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Short communication

Determination of puerarin in rabbit aqueous humor by liquid chromatography tandem mass spectrometry using microdialysis sampling after topical administration of puerarin PAMAM dendrimer complex

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ABSTRACT

To study pharmacokinetic properties of puerarin poly(amido amine) (PAMAM) dendrimer complex, a sensitive liquid chromatography tandem mass spectrometry method (LC-MS/MS) was developed and validated to determine puerarin in rabbit aqueous humor using microdialysis sampling. Astilbin was used as the internal standard. The linear range for puerarin was from 2 to 1000 ng/mL (r = 0.9986) based on 20 μL of aqueous humor. The coefficients of variations for intra-day and inter-day precisions were less than 10.0%, and the relative error of accuracy was within ±6.3%. The mean extraction recovery of puerarin varied from 80.4% to 85.5%. Microdialysis provides a complete concentration versus time profile. A significant difference was observed in main pharmacokinetic parameters of C_{max}, AUC and $t_{1/2}$ between puerarin solution and puerarin PAMAM dendrimer complex. Complex formation resulted in an obvious increase in bioavailability of puerarin after topical administration to rabbit according to the above LC-MS/MS assay method.

1. Introduction

Puerarin is an isoflavone compound isolated from the radix of *Pueraria lobata* (Willd.) Ohwi [1]. It is frequently used as a therapeutic agent for cataracta glauca and ocular hypertension because of its ability to depress intraocular pressure and to improve ocular blood flow [2,3]. A current clinical product is puerarin eye drops (1%, w/v). However, puerarin eye drops typically drain from the ocular surface rapidly, which results in short residence time, short absorption time (only a few minutes), and low bioavailability (less than 5%) [4]. Puerarin poly(amido amine) (PAMAM) dendrimer complex were designed as one of new ocular drug delivery system which had indicated increase in the aqueous solubility and corneal permeability of puerarin and exhibited promise for controlled delivery of prototype drugs [5–7]. Thus, the biological half-life of puerarin could be greatly prolonged by its PAMAM dendrimer complex.

In this study, microdialysis technology was used to sample aqueous humor of rabbits which provides an important advance to the regional sampling and makes a complete concentration versus time profile can be obtained in individual animals [8,9]. Thus, the low concentration of analyte and small sample volumes of microdialysis sampling require very sensitive detection method.

A number of analytical methods have been reported for determination concentration of puerarin in biological samples, such as high performance liquid chromatography (HPLC) using ultraviolet detection (HPLC-UV) [10–12], mass spectrometry detection (LC-MS) [13,14] and tandem MS detection (LC-MS/MS) [15–17]. It is well known, the LC-MS/MS method has high sensitivity and specificity. The LC-MS/MS method reported by Wang et al. [17] had higher sensitivity with the lower limit of quantitation (LLOQ) of 0.39 ng/mL by a linear ion trap mass spectrometer than other methods by an ion trap mass spectrometer with the LLOQ of 2–10 ng/mL [15,16]. This study aimed to develop and validate a sensitive and rapid LC-MS/MS method by tandem quadrupole mass spectrometer to evaluate the pharmacokinetics of puerarin PAMAM dendrimer complex in rabbit aqueous humor with the microdialysis sampling after topical administration.

2. Experimental

2.1. Chemicals and reagents

PAMAM dendrimer with primary amine (G 3) surface groups in methyl alcohol was purchased from Sigma–Aldrich (St. Louis, MO, USA). Puerarin was purchased from Shanghai DND Pharm-Technology Co. (Shanghai, China). Puerarin PAMAM dendrimer complex was synthesized according to previous research [5] in the Pharmaceutical laboratory of Yantai University. Puerarin solu-

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tion (1% (w/v) containing 4% (w/v) polyvinylpyrrolidone K-30 as a solubilizer) was taken as control. Astilbin (Internal standard, IS) was obtained from Chengdu Mansite Pharmaceutical Co., Ltd (Chengdu, China). MAB/3 microdialysis probes (PES membrane 10 mm × 0.6 mm o.d., Microbiotech/se AB, Stockholm, Sweden) were used in the experiment. Acetonitrile was HPLC grade (Merck KgaA, Darmstadt, Germany). All other reagents were analytical grade. De-ionized distilled water was used throughout the experiments.

