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Quantitation of bergenin in human plasma by liquid chromatography/tandem mass spectrometry

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ABSTRACT

This paper reports the development and validation of an assay for quantitation of bergenin in human plasma using liquid chromatography/tandem mass spectrometry (LC–MS/MS). Bergenin and the internal standard (I.S.), 5-bromo-2,4(1*H*,3*H*)-pyrimidinedione (5-BrU), were separated by reversed phase HPLC and quantitated by MS/MS using electrospray ionization (ESI) and multiple reaction monitoring (MRM) in the negative ion mode. The most intense $[M-H]^-$ MRM transition of bergenin at m/z 326.9 → 312.3 was used for quantitation and the transition at m/z 188.9 → 42.2 was used to monitor 5-BrU. Stability issues with bergenin required the addition of ascorbic acid to plasma samples prior to storage and analysis within 10 days storage at −80 °C. The method was linear in the range 3–1000 ng/mL with intra- and inter-day precision of 3.94–5.96 and 1.62–8.31%, respectively, and accuracy <2.33%. The assay was successfully applied to a pharmacokinetic study in healthy volunteers after administration of a single 250 mg oral dose.
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1. Introduction

Bergenin is a *C*-glucoside of 4-O-methylgallic acid isolated from the rhizome of *Rodgersia aesculifolia* Batalin [1]. Its structure is shown in Fig. 1. Bergenin displays a wide range of pharmacological activities and is widely used in Traditional Chinese Medicine (TCM). Not only has it been used for the treatment of stomach hyperacidity and ulcers [2,3] but also as an antiarrhythmic [4], hepatoprotective [5,6], anti-inflammatory [7] and anti-HIV agent [8] as well as for its antitumor [9] and neuroprotective activity [10]. Despite this widespread use, the pharmacokinetics of bergenin in human has not been studied probably because of the lack of a sensitive assay for its determination in biological fluids. As part of the development of an oral formulation of pure bergenin, we now report a simple and sensitive assay suitable for use in clinical pharmacokinetic studies.

Two analytical methods based on reversed phase HPLC methods with UV detection have been applied to the determination of bergenin in rat plasma but they have relatively long run times (about 10 min) and inadequate sensitivity for pharmacokinetic studies of a therapeutic dose in human [11,12]. The method reported here describes an assay based on liquid chromatography tandem mass spectrometry (LC–MS/MS) and its application to a pharmacokinetic study of bergenin in healthy volunteers given a single 250 mg oral dose.

2. Experimental

2.1. Chemicals and reagents

Bergenin (99.0%) and 5-bromo-2,4(1*H*,3*H*)-pyrimidinedione (5-BrU) (99.0%) for use as internal standard (I.S.) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). Acetonitrile was HPLC grade and provided by Fisher Scientific (Fair Lawn, NJ, USA). All other chemicals were of analytical grade and used as received. Blank (drug free) human plasma was obtained from the Changchun Blood Donor Service (Changchun, PR China). Distilled water, prepared from demineralized water, was used throughout the study.

2.2. Preparation of standard and quality control (QC) solutions

A stock solution of bergenin (1 mg/mL) was prepared by dissolving 25 mg in acetonitrile:water (50:50 v/v) in a 25 mL amber volumetric flask. Bergenin standard solutions (6, 20, 60, 200, 600 and 2000 ng/mL) were prepared by dilution of this stock solution

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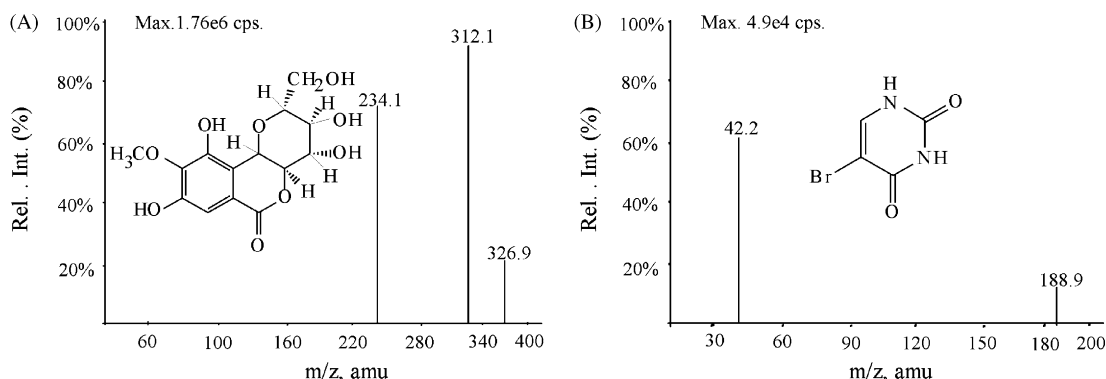

