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Short communication

Development and validation of a high-performance liquid chromatographic method for determination of pinocembrin in rat plasma: Application to pharmacokinetic study

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ABSTRACT

A sensitive and specific reversed-phase high-performance liquid chromatography with ultraviolet detection (RP-UV-HPLC) method has been developed and validated for the identification and quantification of pinocembrin in rat plasma using chrysin as the internal standard. Following protein precipitation with acetonitrile, the analytes were separated by the mobile phase 0.01 M ammonium acetate (pH 4.0)–methanol (35:65, v/v) with an Agilent TC-C18 column (5 μm, 4.6 mm × 150 mm) at a flow rate of 1 ml/min, column temperature 40 °C and detection wavelength 290 nm. A good linear relationship was obtained in the concentration range studied (0.07–133.33 μg/ml, r = 0.9995). The lowest limit of quantification (LLOQ) was 66.7 ng/ml and the lowest limit of detection (LLOD) was 25 ng/ml. Average recoveries ranged from 93.9 to 97.8% in plasma at the concentrations of 0.33 and 33.33 μg/ml. Intra- and inter-batch relative standard deviations were 0.15–2.03 and 1.18–9.96%, respectively. This method was successfully applied to the pharmacokinetic studies in rats after intravenous administration of pinocembrin.

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1. Introduction

Propolis is a resinous substance collected by honeybees from various plant sources. It is one of the most heterogeneous natural products of plant origin. Most propolis samples share considerable similarity in their overall chemical nature. Propolis has been used extensively in folk medicine for many years. Research of the flavonoids in propolis has been prompted by their beneficial effects on health. In propolis, pinocembrin (5,7-dihydroxyflavanone, Fig. 1) is the flavonoid at the highest concentration [1]. As flavonoids with a broad spectrum of pharmacological activities, more and more biological actions of pinocembrin have been reported, including anti-microbial [2,3], anti-oxidant [4], anti-inflammatory [5], endothelium-relaxation effects [6], and neuroprotective effects [7–9].

Up to now, only a few studies regarding the pharmacokinetic profiles of pinocembrin have been described [10,11]. The aim of this study was developing and validating a simple, rapid, sensitive and reproducible HPLC method for the determination of pinocembrin in plasma suitable for the subsequent pharmacokinetic study after intravenous (i.v.) administration to rats.

2. Experimental

2.1. Chemicals and reagents

Pinocembrin (purity >98%) was kindly provided by Prof. Song Wu (Institute of Materia Medica, Chinese Academy of Medical Sciences). Chrysin (purity >98%, internal standard) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol and acetonitrile were of chromatographic grade (Fisher Company Inc., USA). All other reagents were of analytical grade. Milli-Q water (Millipore, Bedford, MA) was used throughout the study.

2.2. Animals

Twelve male Sprague–Dawley rats, weighing 240–270 g, were supplied by Vitalriver Experimental Animal Ltd. (Beijing, China). The rats were housed under controlled environmental conditions (temperature, 23 ± 1 °C; humidity, 55 ± 5%) with a commercial food diet and water freely available. Animal experiments were carried out according to institutional guidelines for the care and the use of

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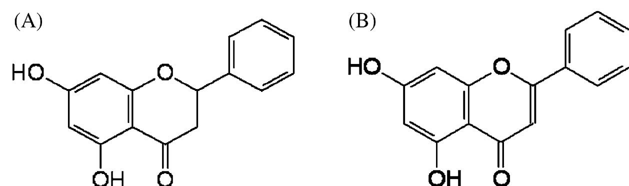

Fig. 1. Chemical structures of pinocembrin (A) and internal standard chrysin (B).

laboratory animals, and approved by the Animal Ethics Committee of Chinese Academy of Medical Sciences.

2.3. Preparation of stock solutions, standards and quality control samples

The methanolic stock solutions of pinocembrin and the internal standard (I.S.) chrysin (1 mg/ml each) were stored at 4 °C in glass bottles until being used. Individual working solutions were prepared by diluting stock solutions with methanol. The concentration of the working solution of the I.S. was 0.25 mg/ml.

