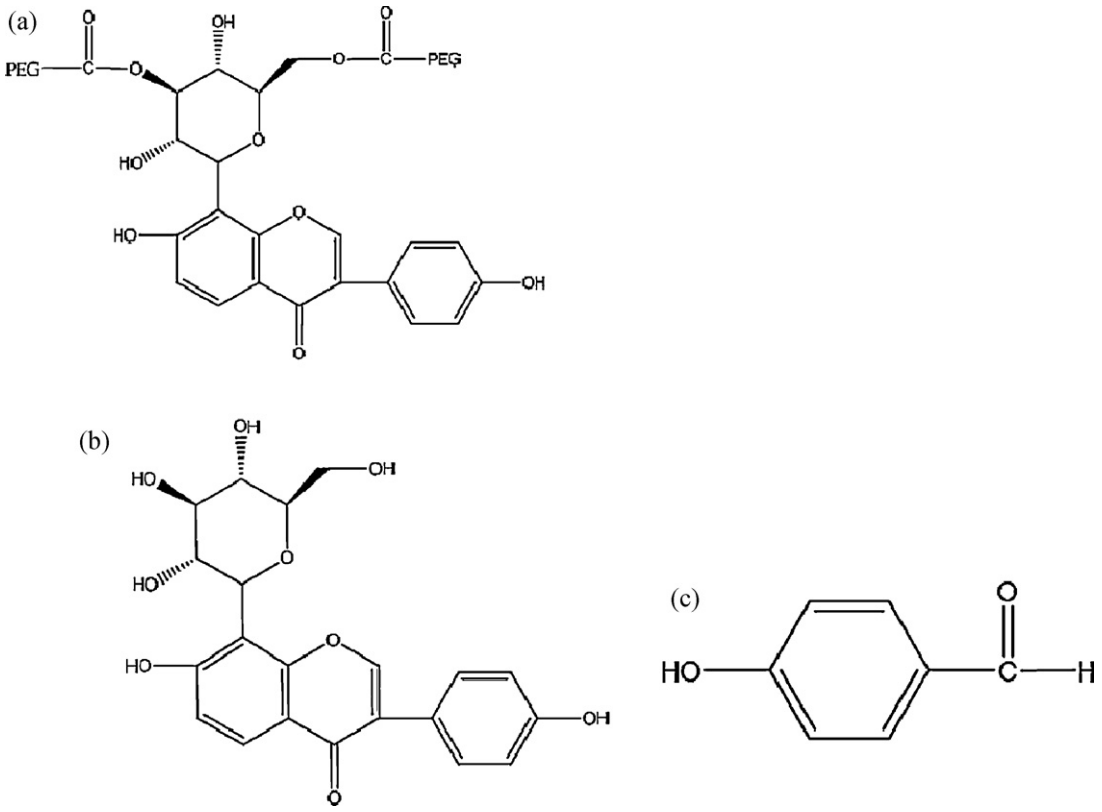

Fig. 1. Chemical structures of PEG-PUE (a), PUE (b) and IS (c).

3.6.2. In vitro release of PUE from PEG-PUE in rat plasma

The method was applied to investigate the *in vitro* release extent and rate of PUE from PEG-PUE in rat plasma. The release percentage-time profile of PUE in rat plasma was shown in Fig. 4. Using Sigma plot 10.0, an optimal mathematical model for the release kinetics of PUE was established. By using this equation, the calculated half-life of the released PUE from PEG-PUE in rat plasma was 152.7 min. The results could provide some evidence that PEG-

