

Validated LC–MS–MS Method for the Determination of Prodrug of Ginkgolide B in Rat Plasma and Brain: Application to Pharmacokinetic Study

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A sensitive, simple and rapid liquid chromatography–tandem mass spectrometry method was developed and validated for the simultaneous determination of ginkgolide B (GB) and the prodrug of ginkgolide B (PGB) in rat plasma and brain tissue. Detection was performed on a triple quadrupole tandem mass spectrometry in multiple reaction monitoring mode using the electrospray ionization technique in negative ionization mode. The total run time was only 2.0 min. Good linearity was found between 1–200 ng/mL ($r > 0.9993$) for plasma samples and 0.5–50 ng/mL ($r = 0.9995$) for the brain tissue samples. The lower limits of quantification of PGB and GB were 1 ng/mL for plasma and 0.5 ng/mL for brain tissue. Intra-day and inter-day precision was less than 11.67%. Intra-day accuracy was in the range of –2.61–10.67%, inter-day accuracy was in the range of –2.36–8.98%. The mean recovery for PGB and GB was between 82.5 and 97.0%. The validated method was successfully applied to a pharmacokinetic study of PGB and GB in rats after intravenous administration.

Introduction

Ginkgo biloba L. is an ancient Chinese tree that has been cultivated and held sacred for its health-promoting properties (1). Previous studies have shown that *Ginkgo biloba* extract possesses antioxidant, anti-ischemic and neuro-protective effects, and is currently used as symptomatic treatment for cerebral and vascular insufficiency, Alzheimer's dementia, aging, cognitive deficits and other age-associated impairments (2, 3). To date, eight terpene lactones have been isolated from *Ginkgo biloba*, including a sesquiterpenoid (bilobalide) and diterpenoids (ginkgolides A, B, C, M, J, K and L) (4). Ginkgolides are potent and selective antagonists of the platelet activating factor (PAF) (5, 6). In particular, ginkgolide B (GB) has been proven to be the most potent PAF receptor antagonist, which has aroused the concern of many researchers (7). However, as the therapeutic drug of cerebrovascular disease, the blood-brain barrier (BBB) permeability of GB is not yet satisfactory.

The BBB is located at the interface between blood and brain. It is designed to regulate brain homeostasis and to permit selective transport of molecules that are essential for brain function (8, 9). Therefore, the penetration of drugs through the BBB is very important for the treatment of cerebrovascular disease. To increase BBB permeability and enhance brain targeting of GB, based on preliminary laboratory studies (10–12), this study concerns one compound of a designed and synthesized prodrug of GB (PGB). The chemical structures of ginkgolide A (GA), GB and PGB are shown in Figure 1.

A sensitive and accurate analytical method for the simultaneous determination of GB and PGB is needed to support the pharmacokinetic study of prodrug. Some reports have been made about the quantitative methods for measuring GB in plasma, such as gas chromatography–mass spectrometry (GC–MS) (13, 14) and liquid chromatography–mass spectrometry (LC–MS) (15–18). However, these methods have certain limitations, including complicated sample preparation, low sensitivity and long chromatographic run times.

This paper describes the development and validation of a relatively simple, sensitive and rapid LC–MS–MS method for the quantitation of PGB and GB in rat plasma and brain. This validated method was successfully applied to the pharmacokinetic study of PGB and GB in rats after intravenous administration, and an evaluation of the brain targeting of PGB.

Experimental

Chemicals and reagents

GB and GA were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The test sample of GB was made in the authors' laboratory [content ($\geq 98\%$) is determined by the area normalization compared with standard sample], PGB was synthesized in the authors' laboratory [the chemical structure of which was confirmed by ¹H and ¹³C nuclear magnetic resonance (NMR) spectroscopy and the purity of which was over 99% by HPLC analysis]. Methanol was of HPLC grade (Tedia, Fairfield, OH). Analytical grade ethyl acetate was from Sinopharm Chemical Reagent Co. (Shanghai, China). Ammonium acetate was from Shantou Xilong Chemical Factory (Guangdong, China). Purified water used in the study was commercially available (Wahaha, Hangzhou, China).