Fig. 1. Full-scan product ion scans of $[M-H]^-$ ions and structures for (A) bergenin and (B) 5-BrU.

with acetonitrile:water (50:50 v/v). QC solutions (20, 200 and 1600 ng/mL) were prepared independently in the same way. A stock solution of I.S. (1 mg/mL) in acetonitrile was diluted with acetonitrile:water (50:50 v/v) to prepare an I.S. working solution (0.5 μg/mL). All solutions were stored at 4 °C when not in use.

2.3. Sample preparation

To a 200 μL aliquot of human plasma in a 1.5 mL Eppendorf tube, 50 μL I.S. working solution, 100 μL acetonitrile:water (50:50, v/v) (or a standard or QC solution of bergenin when 5 mg ascorbic acid was also added) and 500 μL acetonitrile were added. The mixture was vortex-mixed for 30 s and centrifuged at 15000 × *g* for 10 min. The supernatant was transferred to a 10 mL glass tube followed by 100 μL water and 3.0 mL dichloromethane. After vortex-mixing for 30 s and centrifugation at 3500 × *g* for 5 min, an aliquot of the aqueous layer (20 μL) was injected into the LC–MS system.

2.4. LC–MS system

The LC–MS system comprised an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA, USA) coupled to an API 4000 mass spectrometer (Applied Biosystems Sciex, Ontario, Canada) equipped with a Turbo IonSpray source. Data acquisition and integration were controlled by Analyst Software (Applied Biosystems/MDS SCIEX, version 1.3). Chromatographic separation was

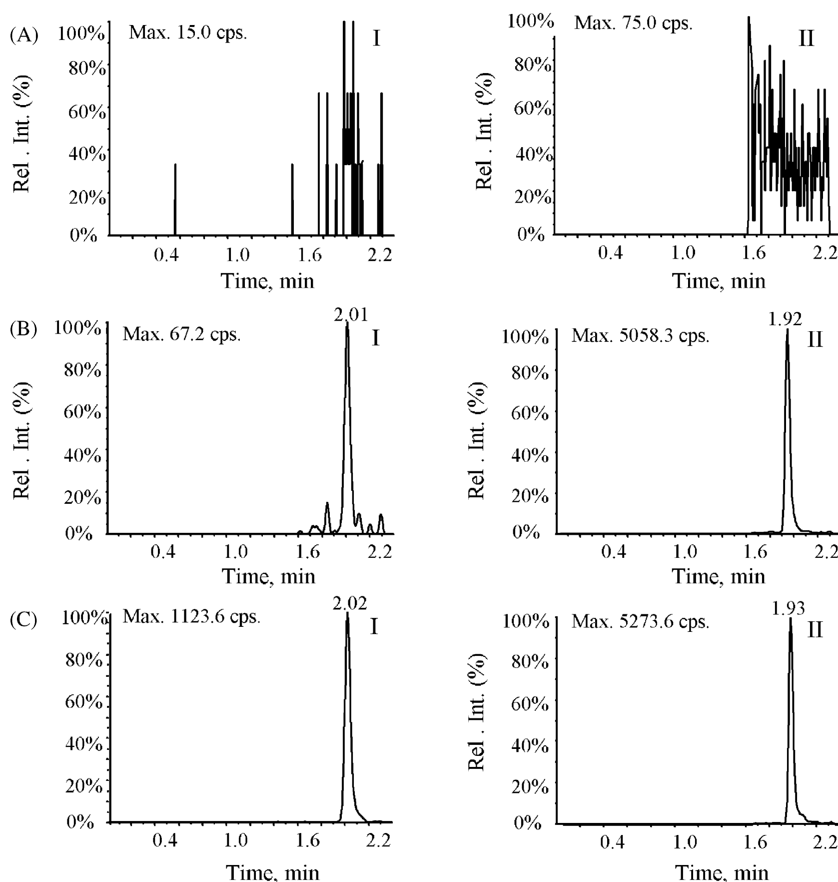

Fig. 2. MRM chromatograms of bergenin and 5-BrU in human plasma. (A) Blank plasma; (B) blank plasma spiked with (I) 6 ng/mL bergenin and (II) 500 ng/mL 5-BrU; (C) blank plasma from a human volunteer 1.5 h after a single 250 mg oral dose of bergenin.