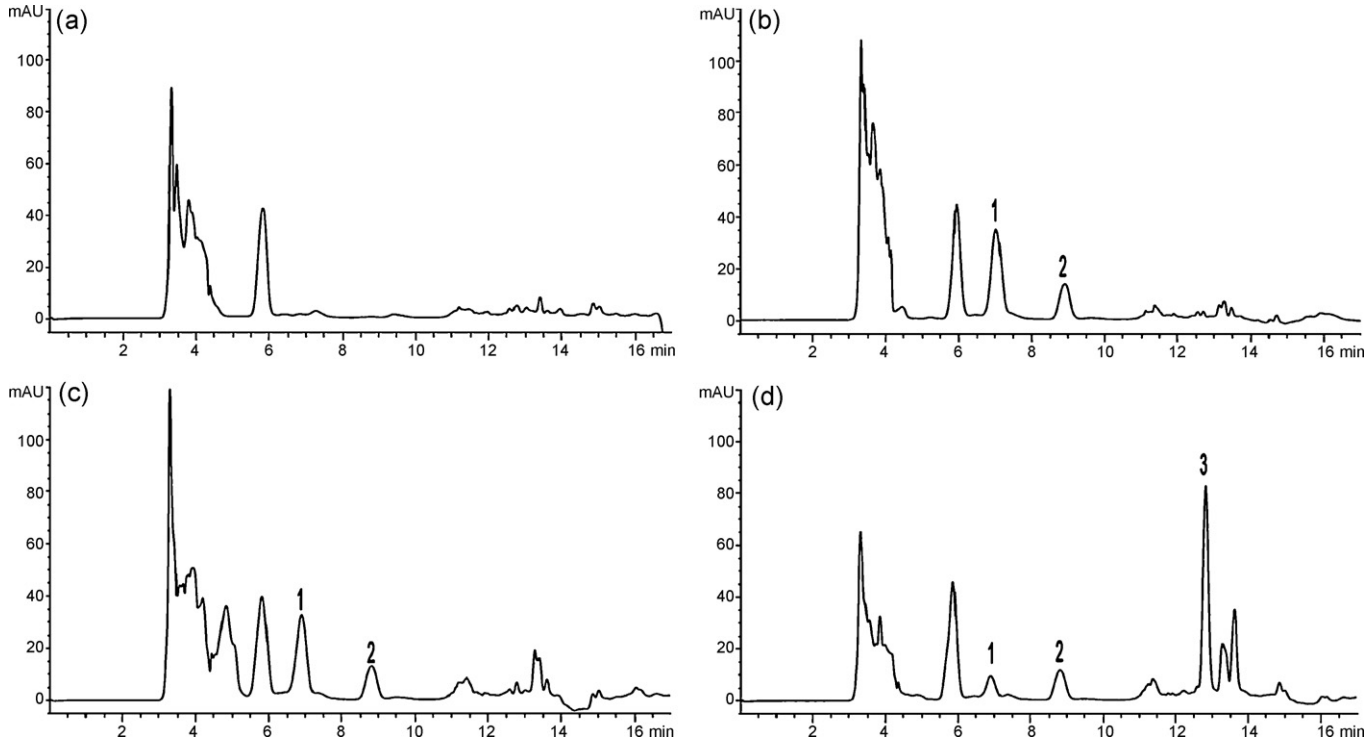

Fig. 2. HPLC-UV chromatograms of (a) a pre-dosing plasma sample, (b) a blank plasma spiked with PUE (1.6 μg/mL) and IS (7.5 μg/mL), (c) a plasma sample after an intravenous administration of PUE and (d) a plasma sample after an intravenous administration of PEG-PUE. Peak 1, PUE (t_R = 6.9 min); Peak 2, IS (t_R = 8.8 min); Peak 3, PEG-PUE (t_R = 12.8 min).

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

Conc. (ng/mL)	Intra-day ($n = 6$)		Inter-day ($n = 3$)	
	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
80	105.2	6.6	101.7	8.3
800	101.0	4.9	98.0	6.5
8000	102.0	2.9	103.8	5.1

Table 2
Stability of PUE in rat plasma.

Stability (% remained)	Spiked concentration (ng/mL)		
	80	800	8000
Post-preparative samples for 24 h	95.9 ± 3.2	96.2 ± 2.1	99.3 ± 4.4
Plasma samples after 3 freeze–thaw cycles	95.1 ± 3.5	96.0 ± 3.2	95.7 ± 2.8
Plasma samples stored at −20 °C for 20 days	95.6 ± 3.3	96.1 ± 3.8	96.2 ± 1.7

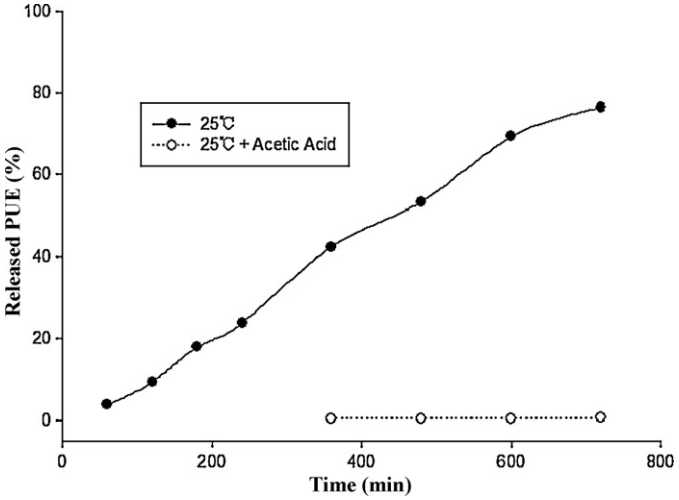

Fig. 3. Time course for *in vitro* prodrug hydrolysis in fresh (real line) or acetic acid treated (broken line) rat plasma at 25 °C ($n = 3$).

PUE extended half-life of PUE and increased the acting time of drug *in vivo*.