Instrument and LC–MS–MS conditions

Liquid chromatography

Liquid chromatography was performed on an Agilent 1100 series (Palo Alto, CA) analyzer consisting of an autosampler. The separation was achieved on a Waters Symmetry shield RP18 column (3.9 × 150 mm, 5 μm) with an XDB-C18 guard column (2.1 × 30 mm, 3.5 μm, Agilent Technologies) at a column temperature of 40°C. The mobile phase consisted of methanol–ammonium acetate (10 mM) (85:15, v/v) at a flow rate of 0.8 mL/min, and the injection volume was 10 μL.

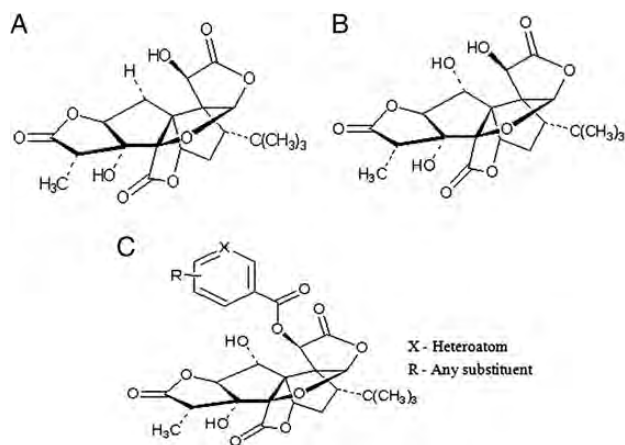


Figure 1. Chemical structures: GA (A); GB (B); PGB (C).

Mass spectrometer conditions

The detection was performed on an API 3200 triple quadrupole mass spectrometer (Applied Biosystems, Ontario, Canada) equipped with an electrospray ionization (ESI) interface, operating in negative ionization mode. The final ESI conditions used were as follows: ionspray voltage was set at $-4,500$ V and ion source temperature was 500°C , nebulizing gas pressure was 40 psi, auxiliary gas pressure was 40 psi and curtain gas pressure was 15 psi. Quantitation was performed using multiple reaction monitoring (MRM) of the transitions of m/z $543.2 \rightarrow 137.0$ for PGB, $423.4 \rightarrow 367.3$ for GB and $407.5 \rightarrow 351.2$ for GA. The Analyst 1.4.1 software package was used for data acquisition and processing.

Preparation of standards and quality control samples

A stock solution of GB and PGB was prepared in methanol ($400 \mu\text{g}/\text{mL}$) and serially diluted with methanol to produce working solutions. A stock solution of GA ($200 \mu\text{g}/\text{mL}$) was also prepared in methanol and the same method was used to produce a final concentration of $4 \mu\text{g}/\text{mL}$.

Calibration standards were prepared by mixing $190 \mu\text{L}$ biological samples with $5 \mu\text{L}$ GB or PGB working solutions and $5 \mu\text{L}$ GA solution ($4 \mu\text{g}/\text{mL}$). The concentration ranges were 1 to $200 \text{ ng}/\text{mL}$ in blank plasma, and 0.5 to $50 \text{ ng}/\text{mL}$ in blank brain homogenates for GB and PGB. The quality control (QC) samples were prepared with plasma and brain homogenates at the concentrations of 1, 25 and $200 \text{ ng}/\text{mL}$ and 0.5, 5 and $50 \text{ ng}/\text{mL}$ for GB and PGB, respectively. All standard samples and QC samples were stored at -20°C until use.