Table 1
Precision and accuracy for the determination of bergenin in human plasma (data are based an assay of six replicates on three different days).

Spiked concentration (ng/mL)	Calculated concentration (mean ± S.D., ng/mL)	Intra-day R.S.D. (%)	Inter-day R.S.D. (%)	Accuracy R.E. (%)
10.0	10.1 ± 0.6	5.06	8.31	1.16
100	97.7 ± 4.4	4.71	1.62	−2.33
800	800 ± 33	3.94	5.68	0.02

performed on a Zorbax SB-C18 column (150 mm × 4.6 mm, 5 μm, Agilent, USA) maintained at 30 °C using a mobile phase of acetonitrile:10 mM ammonium acetate (1% formic acid) (20:80 v/v). The flow rate was 1.0 mL/min and an approximately 1:1 (v/v) split of the mobile phase entering the mass spectrometer was employed.

The electrospray ionization (ESI) source was operated in the negative ion mode. Multiple reaction monitoring (MRM) at unit resolution was employed to monitor the transitions of the deprotonated molecular ions of bergenin at m/z 326.9 → 312.3 (quantifier) and 326.9 → 234.1 (qualifier) (the accurate mass of the bergenin $[M-H]^-$ ion was 327.072156) and of 5-BrU at m/z 188.9 → m/z 42.2. Optimized MS parameters were: Curtain gas, gas 1 and gas 2 (nitrogen) 15, 45 and 60 units, respectively; dwell time 200 ms; IonSpray voltage −4000 V; source temperature 500 °C; declustering potential (DP) −72 V for bergenin and −45 V for 5-BrU; collision energy (CE) −23 eV (m/z 326.9 → 312.3) and −20 eV (m/z 326.9 → 234.1) for bergenin and −35 eV for 5-BrU.

2.5. Assay validation

Linearity was assessed by weighted linear regression ($1/x^2$) of standard curves based on analyte–internal standard peak area ratios prepared in triplicate on three separate days. Intra-day and inter-day precisions [as relative standard deviation (R.S.D.)] and accuracy [as relative error (R.E.)] were determined by analysis of six replicates of each QC sample on three different days. The lower limit of quantitation (LLOQ) was defined as the lowest concentration at which both precision and accuracy were less than or equal to 20%. The limit of detection (LOD) was the concentration with signal-to-noise ratio of 3. Selectivity of the assay was assessed by comparing the chromatograms for six different batches of blank human plasma with those for the corresponding spiked plasma samples. Recoveries of analyte and I.S. were determined by comparing the peak areas of analyte and I.S. in extracted QC samples with those in post-extraction blank samples spiked at the corresponding concentrations. Matrix effects were evaluated by comparing peak areas of post-extraction blank plasma spiked with QC solutions and I.S. with areas of post-extraction water spiked with QC solutions. Stability of bergenin was assessed using QC samples subjected to the following conditions: Storage at room temperature for 6 h; storage at −80 °C for 10 and 18 days; and after three freeze/thaw cycles. Stability in processed samples stored in autosampler vials at room temperature for 12 h was also assessed.

2.6. Pharmacokinetic study

The analytical method was used to determine plasma concentrations of bergenin in 20 healthy male volunteers (mean age 22.7 ± 1.5 years; mean body mass index 20.6 ± 1.2 kg/m^2) after ingesting a single 250 mg oral dose. The clinical protocol was approved by the Ethics Committee of Chengdu Military Provincial Hospital, Kunming, PR China. All volunteers gave written informed consent before entering the study. Blood samples (1.0 mL) were collected before and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 12 and 24 h post-dose. Plasma was prepared by centrifugation at 15000 × g for 10 min after which ascorbic acid (25 mg) was added to each sample. Plasma samples were stored at −80 °C until analysis within 10 days.