2.2. Animals

New Zealand albino rabbits (2.5–3.0 kg) were obtained from the experimental animal center of Luye Pharma (Yantai, Shandong, China). The animals were housed in standard cages in a light-controlled room at 19 ± 1 °C and 50 ± 5% relative humidity and given a standard pellet diet and water. All animals were healthy and free of clinically observable ocular abnormalities. All studies were conducted in accordance with the principles of Laboratory Animal Care (NIH publication no. 92–93, revised in 1985) and were approved by the local ethics committees for animal experimentation.

2.3. LC-MS/MS conditions

The LC-MS/MS system consisted of an 1100 series HPLC system (Agilent Technologies, Waldbronn, USA) and a TSQ Quantum Access tandem mass spectrometer (Thermo Electron Corporation, San Jose, CA, USA) with electrospray ionization (ESI) source. The analytical column C18 (Waters XTerra, 100 mm × 2.1 mm i.d., 3.5 μm) was used. The mobile phase consisted of a mixture of acetonitrile–ammonium acetate (0.3 mM) (40:60, v/v) used at a flow rate of 0.2 mL/min.

The mass spectrometer was operated in negative ionization mode using selective reactions monitoring (SRM) to measure puerarin and IS. The spray voltage was 3 kV. Sheath gas and auxiliary gas pressures were 30 and 5 psi, respectively. The capillary temperature was 350 °C, argon gas pressure was 1.5 mTorr. The collision induced dissociation voltage was 25 V for puerarin and IS. The transitions (precursor to product) monitored were m/z 415.1 → 295.0 for puerarin and m/z 449.1 → 285.0 for IS. The dwell time was 100 ms for each transition. Data acquisition and processing were accomplished using the Xcalibur workstation (version 1.4.1).

2.4. Stock and working solutions

The stock solutions of puerarin and IS were prepared in acetonitrile at the concentration of 1.0 mg/mL, respectively. Different concentrations of standard working solutions of puerarin were obtained by further serially diluting the stock solutions with the mobile phase. The working solution of IS at the concentration of 500 ng/mL were prepared by diluting the IS stock solution with the mobile phase. All solutions were stored under refrigeration at 4 °C.

2.5. Sample preparation

A 10 μL of the IS working solution (500 ng/mL) and 50 μL of 10% acetic acid were added to 20 μL of dialysates, followed by 30 s mixture and 3 min liquid–liquid extraction with 1 mL of ethyl acetate. After centrifugation at 2000 × g for 10 min, the organic layer was separated and evaporated to dryness under a stream of nitrogen at 40 °C. The residue was reconstituted into 100 μL of mobile phase by vortex mixing for 1 min; a 10 μL of the supernatant fluid was injected into the LC-MS/MS system for analysis.

2.6. Method validation

To evaluate linearity, the seven-point calibration curve at concentrations of 2, 5, 20, 50, 200, 500 and 1000 ng/mL was prepared by blank aqueous humor (20 μL) spiked 10 μL of corresponding standard working solutions in duplicate on 3 consecutive days and constructed by plotting peak area ratios (y) of puerarin to IS versus the spiked concentrations (x) by weighted ($1/x^2$) least square linear regression. A blank sample was prepared by adding 10 μL of mobile phase to 20 μL blank aqueous humor. The accuracy and precision were assessed by determining quality control (QC) samples of low, middle and high concentrations (2, 50 and 1000 ng/mL) in five replicates on 3 consecutive days. The concentration of each sample was calculated by calibration curve obtained daily. The accuracy was expressed as the relative error (RE) and the precision as the coefficients of variation (CV). The extraction recovery of puerarin at QC sample concentrations was determined by comparing the peak area ratio of puerarin to IS from blank aqueous humor spiked prior to extraction with those from blank aqueous humor spiked post-extraction, in five replicates, and the IS was added post-extraction to both sets of samples. The total recovery was evaluated by comparing the peak areas obtained from above extraction experiment with those from the corresponding neat standard solutions at QC sample concentrations. The stability of puerarin in aqueous humor was evaluated by QC samples placed at room temperature for 8 h and stored at −20 °C for 3 weeks in triplicate. The stability of reconstituted samples in autosampler vials was assessed at 20 °C for 24 h.

