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Liquid Chromatography-Tandem Mass Spectrometric Determination of Geniposide in Rat Plasma and its Pharmacokinetic Application

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Geniposide is a biologically active ingredient of gardenia fruit. A liquid chromatography-tandem mass spectrometric method was developed and validated for the determination of geniposide in rat plasma. The plasma samples were pretreated by solid-phase extraction and introduced into a BDS Hypersil C_{18} column (50 × 2.1 mm, 5 μm) for chromatographic separation. The mobile phase consisted of 0.1% formic acid and 0.1% formic acid in acetonitrile, and gradient elution was performed at a flow rate of 0.25 mL/min. For mass spectrometric detection, multiple reaction monitoring was performed *via* an electrospray ionization source in positive mode. The calibration curve for geniposide was linear ($r^2 = 0.997$) in the concentration range of 0.005-1 μg/mL. The intra- and inter-day accuracies and precisions fulfilled the required criteria (± 15%). The developed method was subsequently used for pharmacokinetic analysis of geniposide after oral administration to rats at a dose of 50 mg/kg. The mean maximum plasma concentration of geniposide was 0.68 ± 0.29 μg/mL at 0.44 ± 0.13 h, and the mean area under the plasma concentration versus time curve was 1.46 μg·h/mL.

Key Words : Geniposide, LC-MS/MS, Pharmacokinetics, Rats

Introduction

Geniposide, an iridoid glycoside, is one of the major constituents in gardenia fruits.[1] Geniposide is light yellow to white colored, and it is used as a natural water-soluble pigment or is an ingredient of the pigments extracted from gardenia fruits such as Gardenia Yellow, Green, Red, or Blue. Many studies have shown the pharmacological effects of geniposide, which include protective effects against radiation[2] and oxidative damages,[3] and anti-hypoglycemic,[4] anti-thrombotic,[5] anti-inflammatory,[6] and anti-carcinogenic[7] effects.

Several methods have been published for the determination of geniposide in biological samples such as the plasma or serum for pharmacokinetic studies. Those methods generally use high-performance liquid chromatography (HPLC) or liquid chromatography-tandem mass spectrometry (LC-MS/MS).[8-13] However, the HPLC methods have a limitation because of the low sensitivity; these methods can determine geniposide levels greater than 100 ng/mL.[8-10] The improvement in the detection or quantitation limit seems to be achieved by using an LC-MS/MS instrument, which was not completely supported by the provided validation data.[11,12] Wang et al. have described an improved LC-MS/MS method with increased ionization efficiency *via* formation of ammonium or acetate adducts,[13] but the sample preparation procedure is not clearly described in their study. Here, we developed a simple, sensitive, and valid LC-MS/MS method for the determination of geniposide in rat plasma and subsequently used the method for pharmacokinetic analysis of geniposide.

Experimental

Chemicals. Geniposide and formic acid were purchased from Sigma Chemicals (St. Louis, MO). Digoxin was purchased from Wako Pure Chemical Industries (Osaka, Japan). HPLC-grade acetonitrile and methanol were purchased from J. T. Baker (Philipsburg, NJ). HPLC-grade water was prepared using a Milli-Q purification system (Millipore, Bedford, MA). The Oasis HLB cartridge (96-well plate) was purchased from Waters (Milford, MA). A geniposide-containing fraction of gardenia extract (GF) was provided by Prof. Tae Cheon Jeong (Yeungnam Univ., Gyeongsan, Korea).

Preparation of Standard Solutions. Stock solution of geniposide was prepared at a concentration of 1 mg/mL in water. The stock solution was serially diluted with water to obtain working standard solutions at several concentrations. The internal standard (IS) solution was prepared to contain 2 μg/mL of digoxin in methanol. The standard working solutions were stored at −20 °C.

Preparation of Calibration Standards and Quality Control Samples. The standard solution of geniposide (5 μL) was added to 45 μL of blank rat plasma to prepare calibration standards (5-1000 ng/mL) and quality control (QC) samples (10, 100, and 1000 ng/mL). After 5 μL of IS solution was added, the plasma samples were introduced to activated solid-phase extraction cartridges (Oasis HLB 96-well plate). The analytes were eluted with methanol, and the eluent was evaporated to dryness under a stream of nitrogen gas (N_2) at 50 °C. The residue was dissolved in 100 μL of 50% methanol, and a 5-μL aliquot was injected onto the HPLC column for LC-MS/MS analyses.

Sample Preparation. Plasma samples (50 μL) were taken and IS solution (5 μL) was added to them. The sample was then prepared as described above.