3. Results and discussion

3.1. Method development

Methods for the quantitation of bergenin based on HPLC with UV detection report lower limits of quantitation of 0.25 and 0.3 μg/mL [11,12]. Using LC–MS/MS as reported here provides improved sensitivity and selectivity and a technique suitable for pharmacokinetic studies.

ESI was chosen for ion production since bergenin is a relatively polar compound [13]. The negative ion mode was chosen since both bergenin and 5-BrU are weak acids. Full-scan product ion mass spectra of bergenin and 5-BrU are shown in Fig. 1. The transition at m/z 326.9 → 312.3 was more intense than that at m/z 326.9 → 234.1 and was used as the quantifier. The transition at m/z 326.9 → 234.1 was used as a qualifier.

Various mobile phase combinations of acetonitrile and methanol with 10 mM ammonium acetate were investigated to optimize sensitivity, speed and peak shape. Acetonitrile gave a better response than methanol and 10 mM ammonium acetate containing 1% formic acid improved the efficiency of ionization and peak shapes. A number of C18 columns (Zorbax Extend, Zorbax XDB, Nucleosil, and Hypersil) were evaluated with Zorbax SB-C18 giving the best chromatography in a short cycle time of 2.2 min at a flow rate of 1.0 mL/min.

3.2. Sample preparation

In terms of sample preparation, protein precipitation is a relatively simple procedure [11,12] which can save considerable time. However, dilution of analyte by acetonitrile results in reduced sensitivity. We overcame this problem by introducing a subsequent dichloromethane wash which not only removed acetonitrile but also reduced interference from endogenous material and did not compromise the recovery of bergenin. 5-BrU was found to be a suitable I.S. with similar chromatographic behaviour and MS response to bergenin.

3.3. Assay validation

Representative chromatograms of blank plasma, plasma spiked with bergenin at the LLOQ (3 ng/mL) and a study sample taken 1.5 h

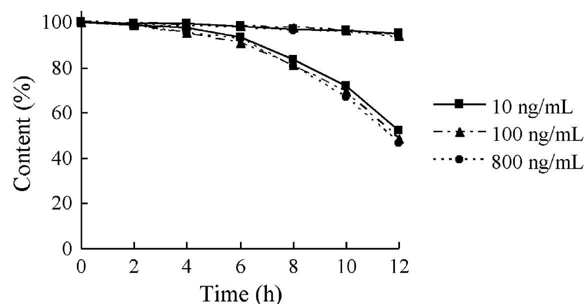

Fig. 3. Stability of bergenin in processed QC samples (n = 3) stored in autosampler vials at room temperature for 12 h without (lower curves) and with (upper curves) the addition of ascorbic acid to the QC samples.

Table 2
Stability of bergenin under various conditions after addition of ascorbic acid to QC samples (data are means ± S.D. of three replicates).

Storage conditions	Initial concentration (ng/mL)	Calculated concentration (ng/mL)
In human plasma at room temperature for 6 h	10.0	9.63 ± 6.44
	100	92.8 ± 6.76
	800	737 ± 2.74
In processed samples at room temperature for 12 h	10.0	9.90 ± 6.16
	100	96.4 ± 7.00
	800	767 ± 3.22
In human plasma after three freeze/thaw cycles	10.0	9.03 ± 3.60
	100	88.2 ± 1.63
	800	742 ± 6.46
In human plasma for 10 days at −80 °C	10.0	9.71 ± 2.59
	100	93.7 ± 5.79
	800	746 ± 1.88
In human plasma for 18 days at −80 °C	10.0	5.20 ± 5.87
	100	48.4 ± 2.38
	800	372 ± 1.64

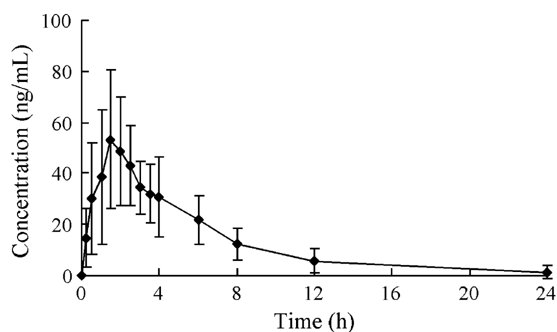

Fig. 4. Plasma concentration–time profile of bergenin after administration of a single 250 mg oral dose. Data are means ± S.D. for 20 healthy male volunteers.