2.7. Microdialysis

The rabbits were kept under anesthesia throughout the experiment by intramuscularly injecting chloral hydrate (3.5 mg/kg). The 23-gauge needle was inserted across the cornea, just above the corneoscleral limbus. A MAB/3 microdialysis probe (cut-off molecular weight of membrane, 50 000 Da) was implanted into the aqueous humor. The relative recovery of puerarin through the membrane was 16.3% *in vitro* determined by HPLC-UV method. Then the probe was perfused with the phosphate buffer (pH 6.8) at a flow rate of 2 μL/min by means of a microinjection pump (CMA/402, CMA/Microdialysis Corporation, Sweden). After probe implantation, the animals were allowed to stabilize for 2 h. Puerarin solution (50 μL) and puerarin PAMAM dendrimer complex solution (50 μL) containing 1% (w/v) of puerarin were instilled into the cul-de-sac of the right and left eyes of each male rabbit, respectively; the eyelids were kept closed for 10 s to prevent the loss of the instilled solution. The dialysates were collected in sample cups at the other end of the polyethylene tube of probe every 20 min until 7 h after instillation and analyzed using the LC-MS/MS method.

2.8. Pharmacokinetic analysis

Drug concentration in aqueous humor was calculated from relative recovery and drug levels in dialysates. Pharmacokinetic parameters were calculated for each subject using non-compartmental methods by the program DAS (Drug and Statistics for Windows, version 2.0, Chinese Pharmacological Association). The data were expressed as mean ± standard deviation (SD).

3. Results and discussion

3.1. Optimization of analytical condition

In this assay, the sensitive and specific LC-MS/MS was implemented with isocratic elution in run time of 3 min per sample. The

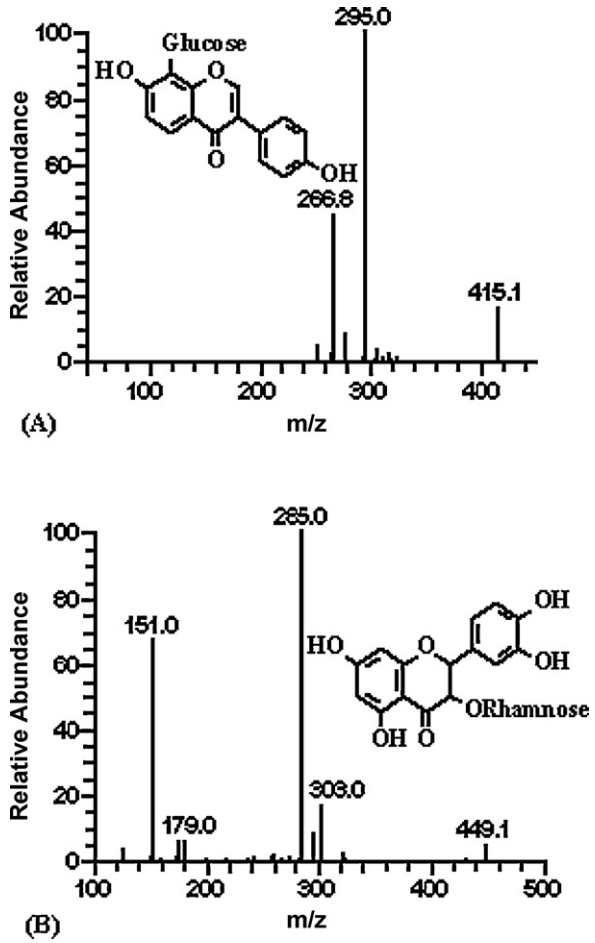

Fig. 1. Product ion spectrum of puerarin (A) and IS (B).

analytical time was reduced at least 1-fold than previous methods [10–17]. The small amounts of ammonium acetate were added to the mobile phase as a modifier that conduces to promote the deprotonation in ESI negative mode. Acetonitrile was used to separate and elute analytes, and provided symmetric chromatographic peaks and lower background noise than methanol. In the mobile phase condition, puerarin and IS produced predominantly deprotonation molecular $[M–H]^-$ in full scan spectrum. The product ion spectrum of puerarin $[M–H]^-$ at *m/z* 415.1 and the IS $[M–H]^-$ at *m/z* 449.1 was shown in Fig. 1. The structure of puerarin and IS was similar, and optimized collision induced dissociation voltage was 25 V for them.

3.2. Specificity

The specificity of the method was assessed by screening analysis of six individual rabbit blank aqueous humor and comparing the chromatograms of blank aqueous humor with the corresponding spiked aqueous humor to check the interference. The retention times of puerarin and IS were 1.6 and 2.0 min, respectively. The typical chromatogram (shown in Fig. 2) showed no significant interference peaks at the retention times of puerarin and IS.

