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Determination of bergenin in rat plasma by high-performance liquid chromatography

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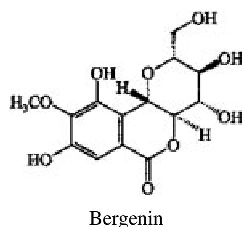
A simple, sensitive, selective and reproducible reversed-phase high-performance liquid chromatography (HPLC) method was developed for the determination of bergenin in rat plasma after intravenous administration. Acetaminophen was successfully used, as internal standard (IS) for calibration. The chromatographic separation was accomplished on a reversed-phase C_{18} column using a mobile phase consisting of methanol-water (20 : 80, v/v, pH 2.50) and a detection wavelength of 275 nm. Retention times of bergenin and acetaminophen were approximately 9.9 and 6.1 min and no interfering peak of the blank plasma chromatograms was observed. Good linearity was achieved in the range of $0.3 \sim 100$ μg/ml ($r^2 = 0.9998$). The extraction recoveries of bergenin from plasma was 70.82%, 69.44%, 70.98% at concentrations of 5, 50, 100 μg/ml. Intra-assay and inter-assay variabilities were $0.92 \sim 2.60\%$ and $2.31 \sim 2.95\%$, respectively. The accuracy was validated by relative error (RE %), which was in the range of $-0.05 \sim 1.74\%$. The capability of the assay to pharmacokinetic studies was demonstrated by the determination of bergenin in plasma after intravenous administration to rats in doses of 7.5 mg/kg, 15.0 mg/kg, and 30.0 mg/kg, using water as the solvent. The half-lives for distribution and elimination are not related to administered doses. A biphasic phenomenon with a rapid distribution followed by a slower elimination phase was observed from the plasma concentration-time curve and the pharmacokinetics was based on first order kinetics.

1. Introduction

Bergenin is a C-glucoside of 4-*O*-methyl gallic acid separated from the rhizome of Saxifragaceae such as *Bergenia guanxianensis* Kuined (Lu and Wang 2003), *Astilbe chinensis* (Sun et al. 2002), *Rodgersia aesculifolia* Batalin (Shen et al. 1987). It has been widely used for over a century in traditional Chinese medicine to treat chronic bronchitis (The Pharmacopoeia of the People's Republic of China 2005) and also has been reported to have antitussive (Xie et al. 1981), hepatoprotective (Lim et al. 2001), anti-inflammatory (Li et al. 2004), neuroprotective (Takahashi et al. 2003) and weak anti-HIV (Piacente et al. 1996) activities. Bergenin is an unstable compound and its shelf life depends mostly on the storage conditions, particularly atmospheric temperature and light (Lu and Wang 2003). In addition, due to its poor water solubility and easily degradation in basic solution, little information is available about the determination of bergenin in biological matrices and pharmacokinetics *in vivo*. As knowledge of the pharmacokinetics can help us explain and predict a variety of events related to the efficacy and toxicity of bergenin, it is important to investigate its pharmacokinetics.

Recently, Shi et al. (2006) reported for the first time about the RP-HPLC determination and pharmacokinetic properties of bergenin in rat plasma. The method was validated with parameters such as linearity, detection and quantification limit, recovery and stability. However, an external standard method was used in this paper and there was no report about internal standard (IS) used in the determination of bergenin, which is essential to acquire appropriate pharmacokinetic data. The basic principle of IS in chemical analysis is based on the addition of a known and fixed amount of one or more elements used as internal standards to all blanks, reference solutions, and samples and it must present chemical and physical properties as similar as possible to those presented by the analyte. To our knowledge, this is the first time that an IS has been used for the determination of bergenin.

The previous HPLC method used 200 μl of plasma sample for the determination of bergenin (Shi et al. 2006). Since the whole volume of blood took $5 \sim 7\%$ of body weight of a rat, it was necessary to develop a HPLC method for the determination of bergenin in small volumes of



Bergenin

rat plasma to minimize the volume of the collected sample and lessen the possible physiological influence on animals. Moreover, the pharmacokinetic study of bergenin after intravenous administration to rats, reported in Shi's paper, was at a single dose of 11.25 mg/kg, using 1,2-propanediolnedistilled water (1 : 5, v/v) as the solvent (Shi et al. 2006). In our study, in order to eliminate the stimulation and physiological influence of 1,2-propanediol, water was used as the intravenous administration solvent.
The objectives of this study were to develop a robust and internal standard based HPLC method for bergenin determination in plasma with a small plasma sample volume and investigate its pharmacokinetics in normal physiological conditions. Acetaminophen was used as IS to improve accuracy and precision for analysis. A one-step extraction procedure with ethyl acetate as extraction solvent was employed without any significant interference. The pH value of the mobile phase was adjusted and the detection wavelength was set at 275 nm to achieve better resolution. Using this method, samples could be applied to HPLC with precise quantitation and a small plasma volume (100 μl) was required. Pharmacokinetic studies of bergenin in rat plasma at three different doses of 7.5 mg/kg, 15.0 mg/kg, and 30.0 mg/kg were characterized for the first time.