Sample preparation

A $190 \mu\text{L}$ volume of rat plasma was placed into an eppendorf tube before $5 \mu\text{L}$ of $4 \mu\text{g}/\text{mL}$ GA solution was added. The mixture was briefly vortexed and extracted with 1 mL of ethyl acetate on a vortex-mixer for 3 min. After centrifugation at $12,000 \text{ rpm}$ for 10 min at 4°C , the upper organic phase was transferred to another tube and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was

Table 1
Mass Parameters for PGB, GB and GA

Analyte	Ion-pairs (m/z)	Declustering potential (V)	Entrance potential (V)	Collision energy (V)	Collision cell entrance potential (V)	Collision cell exit potential (V)
PGB	$543.2 \rightarrow 137.0$	-40	-10	-30	-22	-1
GB	$423.4 \rightarrow 367.3$	-30	-10	-20	-10	-3
GA	$407.5 \rightarrow 351.2$	-15	-10	-22	-20	-5

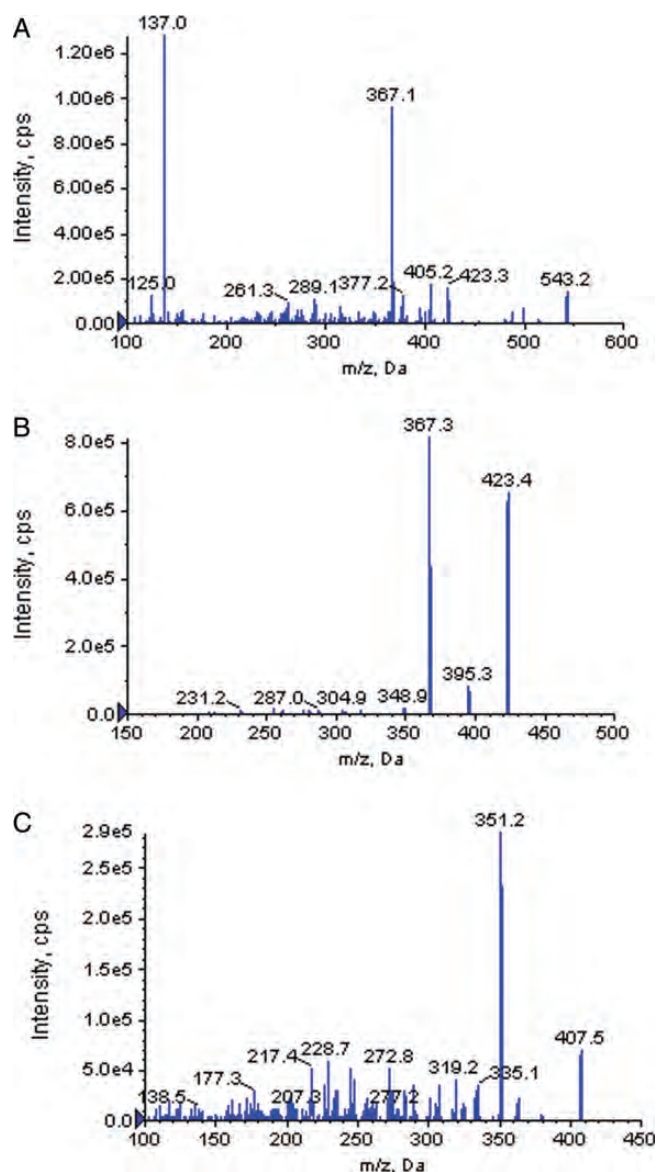


Figure 2. Full-scan production ion spectra of $[\text{M}-\text{H}]^{-}$ ions and fragmentation pathways: PGB (A); GB (B); GA (C).

reconstituted in $400 \mu\text{L}$ mobile phase followed by vortex-mixing, and the mixture was centrifuged at $12,000 \text{ rpm}$ for 10 min. The $300 \mu\text{L}$ supernatant was collected and diluted 10 times, and $10 \mu\text{L}$ was injected into the LC-MS-MS system for analysis.