Rat plasma calibration standards of pinocembrin were prepared by adding 10 μl working solution within the concentration range of 0.07–133.33 μg/ml into 150 μl of drug-free rat plasma. For pinocembrin, concentration points were 0.07, 0.33, 0.67, 3.33, 6.67, 33.33, 66.67, 133.33 μg/ml. These concentration ranges covered the plasma concentrations expected in our experimental studies. Quality control (QC) samples were prepared in the same way as calibration. QC sample concentrations were 0.33, 3.33 and 33.33 μg/ml and stored in glass tubes at −20 °C until analysis.

2.4. Liquid chromatographic conditions

The HPLC system consisted of an Agilent 1100 series (Vacuum Degasser G1379A, Quaternary Pump G1311A, autosampler G1313A, diode-array detector (DAD) and system control unit G1315B) and a Thermostatted Column Compartment WC/09-05. Agilent ChemStation Version B.02.01-SR1 was used for data acquisition and processing.

HPLC separation was achieved using an Agilent TC-C18 column (5 μm, 4.6 mm × 150 mm, Agilent) with a guard column (TC-C18, 4.6 mm × 10 mm, Agilent) maintained at 40 °C. The mobile phase consisted of 0.01 M ammonium acetate (pH 4.0)–methanol (35:65, v/v) at a flow rate of 1 ml/min. The HPLC run time was 10 min. Detection conditions were optimized. The detection wavelength was 290 nm to obtain maximum sensitivity.

2.5. Sample preparation

Each collected blood sample was immediately centrifuged at 4000 rpm for 5 min and then transferred into an Eppendorf tube. A 150 μl volume of blank plasma, calibration standards, QC samples and plasma samples, spiked with I.S. working solution (0.25 mg/ml), were pre-treated by protein precipitation with acetonitrile (0.3 ml). After centrifugation at 15,500 rpm for 10 min at 4 °C twice, the supernatant was transferred immediately to 1.5 ml auto sampler vial for HPLC analysis.

2.6. Method validation

The method validation assays were carried out according to the currently accepted U.S. Food and Drug Administration (FDA) bioanalytical method validation guidance [12].

2.6.1. Sensitivity and specificity

The lowest limit of quantitation (LLOQ) was determined as the minimum concentration that could be accurately and precisely (±5%) quantified. The lowest limit of detection (LLOD on column) was defined as the amount that could be detected with a signal-to-noise ratio of 3. The specificity of the method was evaluated by analyzing blank plasma samples from six rats. Each blank sample was tested for interference using the proposed protein precipitation procedure and HPLC conditions, and compared with those obtained with an aqueous solution of the analyte at a concentration near the LLOQ.

2.6.2. Linearity

Calibration curves of eight concentrations of pinocembrin ranging from 0.07 to 133.33 μg/ml were assayed (weighting factor = $1/C$). Blank plasma samples were analyzed to confirm the absence of interferences but were not used to construct the calibration function.

2.6.3. Precision and accuracy

The precision of the assay was determined from the QC plasma samples by replicate analyses of three concentration levels of pinocembrin (0.33, 3.33 and 33.33 μg/ml). Intra-batch precision and accuracy were determined by repeated analyses of the group of standards on one batch ($n = 5$). Inter-batch precision and accuracy were determined by repeated analyses on three consecutive days ($n = 5$ series per day). The concentration of each sample was determined using the calibration curve prepared and analyzed on the same batch.

2.6.4. Extraction recovery

The recovery of pinocembrin was determined at low, medium and high concentrations. Recoveries were calculated by comparing the analyte/I.S. peak area ratios ($R1$) obtained from plasma samples with those ($R2$) from the standard solutions at the same concentration.

2.6.5. Stability

2.6.5.1. Freeze and thaw stability. QC plasma samples at three concentration levels were stored at the storage temperature (−20 °C) for 24 h and thawed at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions. The freeze–thaw cycles were repeated twice, then the samples were analyzed.

2.6.5.2. Short-term temperature stability. QC plasma samples at three concentration levels were kept at room temperature for a period that exceeded the routine preparation time of the samples (around 6 h).

2.6.5.3. Long-term stability. QC plasma samples at three concentration levels kept at low temperature (−20 °C) were studied for a period of 2 weeks.

2.6.5.4. Postpreparative stability. The autosampler stability was conducted by reanalyzing QC samples kept under autosampler conditions for 12 h.