2. Investigations and results

Typical chromatograms of blank and spiked plasma with bergenin and acetaminophen are shown in Fig. 1. The chromatogram showed a clear and good separation of the peaks, and the retention times were 9.9 min for bergenin and 6.1 min for IS, respectively. No interfering peaks due to plasma components, impurities of internal standard or metabolites were eluted at the retention time of the analytes of interest.
To determine the linearity, blank plasma spiked with the different concentrations of bergenin was analyzed. The slope and intercept of the calibration graphs were calculated by weighted least squares linear regression. The regression equation of the standard curves was:

$$y = (0.0438 \pm 0.0012)x + (0.0583 \pm 0.0018) \quad r^2 = 0.9998 \qquad (1)$$

where y is the peak area ratio of bergenin to the IS, and x is the plasma concentration of bergenin. The concentrations of bergenin in rat plasma after intravenous administration were determined from the peak-area ratios by using the equations for linear regression from the calibration curves.
The limit of detection (LOD) for this method defined as a signal-to-noise ratio of 3 : 1 was 0.15 μg/ml. The LOQ was defined as the lowest drug concentration, which can be determined with an intra-day relative standard deviation RSD $\le$ 20%. The LOQ was estimated as 0.30 μg/ml.
The accuracy was determined by comparing the measured concentrations with the expected concentrations of bergenin in spiked blank rat plasma and expressed as relative error (RE%), which was in the range of $-0.05 \sim 1.7\%$. The precision of the present method was investigated (n = 6) by repeated analysis of bergenin-spiked plasma samples at 5, 50, and 100 μg/ml. The results of intra-assay and inter-assay were reproducible with an intra-day RSD less than 2.60%, and an inter-day RSD less than 2.95% (Table 1). The results demonstrate the acceptable accuracy and precision of the HPLC method developed.
The stabilities of bergenin in rat plasma (n = 5) were investigated both at room temperature (25 °C) and at −20 °C using QC samples of three concentration levels. Data from samples analyzed immediately were compared with those stored at the room temperature (25 °C) for 2, 4 h and at −20 °C for 24 h. Table 2 shows that the concentrations of spiked plasma samples (5, 50, 100 μg/ml) were similar to the values within 2 h at room temperature (25 °C) or 24 h at −20 °C. But after 4 h at room temperature the concentrations of spiked plasma samples declined. This implied that the plasma samples should be treated as soon as possible. No significant degradation was observed with the

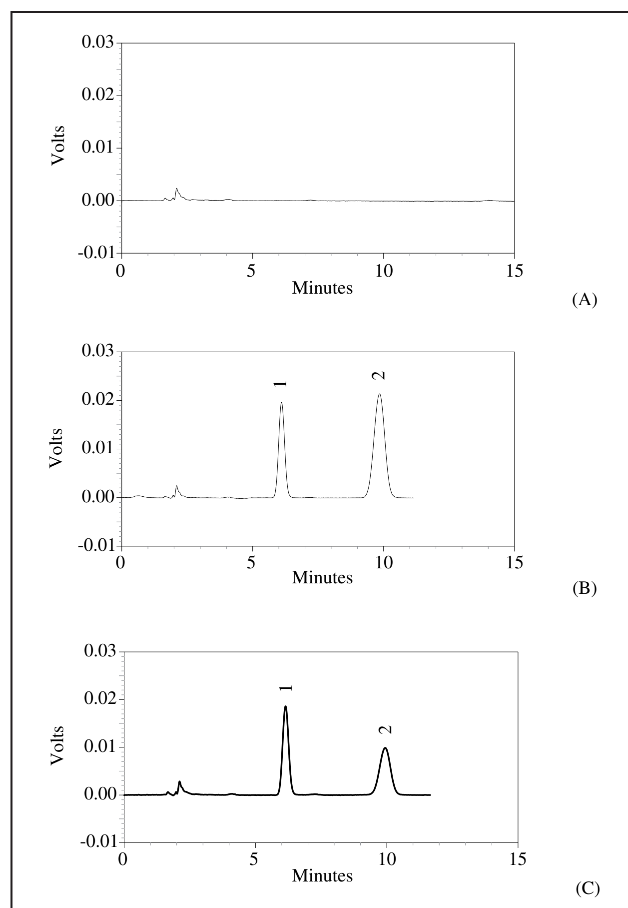


Fig. 1: Representative chromatograms of (A) blank plasma; (B) rat plasma spiked with bergenin (100 μg/ml) and acetaminophen (IS); (C) plasma sample 6 min after i.v. administration (30.0 mg/kg) of bergenin. 1 = acetaminophen, 2 = bergenin