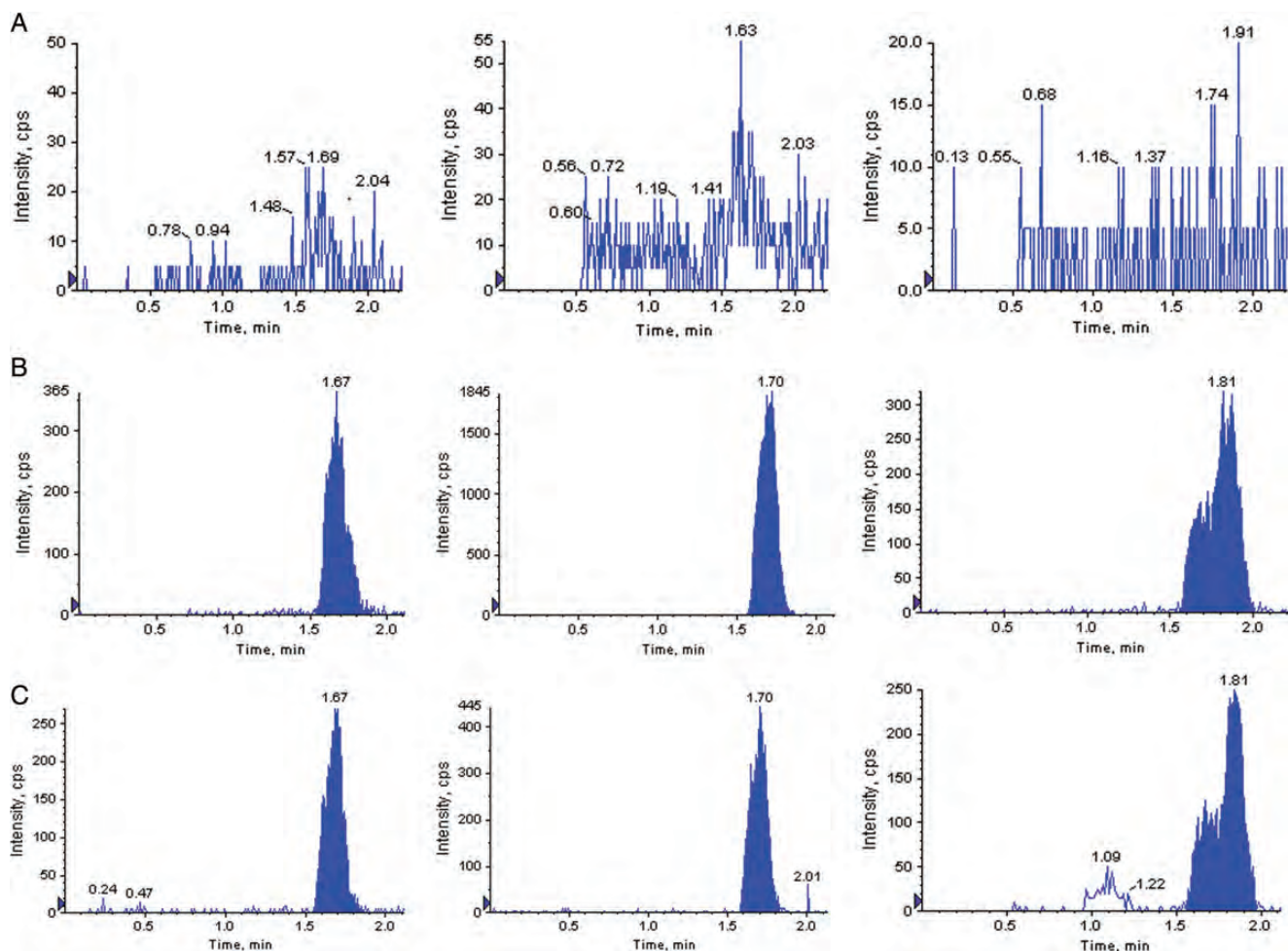


Figure 3. Typical chromatograms: blank plasma (A); blank plasma spiked with PGB (50 ng/mL), GB (25 ng/mL) and GA (4 μ g/mL) (B); plasma sample collected 1.5 h after intravenous administration of PGB and GB (C).

Brain tissue was homogenized with four times of normal saline and then 5 μ L of 4 μ g/mL GA solution was added to 190 μ L of brain homogenate. Subsequent steps were identical to those described previously for the analysis of plasma samples, but supernatant was then analyzed directly using LC-MS-MS without dilution.

Method validation

Method validation was conducted according to the Food and Drug Administration (FDA) guidelines for bioanalytical methods (19).

