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Short Communication

Determination of bullatacin in rat plasma by liquid chromatography–mass spectrometry

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ABSTRACT

A liquid chromatography–mass spectrometry method has been developed and validated for the quantification of bullatacin, a bistetrahydrofuran annonaceous acetogenin, in rat plasma. Squamostatin-A was selected as the internal standard. Analytes were extracted from rat plasma by liquid/liquid extraction using ethyl acetate with high efficiency. The chromatographical separation was performed on an Agilent Zorbax SB-C_{18} column (150 mm × 2.1 mm, 5 μm). The mobile phase consisted of methanol and deionized water (95:5, v/v) containing 0.01% (v/v) formic acid. The chromatographic run time was 7 min per injection and flow rate was 0.2 mL/min. The retention time was 3.22 and 5.23 min for internal standard and bullatacin, respectively. The elutes were detected under positive electrospray ionization and the target analytes quantified by selected ion monitoring mode (645.9 *m/z* for bullatacin and 661.9 *m/z* for squamostatin-A). The method was sensitive with the limit of quantitation at 0.5 ng/mL in 100 μL of rat plasma. Good linearity (r^2 = 0.9998) was obtained covering the concentration of 0.5–2000 ng/mL. The intra- and inter-day assay precision ranged from 3.2 to 8.7% and 2.7 to 9.2%, respectively. In addition, the stability, extraction recovery and matrix effect involved in the method were also validated. This method was applied to measure the plasma bullatacin concentrations after a single tail vein intravenous administration of bullatacin in rats.

1. Introduction

Annonaceous acetogenins are one of the most interesting classes of natural products appearing in the past two decades and exhibit a broad range of biological properties [1–3]. Especially, their potential to inhibit tumor cells that are multiple drug resistant has attracted increasing interest. Bullatacin, a bistetrahydrofuran annonaceous acetogenin known as the most potent inhibitor of the mitochondrial respiratory chain complex I, exhibited 300 times more effective than taxol as tested *in vivo*, and was used to control the quality of standardized extract [4,5]. Mechanism of action studies have shown that annonaceous acetogenins inhibit HIF-1 activation by blocking the hypoxic induction of nuclear HIF-1α protein [6]. However, there is no released data and analytical method about the pharmacokinetic studies of annonaceous acetogenins *in vivo* so far [5]. An accurate and sensitive analytical method is a prerequisite to conduct a series of pharmacokinetic studies [7]. Thus, the aim of this study was to develop an analytical method for the determination of bullatacin in rat plasma using a liquid chromatography couple to a tandem mass spectrometry. The present method was efficient in analyzing large numbers of plasma samples obtained from pharmacokinetic studies following an intravenous injection of bullatacin in rats.

2. Experimental

2.1. Reagents and materials

Bullatacin and squamostatin-A (internal standard, IS) (Fig. 1) were isolated from the seeds of *Annona squamosa* L. in our laboratory. Their structures were characterized based on spectroscopic analysis (^{1}H NMR, ^{13}C NMR, HSQC, ESIMS and EIMS). The purity of each compound reached above 98% detected by HPLC–DAD–ELSD. Methanol (Merck, Germany) was of HPLC grade and deionized water was obtained from the Milli-Q system (Millipore, Bedford, MA, USA). Formic acid was purchased from Sigma (Shanghai, China). Other chemicals and reagents were all of analytical grade.

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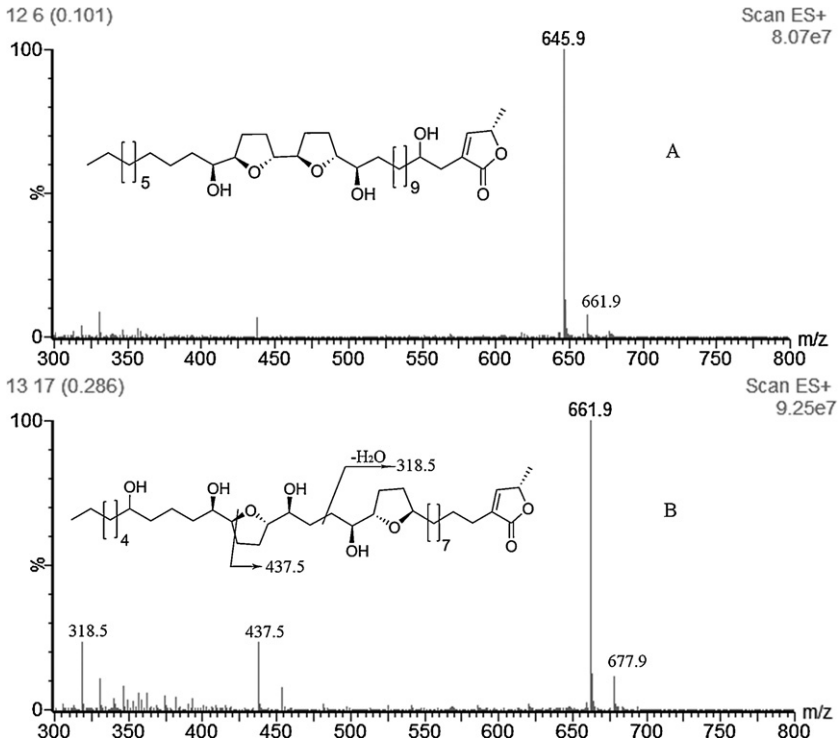