post-dose are shown in Fig. 2. The retention times of bergenin and I.S. were 2.01 and 1.92 min, respectively. The standard curve was linear in the range 3–1000 ng/mL ($y = 0.00412x + 0.00265$, $r > 0.998$) and the precision and accuracy of the method were satisfactory (Table 1). The recoveries of bergenin at 10, 100, 800 ng/mL were 90.9 ± 5.8%, 88.0 ± 7.1% and 87 ± 7.4%, respectively. As regards matrix effects, the percent of nominal concentrations of QC samples at concentrations of 10, 100 and 800 ng/mL were 79.8 ± 8.9, 81.8 ± 4.2 and 85.3 ± 1.7, respectively. The percent of the nominal concentration of the I.S. was 78.5 ± 3.7. The results indicate that ion suppression from the plasma matrix is independent of concentration.

Previous research has shown that stability of bergenin is an issue as expected for a phenolic drug [11]. We observed that without addition of ascorbic acid to plasma, the concentration of bergenin in processed QC samples decreased with time particularly after 6 h (Fig. 3). With addition of ascorbic acid, bergenin was found to be stable in processed QC samples for at least 12 h (Fig. 3). However, even with addition of ascorbic acid, stability of bergenin in plasma remained an issue in relation to storage at −80 °C for more than 10 days (Table 2).

3.4. Pharmacokinetic study

The bergenin plasma concentration–time profile for a single 250 mg oral dose is shown in Fig. 4. The concentration maximum (C_{max}) of 66.6 ± 22.1 ng/mL occurred at 2.0 ± 0.9 h. The elimination half-time ($t_{1/2}$) was 3.7 ± 2.4 h and the mean area under the plasma concentration–time curve (AUC_{0-t}) was 287.7 ± 91.1 ng h/mL There are no pharmacokinetic data for bergenin in human with which to make a comparison but clearly our results indicate high interindividual variability.

4. Conclusion

A LC–MS/MS method for the quantitation of bergenin in human plasma has been developed and validated. The major advantages of the assay are simple sample preparation and a short run time allowing high throughput analysis. The assay has been successfully applied to examine the pharmacokinetics of bergenin in healthy volunteers after a single 250 mg oral dose.

References

[1] X.W. Shen, S.Z. Zhen, Z.S. Fu, Y. Chen, Chem. J. Chin. U. (6) (1987) 528.
[2] K. Abe, K. Sakai, M. Uchida, Gen. Pharmacol. 11 (4) (1980) 361.
[3] R.K. Goel, R.N. Maiti, M. Manickam, A.B. Ray, Indian J. Exp. Biol. 35 (10) (1997) 1080.
[4] H.L. Pu, X. Huang, J.H. Zhao, A. Hong, Planta Med. 68 (4) (2002) 372.
[5] H.K. Lim, H.S. Kim, H.S. Choi, S. Oh, J. Choi, J. Ethnopharmacol. 70 (1) (2000) 69.
[6] H.S. Kim, H.K. Lim, M.W. Chung, Y.C. Kim, J. Ethnopharmacol. 70 (1) (2000) 79.
[7] R.W. Li, D.N. Leach, S.P. Myers, G.D. Lin, G.J. Leach, P.G.A. Waterman, Planta Med. 70 (5) (2004) 421.
[8] S. Piacente, C. Pizza, N. De Tommasi, N. Mahmood, J. Nat. Prod. 59 (6) (1996) 565.
[9] S. Mishima, K. Matsumoto, Y. Futamura, Y. Araki, T. Ito, T. Tanaka, M. Iiluma, Y. Nozawa, Y. Akao, J. Exp. Ther. Oncol. 3 (5) (2003) 283.
[10] H. Takahashi, M. Kosaha, Y. Watanabe, K. Nakade, Y. Fukuyama, Bioorg. Med. Chem. 11 (8) (2003) 1781.
[11] Y.B. Shi, Y.P. Shi, Q.G. Meng, Biomed. Chromatogr. 20 (10) (2006) 1065.
[12] X. Qin, D. Zhou, Z.R. Zhang, Y. Huang, Pharmazie 62 (5) (2007) 323.
[13] J. Wang, Y.W. Wang, J.K. Gu, Y. Wu, Y. Wang, Chem. Res. Chin. U. 21 (2) (2005) 246.