Specificity

Specificity was assessed by comparing the chromatograms of six different batches of blank plasma and brain homogenate samples, blank plasma and brain homogenate samples spiked with the PGB and GB, and rat plasma and brain homogenate samples after intravenous administration of PGB and GB.

Linearity and lower limit of quantification

Calibration curves for PGB and GB were constructed by plotting peak area ratio (y) of analytes to internal standard (IS)

versus concentration of analytes (x), and the results were fitted to linear regression analysis using $1/x^2$ as weighting factor. The lower limit of quantification (LLOQ) was defined as the lowest concentration in the calibration curve that could be determined with a relative error and precision (relative standard deviation, RSD) of less than 20%.

Precision and accuracy

Intra-day precision and accuracy were calculated by analysis of QC samples at three concentrations on the same day, and inter-day precision and accuracy were calculated on three sequential days. The precision was expressed using RSD, and the accuracy was expressed as the percent difference between the averaged measurements and the nominal values described as the relative error (RE). The acceptable precision and accuracy should be within 15%.

Recovery and matrix effect

The recovery and absolute matrix effect of PGB and GB were evaluated using three sets of samples. Set 1: analytes were added to five different lots of blank sample and then extracted; Set 2: analytes were added to five different lots of post-extracted blank sample; Set 3: analytes were dissolved in the

reconstitution solvent. The peak areas obtained in Set 1 were defined as A, the corresponding peak areas in Set 2 as B and in Set 3 as C. The recovery and absolute matrix effect were calculated as follows:

$$\text{Recovery (\%)} = \text{Mean A} / \text{Mean B} \times 100$$

$$\text{Absolute matrix effect (\%)} = \text{Mean B} / \text{Mean C} \times 100$$

Stability

The stability of PGB and GB using QC samples was evaluated under a variety of storage and process conditions. The short-term stability of PGB and GB was determined after exposure of QC samples at 25°C for 4 h. Long-term stability was assessed after storage of QC samples at -20°C for 30 days. The freeze-thaw stability was evaluated at three freeze-thaw cycles.

Pharmacokinetic study

Male and female Sprague-Dawley rats (weighing 200 ± 20 g) were obtained from the Experimental Animal Center of Anhui Pharmaceutical University (Hefei, China). They were maintained in a temperature-controlled environment with a 12 h light/dark cycle breeding room for three days and fasted

overnight before the experiment. All protocols and procedures were approved by the Institutional Animal Care and Use Committee.

The rats were randomly divided into two groups. One group was given GB solution via tail vein at a dose of 10 mg/kg, and another group was given PGB solution via tail vein at a dose of 12.83 mg/kg. After intravenous administration of PGB and GB to rats, blood and brain tissue samples were collected corresponding to 0, 5, 10, 30, 60, 90, 120, 240, 360 and 480 min time points (six rats at each time point). The plasma samples were immediately centrifuged for 10 min at 4,000 rpm and stored at -20°C until analysis. The brain tissue samples were rinsed with normal saline solution (0.9%, w/v), wiped dry and stored at -70°C until sample preparation. The pharmacokinetic parameters were calculated using 3p97 software (Mathematical Pharmacology Professional Committee of China, Shanghai, China). The area under the concentration-time curve (AUC) was calculated from time zero to infinity. To quantitatively evaluate the brain targeting of PGB, drug targeting index (DTI) was introduced as follows:

$$\text{DTI} = \frac{(\text{AUC}_{\text{brain}}/\text{AUC}_{\text{plasma}})_{\text{PGB}}}{(\text{AUC}_{\text{brain}}/\text{AUC}_{\text{plasma}})_{\text{GB}}}$$

Table II

Calibration Curves, Correlation Coefficients and Linear Ranges of PGB and GB in Plasma and Brain Tissue

Analyte	Matrix	Calibration curve	Correlation coefficients (R)	Linear range (ng/mL)
PGB	Plasma	$y = 0.0011x + 0.0205$	0.9993	1–200
	Brain	$y = 0.0180x + 0.0110$	0.9995	0.5–50
GB	Plasma	$y = 0.0017x + 0.0752$	0.9996	1–200
	Brain	$y = 0.0291x + 0.0001$	0.9995	0.5–50