3.3. Linearity and LLOQ

The calibration curves showed linearity over the concentration range of 2–1000 ng/mL based on 20 μL of aqueous humor. The typical calibration curve of puerarin was $y = 0.009132x - 0.001336$ ($r = 0.9986$) by the weighted least square linear regression. The LLOQ of puerarin was 2 ng/mL (chromatogram was shown in Fig. 2). The CV of intra-day and inter-day precisions of LLOQ samples were less than 8.7% and 5.2%, respectively, and the RE of accuracy was within ±4.7% (Table 1).

The higher sensitivity of tandem quadrupole mass spectrometry combined with the sample preparation of liquid–liquid extraction made the LLOQ of 2 ng/mL which was evaluated based on 20 μL of aqueous humor to equal the LLOQ of 0.39 ng/mL calculated by 100 μL of sample volumes analyzed by a linear ion trap mass spectrometer using an on-line solid-phase extraction [17].

3.4. Precision and accuracy

As shown in Table 1, the CV of intra- and inter-day precisions of puerarin were less than 9.6% and 8.1%, respectively, and the RE of accuracy was within ±6.3% at low, middle and high concentration levels. Therefore, the present method has had a satisfactory precision and accuracy, and can be applied to the pharmacokinetic study of the analyte in rabbit aqueous humors.

3.5. Recovery

The results showed that the extraction recovery were 80.4 ± 4.1%, 83.3 ± 2.8% and 85.5 ± 2.3%, and the total recovery were

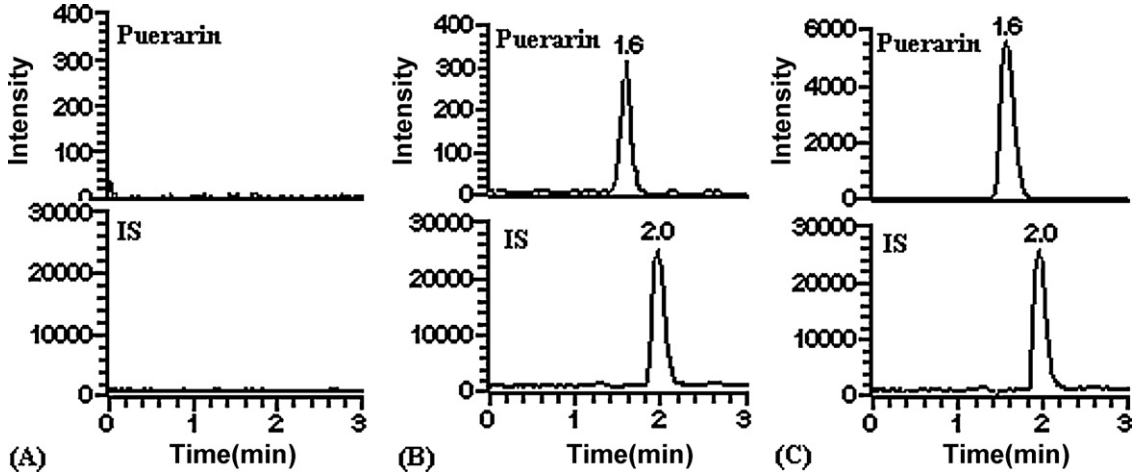

Fig. 2. SRM chromatograms of puerarin and IS for various samples: (A) blank rabbit aqueous humor; (B) blank rabbit aqueous humor spiked with LLOQ of puerarin; (C) a rabbit aqueous humor 3 h after typical administration of puerarin PAMAM dendrimer complex (0.5 mg).

Table 1
Precision and accuracy of puerarin in rabbit aqueous humor (mean ± SD).

Added concentration (ng/mL)	Measured (mean ± SD)	Precision CV (%)	Accuracy RE (%)
Intra-day (n = 5)			
2	2.094 ± 0.166	8.7	4.7
50	53.13 ± 3.75	8.5	6.3
1000	1018 ± 105	9.6	1.8
Inter-day (n = 3)			
2	2.057 ± 0.172	5.2	2.9
50	52.72 ± 4.26	5.2	5.4
1000	999.6 ± 94.4	8.1	−0.04

Table 2
Stability of puerarin in rabbit aqueous humor (mean ± SD) (n = 3).