Fig. 1. Chemical structures and mass scan spectra for bullatacin (A) and IS (B).

2.2. Analytical system

An Alliance 2695 LC system (Waters, Milford, MA, USA) coupled with a triple-quadrupole tandem Waters Quattro Micro mass spectrometer was used for sample analysis. The Mass Lynx 4.1 software was used for instrumental control, acquisition and processing of the data.

LC separation was performed on an Agilent Zorbax SB-C_{18} column (150 mm × 2.1 mm, 5 μm, Agilent Technologies, Wilmington, DE, USA) with a security guard column (12.5 mm × 2.1 mm, 5 μm, Agilent Zorbax SB-C_{18}, DE, USA), kept at 35 °C. The mobile phase, consisting of methanol and deionized water (95:5, v/v) containing 0.01% (v/v) formic acid, was at a flow rate of 0.2 mL/min.

A MS detector with an electrospray ionization (ESI) interface in positive ion mode (ESI^+) was used for quantitative analysis, with acquisition in selected ion monitoring (SIM) mode. The $[M+Na]^+$ ions of bullatacin (*m*/*z* 645.9) and IS (*m*/*z* 661.9) were recorded simultaneously. The optimized electrospray conditions were voltage 3.2 kV, cone voltage 65 V, source temperature 110 °C, desolvation temperature 350 °C and desolvation gas flow (nitrogen) 450 L/h.

2.3. Preparation of standards and quality controls

Stock standard solutions of bullatacin and the IS were prepared in methanol at a concentration of 1.0 mg/mL. Immediately before use, serial dilution of the stock solution with methanol provided bullatacin working solutions covering the concentration from 5.0 ng/mL to 20.0 μg/mL. The working solution of IS was diluted to 10.0 μg/mL. All the solutions were stored at 4 °C.

The plasma calibration standards were prepared at concentrations of 0.5, 1.0, 2.5, 10.0, 25.0, 50.0, 100.0, 250.0, 500.0, 1000.0 and 2000.0 ng/mL for bullatacin and 1000.0 ng/mL for IS by spiking appropriate amount of working solutions to 100 μL of blank rat plasma. Three levels of quality control (QC) samples at concentrations of low (0.5 ng/mL), medium (100.0 ng/mL) and high (1000.0 ng/mL) for bullatacin together with 1.0 μg/mL for IS were prepared by spiking blank plasma samples. The spiked samples were then treated as described in the Section 2.4. The QC samples were frozen and used to evaluate the intra- and inter-day precisions and accuracy of the assay.

2.4. Sample preparation

Liquid/liquid extraction (LLE) by ethyl acetate was applied for the pretreatment of QC samples, calibration standards, and plasma samples. 10 μL IS (10.0 μg/mL in methanol) and 0.9 mL ethyl acetate were added to 100 μL plasma sample. The mixture was vortex-mixed for 3 min, and then centrifuged at 5000 rpm for 5 min. The upper organic layer (0.85 mL) was transferred to another tube and evaporated to dryness in a rotary evaporator (Centrivap console, Labconco Company, USA) at 40 °C. The residuum was dissolved with 100 μL of water:methanol (20:80, v/v), vortex-mixed for 1 min, centrifuged at 12,000 rpm for 3 min, and a 5 μL of the supernatant was then injected onto the LC–MS for analysis.

2.5. Method validation

A thorough and complete method validation of bullatacin determination in rat plasma was done following the US FDA guidelines. The method was validated for specificity, selectivity, linearity, accuracy and precision, recovery, matrix effect and stability.