Results and Discussion

Method development

To improve the sensitivity of the analyte, mass parameters were optimized by infusing a standard solution of each analyte into the mobile phase using a syringe pump. Under the optimal mass parameters, strong and stable signals of PGB, GB and IS were observed in the form of their $[M-H]^-$ molecular ions with

Table III

Precision and Accuracy Data for the Determination of PGB and GB in Rat Plasma and Brain Tissue

Analyte	Matrix	Added (ng/mL)	Intra-day (n = 6)			Inter-day (n = 3)		
			Found (ng/mL)	RSD (%)	RE (%)	Found (ng/mL)	RSD (%)	RE (%)
PGB	Plasma	1	1.02 ± 0.03	2.94	2.00	1.03 ± 0.01	0.97	3.00
		25	25.79 ± 2.06	7.99	3.16	24.93 ± 1.48	5.94	-0.28
		200	221.34 ± 15.15	6.84	10.67	204.51 ± 7.52	3.68	2.26
	Brain	0.5	0.52 ± 0.04	7.69	4.00	0.53 ± 0.02	3.77	6.00
		5	4.97 ± 0.51	10.26	-0.60	5.03 ± 0.15	2.98	0.60
		50	52.43 ± 6.12	11.67	4.86	54.49 ± 3.95	7.25	8.98
GB	Plasma	1	0.99 ± 0.04	4.04	-1.00	0.99 ± 0.07	7.07	-1.00
		25	25.10 ± 0.69	2.75	0.40	24.41 ± 1.19	4.88	-2.36
		200	194.78 ± 4.78	2.45	-2.61	202.66 ± 5.26	2.60	1.33
	Brain	0.5	0.53 ± 0.01	1.89	6.0	0.51 ± 0.01	1.96	2.00
		5	5.04 ± 0.17	3.37	0.80	4.98 ± 2.06	1.20	-0.40
		50	50.36 ± 0.75	1.49	0.72	50.27 ± 0.43	0.86	0.54

Table IV

Recovery and Absolute Matrix Effect of PGB and GB in Rat Plasma and Brain Tissue

Matrix	Concentration (ng/mL)	Recovery (%)				Absolute matrix effect (%)	
		PGB	IS	GB	IS	PGB	GB
Plasma	1	82.5 ± 4.3		91.3 ± 2.7		105.3 ± 2.7	101.5 ± 3.9
	25	85.7 ± 1.8	88.3 ± 1.9	96.9 ± 6.0	95.3 ± 1.6	99.1 ± 1.4	101.2 ± 0.5
	200	87.4 ± 2.1		97.0 ± 5.1		97.2 ± 3.0	98.8 ± 0.8
Brain	0.5	88.1 ± 2.6		89.4 ± 2.5		100.5 ± 1.4	99.2 ± 1.3
	5	90.3 ± 1.9	92.1 ± 3.2	90.3 ± 4.2	92.1 ± 3.5	102.6 ± 2.1	99.5 ± 2.0
	50	91.8 ± 2.0		88.7 ± 1.9		96.3 ± 3.5	103.4 ± 3.6

Table V
Stability of PGB and GB in Rat Plasma and Brain Tissue

Analyte	Matrix	Concentration (ng/mL)	RE (%)		
			4 h (25°C)	30 days (-20°C)	Three freeze-thaw cycles
PGB	Plasma	1	1.9	2.8	1.7
		25	2.3	1.6	2.1
		200	4.4	3.3	3.5
	Brain	0.5	2.1	3.2	2.6
		5	2.0	1.8	2.8
GB	Plasma	1	4.1	4.6	3.1
		25	2.5	3.0	3.3
		200	2.7	2.9	4.0
	Brain	0.5	1.8	1.7	2.7
		5	4.1	2.9	1.7
		50	2.7	3.5	3.3
		50	3.4	4.3	2.8