2.6.5.5. Stock solution stability. The stability of pinocembrin and the I.S. working solutions were evaluated at room temperature for 4 days.

2.6.6. Standard curves and quality control samples in each batch

Standard curves were used to calculate the concentrations of pinocembrin in the unknown samples. Quality control samples (five duplicates at three concentrations) were analyzed after every five unknown samples.

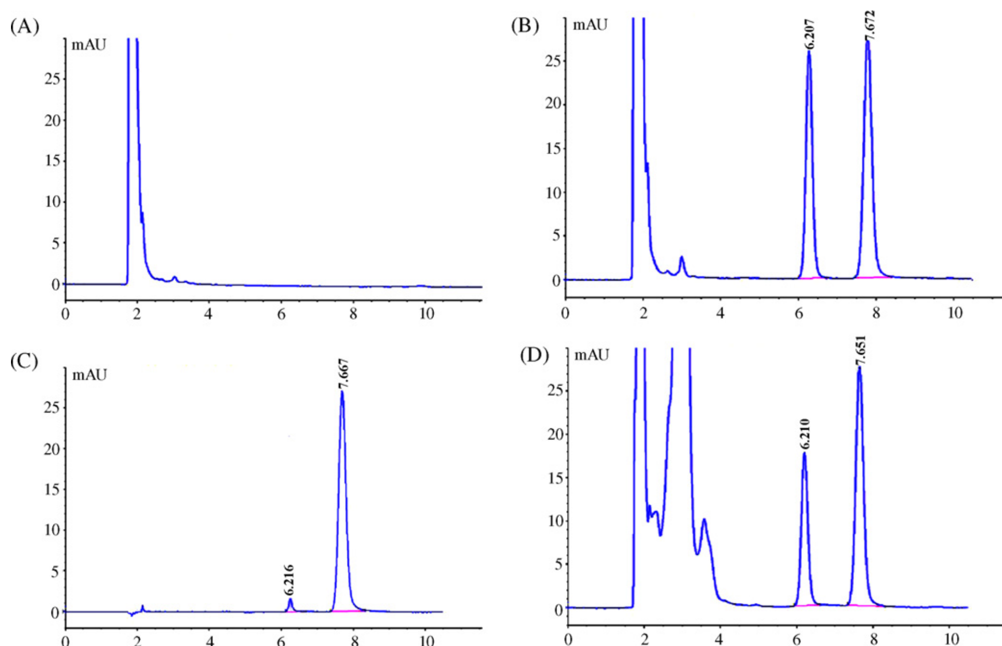

Fig. 2. HPLC chromatograms of (A) blank plasma; (B) blank plasma spiked with pinocembrin (1.25 μg/ml) and chrysin (1 μg/ml); (C) LLOQ standard; (D) a rat plasma sample 60 min after i.v. administration of pinocembrin (67.5 mg/kg).

2.7. Application to pharmacokinetic study

The developed HPLC assay method was used in the pharmacokinetic study after i.v. 22.5 and 67.5 mg/kg administration of pinocembrin to rats. Twelve male Sprague–Dawley rats (240–270 g), were obtained from Vitalriver Experimental Animal Ltd. (Beijing, China). The rats were housed under controlled environmental conditions (temperature, 23 ± 1 °C; humidity, 55 ± 5%) with a commercial food diet with free access to water. The rats were acclimatized to the facilities for 5 days, and then fasted with free access to water for 12 h prior to experiment.

Pinocembrin powder dissolved in isotonic saline was delivered using a 1 ml syringe into a rat's femoral vein. The preparations were made immediately before drug administration. About 0.3 ml blood samples via the post-orbital venous plexus veins were collected in heparinized tubes at 3, 5, 10, 15, 30, 45, 60, 90 and 120 min after i.v. 22.5 and 67.5 mg/kg administration, respectively. The blood sample was transferred into a heparinized eppendoff tube, mixed gently, and then centrifuged (4000 rpm, 5 min) to obtain 150 μl plasma, which was kept at −20 °C until analysis.

To determine the pharmacokinetic parameters of pinocembrin, the concentration–time data were analyzed by non-compartmental method using the DAS Software (version 1.0, Medical College of Wannan, China). The elimination half-life ($T_{1/2}$) was determined by linear regression of the terminal portion of the plasma concentration–time data. The area under the plasma concentration–time curve from zero to the last measurable plasma concentration point (AUC0–τ) was calculated by the linear trapezoidal method.