Blank plasma samples obtained from five rats were screened to determine specificity. Each blank plasma sample was tested using the extraction procedure and chromatographic conditions described above to ensure no interference of bullatacin and IS from plasma. The linearity was observed from five calibration curves prepared and run on five different days over the range of 0.5–2000.0 ng/mL for bullatacin. The intra- and inter-day assay precisions and accuracy were estimated using a calibration curve to

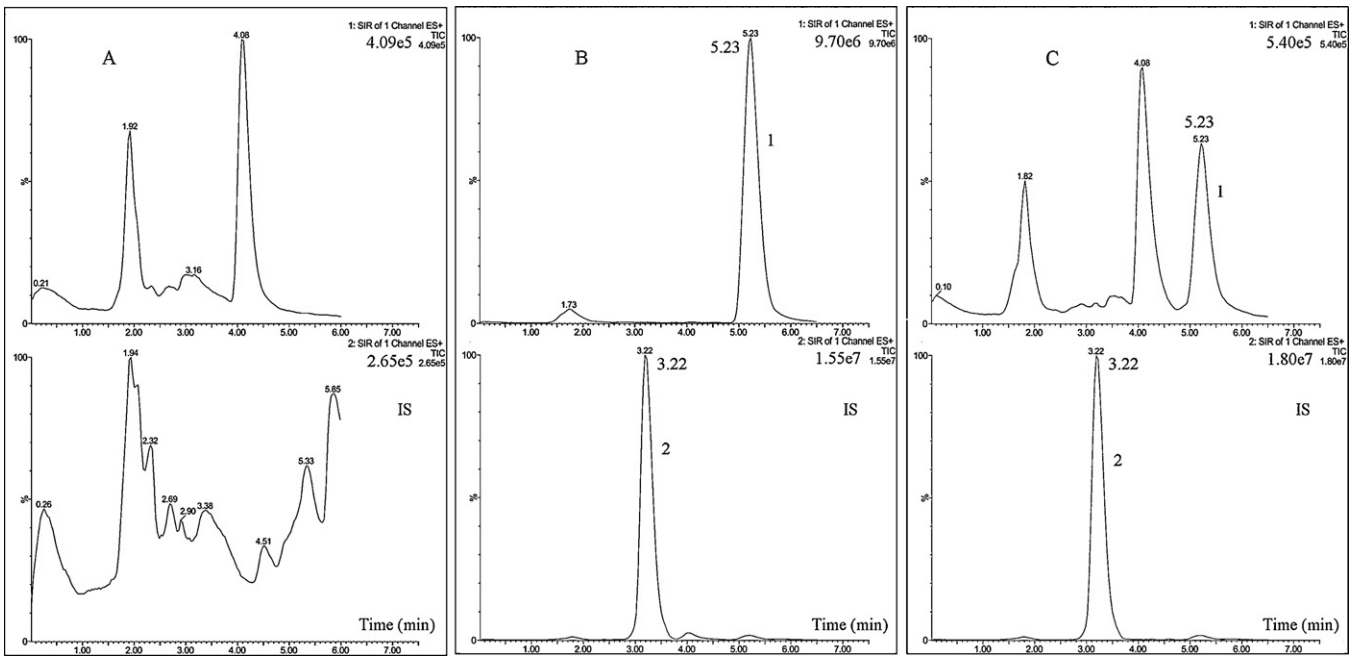

Fig. 2. Representative chromatograms of bullatacin and IS in rat plasma: (A) blank plasma; (B) blank rat plasma spiked with bullatacin (100.0 ng/mL) and IS (1000.0 ng/mL); and (C) plasma at 5 h after intravenous administration of bullatacin at 2.0 mg/kg. Retention time for bullatacin (1) and IS (2) was 5.23 min and 3.22 min, respectively.

predict the concentration of the quality controls. The matrix effect was assessed by comparing the mean peak areas of QC samples spiked after extraction with those of standard solutions. The extraction recovery was determined by comparing the mean peak areas of quality controls spiked before LLE to those spiked after the pretreatment.

2.6. Stability

Stability experiments were performed to evaluate the stability of the analyte in plasma under different conditions. Short-term stability was determined by keeping the QC samples at room temperature for 6 h. Long-term stability was evaluated by analyzing samples stored at −20 °C for 30 days and three freeze–thaw stability studies. Post-preparative stability was assessed by reanalyzing post-extraction samples kept in the autosampler at 10 °C for 24 h. The stability was acceptable when 85–115% of the initial analytes were found.