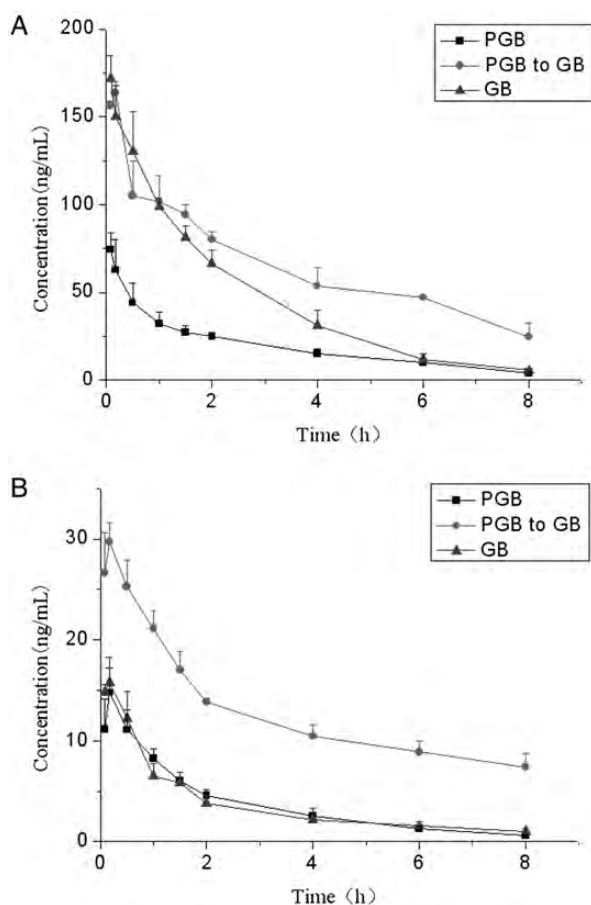


Figure 4. Concentration-time curves of PGB and GB decomposed from PGB and GB (original drug) after intravenous administration of PGB and GB to rats (mean \pm SD) ($n = 6$): plasma concentration-time curve (A); brain concentration-time curve (B).

mass to charge ratios of m/z 543.2, 423.4 and 407.5, respectively. The detailed parameters of the analytes are shown in Table I. Full-scan production ion spectra of $[M-H]^-$ ions and fragmentation pathways of PGB, GB and GA are shown in Figure 2.

Various extraction solvents and protein precipitants were investigated for the optimal recovery. Among them, ether, ethyl

acetate, chloroform and *n*-hexane were used as extraction solvents, and acetonitrile, methanol and ammonium sulfate were used as protein precipitants. The results showed that protein precipitant has little effect on the extraction recovery. Among the solvents, ethyl acetate offered the most the satisfactory recovery. Finally, ethyl acetate was selected for extraction solvent without protein precipitation.

Method validation

Specificity

The selectivity was evaluated by comparing the chromatograms of blank plasma and brain homogenate samples, blank plasma and brain homogenate samples spiked with PGB and GB, and rat plasma and brain homogenate samples after intravenous administration of PGB and GB, which showed no endogenous interference at the retention times of PGB, GB and GA. The typical chromatograms are shown in Figure 3 (plasma samples are shown as an example). The analytes and IS were separated from the biological background with retention times of 1.81, 1.70 and 1.67 min, respectively. The total analysis time for each run was 2.0 min.

Linearity and LLOQ

The standard curves of the peak area (y) to the concentration (x) were constructed using a $1/x^2$ weighted linear least-squares regression model. The calibration curves, correlation coefficients and linear ranges of PGB and GB in plasma and brain tissue are listed in Table II. The standard curves showed good linearity in the concentration range for PGB and GB. The LLOQ was 1 ng/mL for plasma and 0.5 ng/mL for brain tissue.

Precision and accuracy

Precision and accuracy were satisfactory at the three investigated concentrations (Table III). Intra-day and inter-day precisions were less than 11.67%. Intra-day accuracy ranged from -2.61 to 10.67% and inter-day accuracy ranged from -2.36 to 8.98%. The preceding results demonstrate that all of these values were within the acceptable range of $\pm 15\%$ and the method was accurate and precise.

Recovery and matrix effect

The recovery and absolute matrix effect data of PGB and GB are shown in Table IV. The mean recovery for PGB, GB and IS were between 82.5 and 97.0%, and no significant matrix effect was observed for PGB and GB.