3. Results and discussions

3.1. Selection of internal standard

Chrysin was chosen for quantification as the I.S. due to its similarity with pinocembrin in structure, chromatographic behaviour, and high stability.

3.2. Liquid chromatography

Representative chromatograms for pinocembrin and chrysin in actual plasma samples are presented in Fig. 2. The shapes of peaks were found to be fine. There were no endogenous compounds or other impurities interfering with the assay. The retention time of pinocembrin and chrysin were approximately 6.2 and 7.6 min, respectively. The overall chromatographic run time was less than 10 min.

3.3. Method validation

3.3.1. Linearity

The linear regression analysis of pinocembrin was constructed by plotting the peak-area ratio of pinocembrin against the I.S. (R) versus analyte concentration (μg/ml) in spiked plasma samples (C). The calibration curves were constructed in the range of 0.07–133.33 μg/ml. The average regression equation of these curves and their correlation coefficients (r) were as follows: $R = 0.1072C–0.0013$ ($r = 0.9995$, $n = 5$). It showed good linear relationships between the peak areas and the concentrations. For pinocembrin, the LLOQ was 66.7 ng/ml and the LLOD was 25 ng/ml.

3.3.2. Accuracy and precision

The intra-batch precision is shown in Table 1. The precision for concentrations of 0.33, 3.33 and 33.33 μg/ml pinocembrin were 0.15, 0.23 and 2.03%, respectively. The accuracy ranged from 99.13 to 104.71% throughout the three concentrations examined. The inter-batch precision was studied over 5 days, and the results are also given in Table 1. The precision ranged from 1.18 to 9.96%, and the accuracy reached from 98.45 to 100.88% throughout the three concentrations examined.

3.3.3. Recovery

The recoveries of pinocembrin added to rat plasma were from 93.94 to 97.81% (Table 1). The results showed that there was no significant difference in the signals of analytes extracted from rat plasma and from the mobile phase.

Table 1
Accuracy, precision, recovery and stability of pinocembrin in rat plasma.

	Spiked concentration (μg/ml)		
	0.33	3.33	33.33
Accuracy and precision			
Intra-batch precision (n = 5)			
Measured concentration (μg/ml)	0.35 ± 0.01	3.30 ± 0.01	33.14 ± 0.05
Accuracy (%)	104.71 ± 2.13	99.13 ± 0.23	99.42 ± 0.14
R.S.D. (%)	2.03	0.23	0.15
Inter-batch precision (n = 5)			
Measured concentration (μg/ml)	0.33 ± 0.03	3.28 ± 0.04	33.12 ± 0.78
Accuracy (%)	100.88 ± 9.66	98.45 ± 1.14	99.38 ± 2.34
R.S.D. (%)	9.96	1.18	2.35
Recovery			
Measured concentration (μg/ml)	0.31 ± 0.01	3.21 ± 0.01	32.60 ± 0.10
Recovery (%)	93.94 ± 1.68	96.19 ± 0.38	97.81 ± 0.31
Stability of samples (n = 5)			
Freeze–thaw (three cycles) (%)	105.62 ± 7.13	103.84 ± 5.97	98.66 ± 6.03
Short-term (room temperature, 6 h) (%)	98.95 ± 3.09	101.55 ± 4.90	102.01 ± 3.66
Long-term (−20 °C, 2 weeks) (%)	101.75 ± 4.13	97.10 ± 4.60	98.75 ± 2.80
Postpreparative (12 h) (%)	99.27 ± 2.05	100.6 ± 1.71	102.95 ± 1.01
Stock solution (room temperature, 4 days) (%)	100.87 ± 1.33	102.75 ± 2.42	99.67 ± 1.86

Table 2
Pharmacokinetic parameters of pinocembrin after i.v. administration of 22.5 and 67.5 mg/kg, each value represents the mean ± S.D. (n = 5).