2.7. Pharmacokinetic application

Six Sprague-Dawley rats (male, weighing 200 ± 20 g) were obtained from the Laboratory Animal Center of Nanjing Medical University (Nanjing, China) and kept in an environmentally controlled breeding room for at least 3 days before experimentation. Animal welfare and experimental procedures were strictly in accordance with the guide for the care and use of laboratory animals (National Research Council of USA, 1996). After an overnight fast, the rats were given a single tail vein intravenous dose of 2.0 mg/kg bullatacin. Heparinized blood (0.3 mL) was serially taken from fossa orbitalis vein up to 7 h after drug administration. Plasma was separated and stored at −20 °C until analyzed. The plasma concentrations–time data was analyzed using a standard noncompartmental model with Kinetica 4.4 pharmacokinetics software (Thermo Electron Scientific Instruments Corp.). The major pharmacokinetic parameters were obtained using the software.

3. Results and discussion

3.1. Sample preparation and mass spectrometry

To achieve better extraction efficacy and less interference, various sample processing approaches including direct protein precipitation and LLE with different solvents (aether, cyclohexane, dichloromethane and ethyl acetate) were investigated respectively. After comprehensively evaluating extraction and total processing time, a simple LLE method was utilized for extraction from plasma samples and ethyl acetate was chosen for its good extraction efficiency. The instrument was operated in ESI^+ with acquisition in SIM mode. Under this condition, the most sensitive signal with m/z at 645.9 and 661.9 was observed and identified as $[M+Na]^+$ for bullatacin and IS (Fig. 1), respectively.

3.2. Liquid chromatography

To develop a simple separation process with a short run time, chromatographic analysis of bullatacin and IS was initiated under isocratic conditions. SB-C_{18} and TC-C_{18} columns, various mixtures of solvents using different buffers (ammonium acetate, ammonium formate and formic acid), along with altered flow rates (in the range of 0.2–0.4 mL/min) were tested to achieve ideal separation. Compared with the TC-C_{18}, the separation by SB-C_{18} showed better peak shape. Addition of 0.01% (v/v) formic acid to the mobile phase was found to facilitate optimum separation and enhance detection under the positive ionization mode. Methanol and deionized water (95:5, v/v) containing 0.01% (v/v) formic acid was finally used as the mobile phase. A flow rate of 0.2 mL/min permitted a run time of 7 min.

3.3. Specificity, linearity, accuracy and precision

Fig. 2 presented typical chromatograms for the blank plasma, plasma spiked with 100.0 ng/mL of bullatacin plus 1000.0 ng/mL

Table 1
Precision and accuracy validation results for bullatacin determination ($n = 5$).

Concentration (ng/mL)	Intra-day		Inter-day	
	Precision (RSD%)	Accuracy (mean ± SD%)	Precision (RSD%)	Accuracy (mean ± SD%)
0.5	8.7	99.5 ± 3.6	9.2	96.3 ± 4.9
100	5.6	104.0 ± 5.3	8.4	103.3 ± 4.2
1000	3.2	99.3 ± 2.8	2.7	98.5 ± 3.3

Table 2
Stability of bullatacin under different storage conditions (mean ± SD%, $n = 5$).

Concentration (ng/mL)	At room temperature for 6 h	Freeze–thaw stability	At 10 °C in the autosampler for 24 h	At −20 °C for 30 days
0.5	106.2 ± 3.4	100.4 ± 5.3	99.3 ± 2.5	105.3 ± 3.9
100	107.7 ± 3.1	101.3 ± 3.7	101.4 ± 4.2	104.2 ± 2.1
1000	99.8 ± 4.1	98.3 ± 1.4	98.3 ± 1.5	100.4 ± 4.8

IS and a rat plasma sample. Under the optimized chromatographic conditions and sample processing procedure, the retention time was 3.22 and 5.23 min for IS and bullatacin, respectively. The chromatograms showed a clear and excellent separation between bullatacin, IS and endogenous interferences from plasma. The calibration curves provided a reliable response for bullatacin ($y = 4.669x + 0.001$, $r^2 = 0.9998$, 0.5–2000.0 ng/mL). The ratio of the peak area of bullatacin relative to that of IS was correlated with the corresponding plasma concentration, and good linearity was observed. The detection limit for bullatacin was 0.5 ng/mL. The estimates of the intra- and inter-day precisions and accuracy of the assay are presented in Table 1. The relative standard deviations of the intra- and inter-day assay precisions were less than 8.7% and 9.2%, respectively. The intra- and inter-day assay accuracy was 99.3–104.0% and 96.3–103.3%, respectively.