LC-MS/MS Analyses. The LC-MS/MS system consisted of an Agilent 1260 Infinity HPLC system (Agilent Technologies, Palo Alto, CA) and an Agilent Triple-Quad mass spectrometer (Agilent Technologies, Palo Alto, CA) equipped with a turbo ion spray source. Chromatographic separation was achieved using a BDS Hypersil column (50 mm × 2.1 mm ID, 5 μm, Thermo Scientific, Waltham, MA), and oven temperature was maintained at 40 °C. The mobile phase consisted of 0.1% formic acid (solvent A) and 90% acetonitrile with 0.1% formic acid (solvent B). A gradient program was used at a flow rate of 0.25 mL/min. The initial composition of the mobile phase was 10% solvent B, which was changed to 90% of solvent B to 1 min, maintained for 4 min, and returned to the initial composition. Total run time was 8 min. Electrospray ionization (ESI) was performed in negative ion mode. The mass transitions monitored in multiple reaction monitoring (MRM) detection were 433 → 225 for geniposide and 825 → 779 for the IS (digoxin).

Method Validation. The analytical method was validated according to the "Guidance for Industry, Bioanalytical Method Validation" described by the US Food and Drug Administration.[14] The calibration curve of geniposide in rat plasma was generated by plotting the peak area ratio for geniposide to IS versus the concentration of geniposide in the standard-spiked plasma by least-square linear regression. The calibration curves were obtained using 8 calibration standards in triplicate in the concentration range of 5-1000 ng/mL.

The intra-day precision and accuracy were evaluated by analyzing 5 plasma samples spiked with 3 concentrations of QC samples. The inter-day precision and accuracy were assessed at the same concentrations and the tests were repeated for 5 different days. Accuracy was expressed as a percentage of the nominal concentration and precision was represented as a relative standard deviation (%RSD).

The matrix effect, recovery, and process efficiency for geniposide and digoxin in rat plasma were evaluated at 2 concentrations (10 and 500 ng/mL). The peak areas of geniposide or digoxin were measured in the mobile phase (A) and in the samples spiked after (B) and before (C) extraction. The matrix effect, recovery, and process efficiency were calculated as follows: Matrix effect (%) = B/A × 100; Recovery (%) = C/B × 100; Process efficiency (%) = C/A × 100.

The stability of geniposide in rat plasma was evaluated in triplicates using 2 concentrations (10 and 500 ng/mL). For short-term stability studies, the QC samples were stored at room temperature for 6 h and then were analyzed. For long-term stability studies, the QC samples were stored at −20 °C for 14 days and then were analyzed. For freeze-thaw stability, the QC samples were stored at −20 °C for 24 h and thawed at room temperature. The freeze/thaw cycle was repeated 2 more times and then the samples were analyzed. For post-preparative stability studies, the QC samples were analyzed after storing in the autosampler (4 °C) for 24 h.

Animal Experiments. Male Sprague-Dawley rats (8 weeks old, weighing approximately 280-300 g) were purchased from Nara Bio (Seoul, Korea). Rats were housed in a room with controlled temperature (23 ± 2 °C) and moisture (55% ± 10%), exposed to a controlled 12-h light/12-h dark cycle, and allowed access to food and water ad libitum. Rats were fasted overnight before oral administration. GF was used for administration of geniposide due to unavailability of geniposide standard. GF was standardized to contain 40% geniposide and the content of geniposide was determined using the developed LC-MS/MS method. Rats were orally administered a geniposide-containing fraction of gardenia extract (GF) at a concentration of 125 mg/kg (50 mg/kg as geniposide). The blood samples were collected at 0.08, 0.5, 1, 2, 4, 6, 8, 12 and 24 h from carotid artery. The samples were centrifuged at 13,000 rpm for 5 min, and plasma samples were collected and stored at −20 °C before analysis.

Pharmacokinetic Analysis. Plasma concentration data for individual rats were analyzed, and pharmacokinetic parameters were estimated by a non-compartmental method using WinNonlin™ 5.2 (Pharsight, Sunnyvale, CA). The area under the plasma concentration-time curve (AUC) was calculated using the trapezoidal rule extrapolated to infinite time. The terminal elimination half-life ($t_{1/2}$), the peak plasma concentration (C_{max}), and the time to reach C_{max} (T_{max}) were obtained directly from the experimental data.