Table 1: Precision and accuracy of the method to determine bergenin in rat plasma (n = 15)

Concentration (μg/ml)		Intra-day		Inter-day
Added	Found	RE (%)	RSD (%)	RSD (%)
5	5.087	1.74	1.94	2.95
50	49.975	−0.05	0.92	2.54
100	100.900	0.90	2.60	2.31

Table 2: Stability of bergenin in rat plasma (n = 5)

Time and condition of storage	Nominal concentration (μg/ml)	Percent of nominal (%)	RSD (%)
25 °C, 2 h	5	99.64	2.66
	50	97.41	2.68
	100	96.66	3.17
25 °C, 4 h	5	86.43	2.87
	50	83.77	2.15
	100	84.90	1.93
−25 °C, 24 h	5	98.25	2.21
	50	97.71	2.54
	100	96.19	3.51

samples stored at −20 °C, which indicated that the processed samples were stable enough under these storage conditions for 24 h.

The extraction recoveries were assessed (n = 5) at three concentration levels (5, 50, 100 μg/ml) and were calculated by comparing the peak areas of the prepared standard samples with those of the standard solutions. The mean extraction recoveries of bergenin at three concentrations were within accepted values 70.8%, 69.4% and 70.9%, respectively. The recoveries of IS were 89.94%, 90.61% and 90.99%, respectively. The results are shown in Table 3.

Three different doses (7.5, 15.0, and 30.0 mg/kg) were selected by mathematical conversion of doses between different animals (from human to rat). The work elucidated

Table 3: Recoveries of bergenin and acetaminophen (n = 5)

Concentration (μg /ml)	IS		Bergenin	
	Mean ± SD (%)	RSD %	Mean ± SD (%)	RSD %
5	89.94 ± 0.99	1.10	70.82 ± 1.34	1.89
50	90.61 ± 1.01	1.11	69.44 ± 0.74	1.06
100	90.99 ± 2.27	2.49	70.98 ± 1.59	2.24

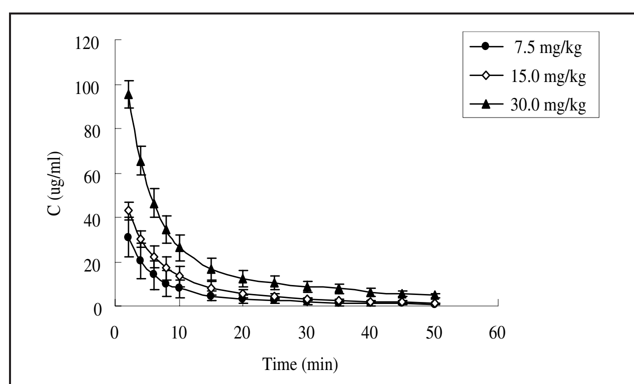


Fig. 2: Mean plasma concentration-time curves after i.v. administration of bergenin to rat at doses of 7.5(–●–), 15.0(–□–), and 30.0(–▲–) mg/kg

the basic pharmacokinetic parameters of bergenin after i.v administration to rats. Pharmacokinetic parameters were determined by fitting pharmacokinetic models to the plasma concentration-time profiles for each rat after intravenous dosing. From the results of kinetic analysis, a two-compartment open model with 1/cc weighting coefficient was considered the most appropriate pharmacokinetic model.