3.6.3. Pharmacokinetic study

The developed assay was applied to a pharmacokinetic study after intravenous administration of either PEG-PUE or PUE to rats

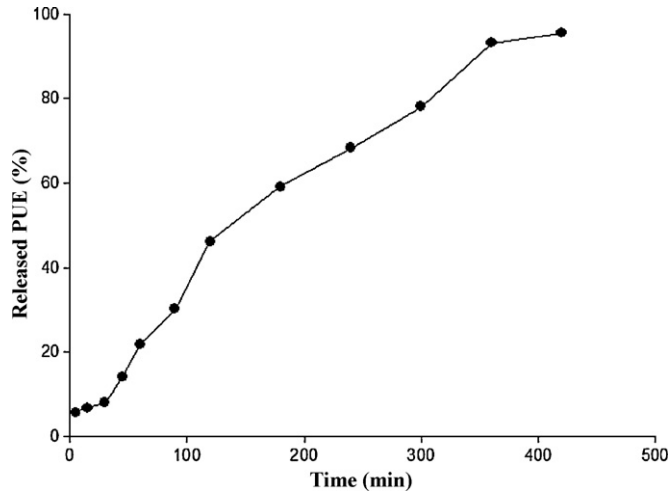

Fig. 4. *In vitro* release of PUE from PEG-PUE in rat plasma at 37 °C ($n = 3$).

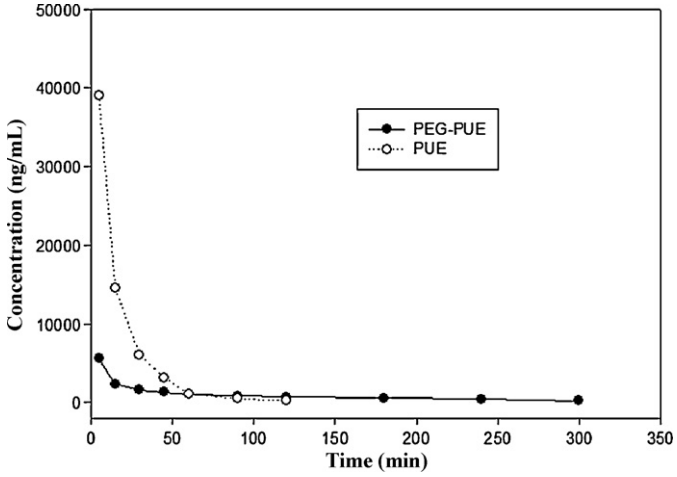

Fig. 5. The plasma concentration-time profiles of PUE after single intravenous administration of PUE (broken line) or PEG-PUE (real line) to rats at a dose of 20 mg/kg (PUE equivalent) ($n = 7$).

Table 3
Pharmacokinetic parameters of PUE and PEG-PUE.

Compound	$T_{1/2}$ (min)	MRT (min)	K10 (min^{-1})
PUE	26.9	15.7	0.073
PEG-PUE	139.3	73.6	0.029

at a dose of 5 mg/kg (PUE equivalent). A two-compartment model was proposed and validated through DAS 2.1 program. The mean plasma concentration-time profiles of PUE following intravenous administration of PEG-PUE or PUE were shown in Fig. 5, and pharmacokinetic parameters calculated from the data were summarized in Table 3. Plasma level of PUE declined rapidly, with a half-life of 26.9 min, which was consistent with the result of Gou et al. [3], whereas PEG-PUE showed a 5.2-fold increase in half-life of PUE and a 4.7-fold increase in mean residence time. These results suggested that the PEG-PUE could provide prolonged plasma level of PUE, resulting in substantial improvement in plasma exposure for better access of PUE to remote targets. However, The plasma concentration of PUE was relatively low, for the released PUE from PEG-PUE was eliminated very quickly in the metabolic process.

4. Conclusion

A sensitive and reproducible HPLC-UV method was developed and validated for the quantitative determination of PUE regenerated from its prodrug in rat plasma. Additionally, the relatively

low concentration of regenerated PUE in the presence of much higher concentration of the prodrug *in vivo* requires special sample preparation and storage methods, which prevent the degradation of PEG-PUE *in vitro*. Both of these challenges were resolved by the method reported here. This reliable method had been successfully applied in the comparative study of pharmacokinetics of PEG-PUE versus PUE in rats. Pharmacokinetic experiments revealed that PEG-PUE provided longer circulating life of PUE than PUE injection. It is probably due to a slow release from target tissue where the polymer-drug conjugate acts as a reservoir where PEG-PUE is slowly hydrolyzed. Further study on drug efficacy and safety of PEG-PUE was under way to evaluate whether the improved half-life and sustained release of PUE were sufficient for the treatment of cardiovascular diseases.

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