Results and Discussion

Chromatography and Spectrometry. We evaluated the mass response of geniposide in ESI in both positive and negative ionization modes. In the positive mode, geniposide

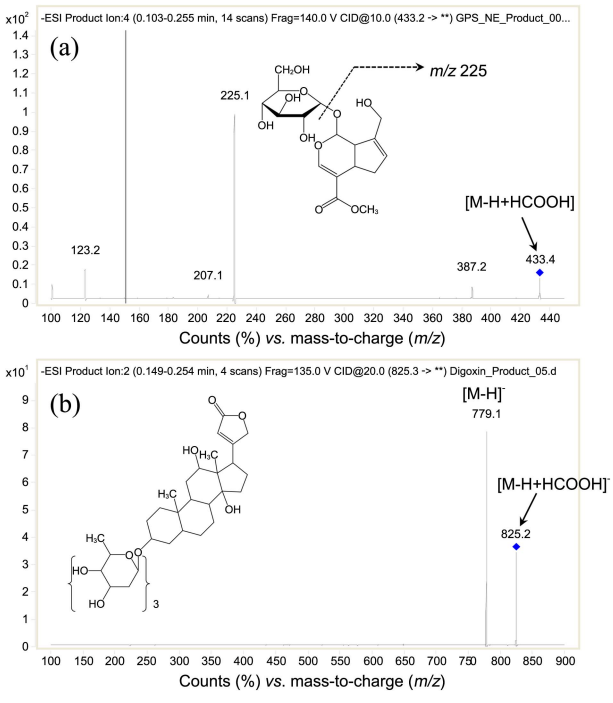

Figure 1. Product ion spectra of geniposide (a) and digoxin (b).

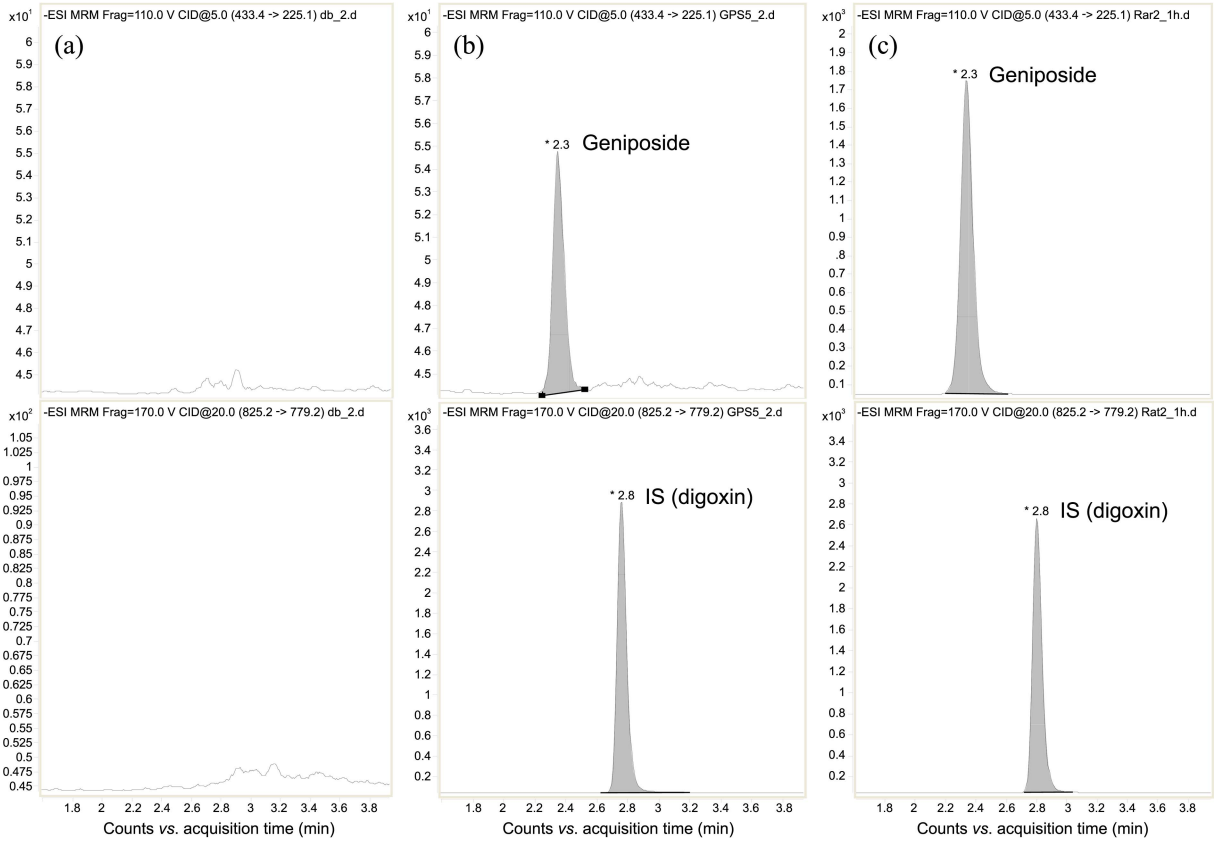

Figure 2. Typical MRM chromatograms of (a) blank rat plasma, (b) blank rat plasma spiked with geniposide (0.005 μg/mL) and IS and (c) the rat plasma sample taken 1 h after oral administration of GF.