3.4. Recovery and matrix effect

The extraction recovery of bullatacin was calculated by analyzing five replicates at 0.5, 100.0 and 1000.0 ng/mL. The extraction recoveries (mean ± SD%) were 102.1 ± 3.2%, 98.3 ± 5.4% and 90.0 ± 6.3% for the assays, respectively. The matrix effects (mean ± SD%) for plasma determined at concentrations of 0.5, 100.0 and 1000.0 ng/mL for bullatacin were 98.3 ± 3.5%. No significant matrix effects were evident.

3.5. Stability

Stability of bullatacin under various storage conditions was investigated at three different concentrations (Table 2). The results indicated that bullatacin in the plasma was stable under the various conditions evaluated. Thus the samples were stable during the whole process of analysis.

3.6. Application of the method

The validated method was used to evaluate the pharmacokinetics of bullatacin in rats. Fig. 3 showed the mean plasma concentrations after a single intravenous dose of 2.0 mg/kg bullatacin in rats. The major pharmacokinetic parameters (mean ± SD) of bullatacin were calculated using a noncompartment model based on statistical moment. C_{max} (μg/mL), $T_{1/2}$ (h), Vd (L), CL (L/h), AUC_{0-t} (ng h/mL) and $AUC_{0-\infty}$ (ng h/mL) were 2.39 ± 0.11, 5.04 ± 0.17, 28.05 ± 10.17, 9.08 ± 0.99, 3.75 ± 0.14 and 4.80 ± 0.19, respectively.

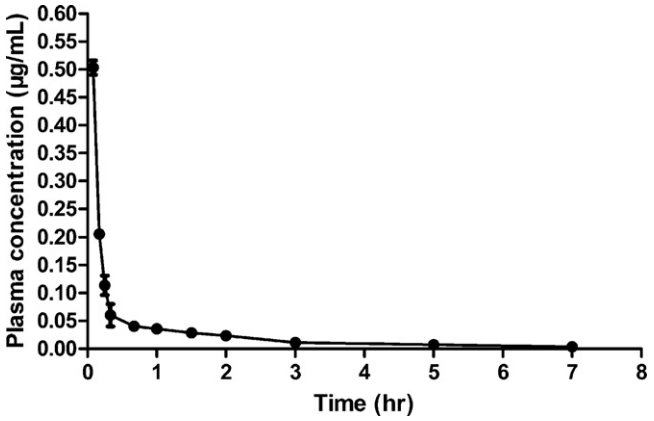

Fig. 3. Time course of mean plasma bullatacin concentrations (±SD) after a single intravenous administration of 2.0 mg/kg bullatacin in rats ($n = 6$).

4. Conclusions

The described LC–MS validated analytical methodology enables the rapid and selective assay of bullatacin in rat plasma. The propose method presents high sensitivity, accuracy, precision, recovery and stability combined with high accuracy mass measurement, thus being suitable for pharmacokinetic studies of the compound *in vivo*.

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References

[1] A. Yazbak, S.C. Sinha, E. Keinan, J. Org. Chem. 63 (1998) 5863.
[2] A. Bermejo, B. Figadere, M.C. Zafra-Polo, I. Barrachina, E. Estornell, D. Cortes, Nat. Prod. Rep. 22 (2005) 269.
[3] L.A.R. Lima, L.P.S. Pimenta, M.A.D. Boaventura, Food Chem. 122 (2010) 1129.
[4] J.L. McLaughlin, J. Nat. Prod. 71 (2008) 1311.
[5] C.C. Liaw, T.Y. Wu, F.R. Chang, Y.C. Wu, Planta Med. 76 (2010) 1390.
[6] V. Coothankandaswamy, Y. Liu, S.C. Mao, J.B. Morgan, F. Mahdi, M.B. Jekabsons, D.G. Nagle, Y.D. Zhou, J. Nat. Prod. 73 (2010) 956.
[7] K. Noh, J.H. Park, J.H. Park, M. Kim, M. Jung, H. Ha, K. Kwon, H.J. Lee, W. Kang, J. Pharm. Biomed. Anal. 56 (2011) 114.