Pinocembrin dose (mg/kg)	AUC^{0-120} (mg/l min)	MRT^{0-120} (min)	$T_{1/2}$ (min)	CL (l/min/kg)
22.5	340.37 ± 69.05	11.69 ± 2.67	14.61 ± 3.74	0.07 ± 0.02
67.5	1698.55 ± 335.95	15.06 ± 1.39	13.93 ± 5.02	0.04 ± 0.01

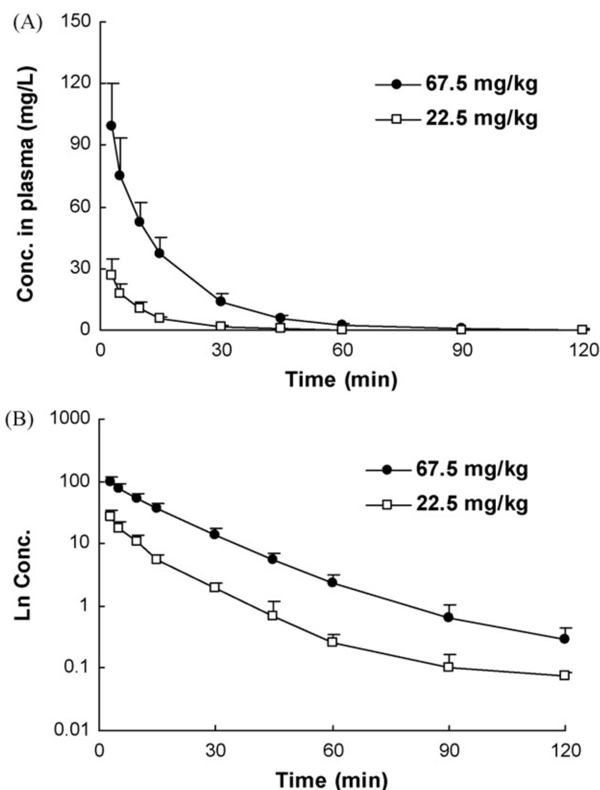

Fig. 3. Mean plasma concentration–time profiles of pinocembrin in six male rats after i.v. administration of 22.5 and 67.5 mg/kg, each point and bar represents the mean ± S.D. (n = 6). (A) Y-axis: conc. (B) Y-axis: Ln conc.

3.3.4. Stability

Stability of pinocembrin during sample handling (freeze–thaw, short-term temperature, long-term, postpreparative and stock solution) is shown in Table 1. Pinocembrin was stable for at least 6 h at room temperature in plasma samples, and mean recoveries from the nominal concentration were more than 98%, respectively, at 0.33, 3.33 and 33.33 μg/ml. Pinocembrin was stable in plasma samples when stored at −20 °C for a 2-week period and mean recoveries from nominal concentration were all more than 97%, and the working solutions were found to be stable within 4 days.

3.3.5. Pharmacokinetic study of pinocembrin in rats

The method described above was successfully applied to the pharmacokinetic study in which plasma concentrations of pinocembrin were determined for 120 min after i.v. administration of 22.5 and 67.5 mg/kg. The pharmacokinetic parameters are listed in Table 2 and the pharmacokinetic profiles of pinocembrin are shown in Fig. 3. The pharmacokinetic parameters of pinocembrin were estimated as in Section 2.7. The area under the plasma concentration ($AUC^{0-120\,min}$) of pinocembrin after i.v. 22.5 and 67.5 mg/kg were 340.37 ± 69.05 and 1698.55 ± 335.95 mg/(l min), respectively. The $T_{1/2}$ were 14.61 ± 3.74 and 13.93 ± 5.02 min. The LLOQ of 66.7 ng/ml was sensitive enough for the pharmacokinetics research of pinocembrin.

The rapid decline in the plasma concentration of pinocembrin after i.v. administration to rat is due to fast excretion or/and extensive metabolism. Pinocembrin is known to exist in plasma partly as the glucuronide and sulphate conjugate [11,13]. Since these metabolites cannot pass the blood brain barrier, we were interested in free pinocembrin concentration only for our planned study regarding the neuroprotective action of this drug.

4. Conclusion

A reliable and sensitive HPLC method for the analysis of pinocembrin in rat plasma had been successfully developed and

validated. The method was successfully applied to a pharmacokinetic study of pinocembrin in rats. To our knowledge, this is the first report of short runtime HPLC method on the determination of free pinocembrin with low detection limit in the ng/ml range in rats. The pharmacokinetic parameters obtained from this study can give some useful information for further research of pinocembrin.

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