Storage conditions	Concentration (ng/mL)					
	2		50		1000	
	Measured	RE%	Measured	RE%	Measured	RE%
8 h at room temperature	2.090 ± 0.164	4.5	49.02 ± 3.87	−2.0	943.0 ± 16.6	−5.7
24 h at autosampler	1.869 ± 0.102	−6.6	50.88 ± 1.51	1.8	1071 ± 49.9	7.1
3 weeks at −20 °C	1.911 ± 0.129	−4.5	51.36 ± 1.74	2.7	1014 ± 46.6	1.4

76.4 ± 6.6%, 77.3 ± 3.3% and 80.2 ± 2.3% at the three concentration (2, 50 and 1000 ng/mL) levels, respectively. Under the same experiment condition, the extraction recovery and total recovery of IS were 81.1 ± 2.6% and 81.7 ± 3.5%, respectively.

3.6. Stability

As shown in Table 2, the QC samples were stable after placement at room temperature for 8 h (RE < 5.7%), storage at −20 °C for 3 weeks (RE < 4.5%). Processed samples were stable up to 24 h at the autosampler tray (RE < 7.1%). These indicated that a great quantity of samples could be processed and analyzed in each analytical batch.

3.7. Pharmacokinetic parameters

The puerarin solution and puerarin PAMAM dendrimer complex solution were typically administered to rabbits at a dose of 0.5 mg. The mean aqueous humor concentration–time profiles of the two formulations are presented in Fig. 3. The pharmacokinetic parameters (Table 3) showed the significant difference between puerarin solution and puerarin PAMAM dendrimer complex (P < 0.05). After instillation of puerarin dendrimer complex solution, the C_{max} and AUC of puerarin were 1.3-fold and 2-fold higher than those after instillation of puerarin solution, respectively; the $t_{1/2}$ of drug eliminated from the aqueous humor was 2.7-fold longer than that of the puerarin solution.

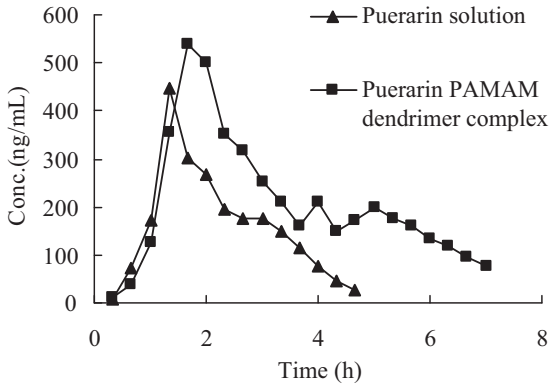

Fig. 3. Mean concentration–time profiles of puerarin in rabbit aqueous humor after typical administration of puerarin solution or puerarin PAMAM dendrimer complex at dose of 0.5 mg (n = 5).

Table 3
Pharmacokinetic parameters of puerarin in aqueous humor after instillation (mean ± SD) (n = 5).

Parameters[a]	Puerarin solution	Puerarin PAMAM dendrimer complex
C_{max} (ng/mL)	447.2 ± 37.7	582.8 ± 67.0
AUC_{0-t} (ng·h/mL)	734.6 ± 46.2	1439.2 ± 185.0
$AUC_{0-\infty}$ (ng·h/mL)	754.6 ± 49.8	1589.3 ± 163.6
$t_{1/2}$ (h)	0.48 ± 0.03	1.30 ± 0.19
MRT (h)	2.18 ± 0.12	3.20 ± 0.16
T_{max} (h)	1.33	1.67

[a] C_{max}: the maximum aqueous humor concentration; AUC_{0-t}: area under the curve from time zero to the last sampling time point; $AUC_{0-\infty}$: area under the curve from time zero to the infinity; $t_{1/2}$: elimination half-live; MRT: mean residence time; T_{max}: time to peak value.

4. Conclusions

A highly sensitive and rapid LC-MS/MS method for the quantitation of puerarin has been developed. The method has been validated with a LLOQ of 2 ng/mL based on 20 μL of aqueous humor. Using this method, pharmacokinetics of puerarin solution and puerarin PAMAM dendrimer complex were investigated after topical administration to rabbits. The puerarin PAMAM dendrimer complex significantly improved the availability of puerarin and delayed its half-life in aqueous humor of rabbits. PAMAM dendrimers are thus a promising ocular drug carrier that may improve drug bioavailability and efficacy.

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