A biphasic phenomenon with a rapid distribution followed by a slower elimination phase was observed from the plasma concentration-time curves (Fig. 2). Bergenin was rapidly distributed with very short and similar distribution half-lives ($t_{1/2\alpha}$) over the dose range investigated (Table 4). The terminal elimination half-life ($t_{1/2\beta}$) of bergenin at the 7.5 mg/kg dose level was 32 ± 18 min and not significantly different from the values obtained at the 15.0 and 30.0 mg/kg dose levels ($P > 0.05$). The AUC, an indicator of the body's exposure to a drug, increased proportionately to the intravenous dose of bergenin administered. Statistical analysis of the terminal elimination half-lives, volumes of distribution and clearance values between doses indicate that bergenin exhibits dose-independent kinetics ($P > 0.05$). In the range of the dose examined, the pharmacokinetics of bergenin in rat was based on first order kinetics. The plasma concentration-time curves could be described adequately by Eqs. (2)–(4):

Analysis of data after i.v. injection of bergenin at 7.5 mg/kg yields Eq. (2)

$$C = 45.63e^{-0.275t} + 5.697e^{-0.037t} \quad (2)$$

Analysis of data after i.v injection of bergenin at 15.0 mg/kg yields Eq. (3)

$$C = 55.31e^{-0.212t} + 7.834e^{-0.032t} \quad (3)$$

Analysis of data after i.v injection of bergenin at 30.0 mg/kg yields Eq. (4)

$$C = 124.238e^{-0.235t} + 18.637e^{-0.023t} \quad (4)$$

3. Discussion

Chromatographic conditions such as pH value of mobile phase and detection wavelength were investigated in the present research. The preliminary study showed that bergenin displayed large polarity and little retention on the C_{18} column with the mobile phase of a neutral pH value. A reasonable retention time and resolution between bergenin and IS were achieved when pH was adjusted to pH 2.5. Bergenin in the mobile phase exhibits maximum absorption at 220 and 275 nm. Since interference peaks were observed at 220 nm, the wavelength of 275 nm was more suitable for the assay. The chromatographic conditions could achieve satisfactory resolution, reasonable retention and symmetric peak shapes for bergenin and IS.

Table 4: Pharmacokinetic parameters of bergenin (7.5, 15.0, 30.0 mg/kg, i.v., n = 6) in rats after intravenous administration

Parameter	Estimate (7.5 mg/kg)	Estimate (15.0 mg/kg)	Estimate (30.0 mg/kg)
$t_{1/2\alpha}$ (min)	2.99 ± 1.28	3.87 ± 1.76	2.96 ± 0.14
$t_{1/2\beta}$ (min)	32.98 ± 18.30	34.10 ± 17.38	33.23 ± 11.75
$AUC_{0\to\infty}$ (μg · min/ml)	423.19 ± 181.42	638.47 ± 68.49	1580.23 ± 235.20
Vol (l/kg)	0.18 ± 0.083	0.24 ± 0.057	0.21 ± 0.009
Cl (l/min/kg)	0.021 ± 0.008	0.024 ± 0.003	0.019 ± 0.003
$MRT_{0\to t}$ (min)	8.41 ± 1.49	9.41 ± 1.15	10.11 ± 1.17

Data are expressed as mean ± SD; $t_{1/2\alpha}$ = distribution half-life; $t_{1/2\beta}$ = elimination half-life; $AUC_{0\to\infty}$ = area under the concentration-time curve; Vol = volume of distribution; Cl = clearance; MRT = mean residence time

Internal standard method is a preferred method especially in biological samples. The addition of IS would offset the errors caused by instability of the instruments, inaccuracy of the injection volume, and so on. Hence, our attention was mainly focused on the optimization of IS. An ideal IS should have similar chromatographic and spectral properties as the compounds to be analyzed. Several substances had been tried such as chlorphenamine and ρ-aminobenzoic acid, acetaminophen was chosen at last since under the HPLC conditions applied it presented a good peak shape, satisfactory resolution, and was commercially available. In addition, the separation factor of acetaminophen to bergenin was 7.68; the ratio of bergenin to acetaminophen remained constant through the sample processing; the suitability of acetaminophen as IS was further proven by recovery studies. Both the range of relative standard deviation (RSD %) and relative error (RE %) were more narrow compared with the external standard method established by Shi et al. (2006). Good linearity and precision were also obtained. The injection volume is only 10 μl which is suitable for routine analysis.

Complex sample pretreatment would be required to prevent interference from other substances for UV determination, especially in biological fluids. During treatment of plasma samples, various solvents were tested to extract bergenin from plasma, such as ether and chloroform. However, the recoveries of bergenin were unsatisfactory (<50%). Methanol was also used according to the method reported by Shi et al. (2006), but the recovery was not satisfactory either, moreover, much chromatographic interference similar to their report were observed. Acetic ether was employed as a deproteinizing agent and an extracting agent. The extraction recoveries for both bergenin and IS were satisfactory without any significant interference. With regard to plasma samples, the extraction method is simpler and smaller volumes of plasma sample were required than with other previous reports.