Stability

The stability of PGB and GB in plasma and brain tissue was investigated at different concentrations under a variety of storage conditions. The analytical results showed that PGB and GB were stable under various conditions (Table V).

Pharmacokinetic study

The validated method was successfully applied to the pharmacokinetic study in rat after intravenous administration of 12.83 mg/kg of PGB and 10 mg/kg of GB. The mean plasma concentration-time curve and mean brain concentration-time curve are shown in Figure 4. The primary pharmacokinetic

Table VI

Pharmacokinetic Parameters of PGB, GB (Decomposed from PGB) and GB (Original Drug) in Plasma and Brain Tissue after Intravenous Administration of PGB and GB

Matrix	Analyte	Parameters					
		AUC _{0-∞} (μg/L/h)	AUC _{0-t} (μg/L/h)	T _{1/2} (h)	MRT (h)	Vd (L/kg)	CL (L/h/kg)
Plasma	PGB	164.14 ± 24.69	150.60 ± 22.46	2.40 ± 0.16	2.46 ± 0.21	145.49 ± 36.66	78.17 ± 13.20
	GB (decomposed from PGB)	648.78 ± 286.30	518.20 ± 27.65	3.68 ± 0.56	2.95 ± 0.08	67.37 ± 6.10	19.78 ± 5.92
	GB(original drug)	390.87 ± 26.44	378.00 ± 29.94	1.64 ± 0.03	2.00 ± 0.01	55.72 ± 6.54	26.46 ± 2.08
Brain	PGB	31.08 ± 4.12	29.13 ± 4.03	2.15 ± 0.40	2.11 ± 0.02	947.01 ± 236.45	412.75 ± 51.88
	GB (decomposed from PGB)	234.03 ± 79.81	101.37 ± 8.88	12.39 ± 3.12	3.06 ± 0.12	426.48 ± 53.08	54.82 ± 21.03
	GB(original drug)	34.05 ± 8.50	28.74 ± 2.76	3.72 ± 1.07	2.16 ± 0.22	574.01 ± 16.82	293.67 ± 105.27

Vd: apparent volume of distribution; CL: Clearance

parameters of PGB and GB in plasma and brain tissue are listed in Table VI.

The results showed that in the pharmacokinetics of PGB, GB decomposed from PGB and GB was described with a two-compartment model. The elimination half-life ($T_{1/2}$) of PGB, GB decomposed from PGB and GB in plasma was 2.40, 3.68 and 1.64 h, respectively. The mean residence time (MRT) of PGB, GB decomposed from PGB and GB in plasma was 2.46, 2.95 and 2.00 h, respectively. These results suggested that, compared with administration of the original drug of GB, the decreased clearance rate of GB in plasma and brain tissue was decreased by the prodrug method. The brain concentration-time profiles show that concentrations of GB decomposed from PGB reached maximum at 10 min and maintained a high concentration at 8 h. Compared with GB (original drug), the C_{max} value of GB decomposed from PGB was 1.89-fold. The $T_{1/2}$ and MRT of PGB, GB decomposed from PGB and GB in brain tissue were 2.15, 12.39 and 3.72 h and 2.11 and 3.06 and 2.16 h, respectively. These results indicated that PGB could be transported into the brain and then quickly decomposed to GB.

The calculated DTI value of PGB was 2.17, which suggested that the brain targeting was improved after intravenous administration of PGB.

Conclusion

A simple, rapid, sensitive and selective LC-MS-MS method has been developed and validated for the determination of PGB in rat plasma and brain tissue. With this method, the total analysis time for each run was 2.0 min, and LLOQs of PGB and GB were 1 ng/mL for plasma and 0.5 ng/mL for brain homogenate. The calculated DTI value of PGB was 2.17, and brain targeting of PGB was significantly improved. The method was successfully applied to a pharmacokinetics study of PGB and GB after intravenous administration, and the result showed that the method is suitable for routine high throughput analyses, which developed the foundation for the further development of prodrugs of GB.

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