We found during the experiment that bergenin was poorly dissolved in water at room temperature, while dissolved easily at 60 °C without affecting its properties, and would not precipitate when cooled down again to room temperature. In the present study, 1,2-propanediol-water was also used to be the solvent for i.v. administration but the results showed the distribution and elimination half-life of bergenin is longer than when water was used. According to the reports of Shi et al. (2006), the mean value of distribution half-life $t_{1/2\alpha}$ was 6.6 min and elimination half-life $t_{1/2\beta}$ was 247.8 min. But in our research, $t_{1/2\alpha}$ and $t_{1/2\beta}$ were 3 min and 33 min, respectively, which implied that 1,2-propanediol may change the pharmacokinetics of bergenin *in vivo*.

In summary, the parameters derived from this study represent, to our knowledge, the first account of the pharmacokinetic character of intravenous bergenin in rats which was most close to physiological conditions. The exact procedure of metabolism following i.v. administration and absorption after oral administration deserves further investigation.

4. Experimental

4.1. Chemicals and reagents

Bergenin was purchased from Sichuan Dihon Medical Development Co.Ltd (Chengdu, China). Acetaminophen used as IS was obtained from the National Institute for the Control of Pharmaceutical and Biological Products. Methanol (HPLC grade) was from Yucheng Chemical Plant of Shandong Yuwang Industrial Ltd. Phosphoric acid and ethyl acetate were of analytical reagent grade and obtained from Chengdu Fangzhou Reagent Company. Water was prepared in ultra pure water system.

4.2. Chromatographic system

HPLC determinations were performed with a Shimadzu model 10 Avp liquid chromatographic system (Tokyo, Japan), consisting of a LC-10AT pump and an UV/Vis detector (SPD-10A). The system was controlled by a system controller (SCL-10A) and a personal computer. An analytical column, Diamonsil C_{18} reverse phase column (150 × 4.6 mm, 5 μm) from Dikma Technologies, and a Dikma EasyGuard 6101 C_{18} kit guard column were used for chromatographic separation.

The mobile phase consisted of methanol-water (20 : 80, v/v), which had been adjusted to pH 2.50 with orthophosphoric acid, and then filtered and degassed under reduced pressure prior to use. Chromatography was performed at temperature of 30 °C and a flow rate of 1.0 ml/min, with the detection wavelength set at 275 nm.

4.3. Preparation of calibration standards

A plasma calibration curve was constructed with ten different standards covering the expected concentration range (0.3 ~ 100 μg/ml). Stock solution of bergenin (1 mg/ml) was prepared with water and further diluted into working solutions ranging from 3 ~ 1000 μg/ml. Different plasma standard samples containing 0.3, 0.5, 2, 5, 10, 30, 50, 70, 90, 100 μg/ml were prepared by spiking 180 μl of drug free plasma with 20 μl of standard working solutions. Acetaminophen was diluted to 100 μg/ml. All stock and working solutions were stored at 4 °C. Quality control (QC) samples to determine accuracy and precision of the method were prepared at low (5 μg/ml), medium (50 μg/ml) and high (100 μg/ml) concentrations in the same way as the plasma sample for calibration. The IS was added to each standard sample just prior to sample processing.

4.4. Drug administration and plasma sample collection

Male Wistar rats (200 ± 20 g) were obtained from Laboratory Animal Center of Sichuan University. These animals were allowed to freely access to food and water, and fasted overnight before use (the environment temperature maintained at 25 ± 1 °C).

The rats were given bergenin through the tail vein at three different doses (7.5, 15.0, 30.0 mg/kg, respectively), serial blood samples (0.3 ml) were obtained from the tail into heparinized tubes at 0, 2, 4, 6, 8, 10, 15, 20, 25, 30, 35, 40, 45 and 50 min after drug administration. Plasma samples were obtained following centrifugation at 12000 × g for 10 min.

4.5. Sample preparation

100 μl of plasma was vortexed with 25 μl of the IS solution. 400 μl of ethyl acetate was added and vortexed for 2 min, then sonic oscillated to get complete extraction. The extracts were centrifuged at 5000 × g to get better phase separation. This procedure was repeated three times and all of the supernatant was mixed and dried under vacuum. The residues were reconstituted with 100 μl mobile phase, and then centrifuged at 5000 × g for 2 min. A 10 μl aliquot of the supernatant was injected into the HPLC system.

4.6. Pharmacokinetics data analysis

The data were analyzed using non-linear regression analysis by the computer program DAS 2.0 (Drug and Statistics, Anhui, China).

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