was observed as sodium adduct ions but the formation of the adduct ions was somewhat unstable. In the negative mode, geniposide was ionized to yield formic acid adduct ions ([M-H+HCOOH]$^-$) in the presence of 0.1% formic acid as a mobile phase modifier. In addition, digoxin (IS) showed the same pattern of ion formation as that of geniposide. Thus, the pseudomolecular ions of geniposide and digoxin (IS) were found at *m/z* 433 and 825, respectively. When the product ions were scanned for those formic acid adduct ions (Figure 1), the main product ion for geniposide was observed at *m/z* 225, which can be explained by the neutral losses of glucose and formic acid molecules. Digoxin showed the main product ion at *m/z* 779 with removal of the adducted formic acid. Consequently, the MRM transitions for geniposide and digoxin were optimized to be 433 → 225 and 825 → 779, respectively.

Optimization of chromatographic separation was performed on a Hydrosphere C18 column with gradient elution. Addition of 0.1% formic acid to both aqueous and organic solvents produced good resolution and peak shape in the chromatogram as well as improved the ionization of the analytes by formation of formic acid adducts. The retention times of geniposide and digoxin were 2.3 and 2.8 min, respectively (Figure 2).

Method Validation.

Specificity: The specificity of the method was evaluated by comparing the MRM chromatograms of geniposide and digoxin as a blank, a standard spiked plasma sample, and a plasma sample collected after oral administration of geniposide (Figure 2). No interference from the matrix was observed at the retention times of geniposide or digoxin.

Linearity and Lower Limit of Quantitation: The calibration curve of geniposide was obtained in the concentration range of 5-1000 ng/mL (n = 3). The correlation coefficient (r^2) was greater than 0.997, which indicated a good linearity. The lower limit of quantitation (LLOQ), defined as the lowest concentration analyzed with accuracy between 80% and 120% and precision (%RSD) < 20%, was 5 ng/mL using our analytical method. The limit of detection (LOD) was 1 ng/mL on the basis of the signal to noise ratio of 3.

Precision and Accuracy: The precision and accuracy of the method were determined by replicate analyses (n = 5) of the QC samples of 3 concentrations (10, 100, and 1000 ng/mL). The intra-day precision was lower than 8.8% and the accuracy remained between 96.2% and 103.7% at all concentrations tested. The inter-day precision was lower than 7.9% and the accuracy remained between 101.0% and 108.4% (Table 1). These results indicated that the developed method was precise, reproducible, and accurate enough for the quantification of geniposide in rat plasma.

Matrix Effect, Recovery, and Process Efficiency: The average matrix effect, recovery, and process efficiency of geniposide at 2 different concentrations (10 and 500 ng/mL) were 85.6%, 91.8%, and 78.6%, respectively. The matrix

Table 1. Intra- and inter-day accuracy and precision for determination of geniposide in rat plasma

Nominal Concentration (ng/mL)	Concentration found (ng/mL)	Accuracy (%)	Precision (%)
Intra-day (n = 5)			
10	10.37 ± 0.40	103.7	3.9
100	96.19 ± 5.04	96.2	5.2
1000	996.3 ± 88.0	99.6	8.8
Inter-day (n = 5)			
10	10.84 ± 0.69	108.4	6.3
100	103.4 ± 5.8	103.4	5.6
1000	1010 ± 80	101.0	7.9

Table 2. Matrix effect, recovery and process efficiency data for geniposide and IS (digoxin) in rat plasma (n = 6)

Concentration (ng/mL)	Matrix effect (%)	Recovery (%)	Process efficiency (%)
	Mean ± SD	Mean ± SD	Mean ± SD
Geniposide			
10	84.5 ± 2.7	93.4 ± 3.9	78.9 ± 2.8
500	86.6 ± 1.6	90.2 ± 1.1	78.2 ± 1.4
IS (digoxin)			
200	72.5 ± 2.3	92.0 ± 4.3	66.6 ± 2.6

effect, recovery, and process efficiency of the IS were 72.5%, 92.0%, and 66.6%, respectively. Some degree of ion suppression by the matrix was observed in both analytes. However, the recovery of both compounds was more than 90% and the %RSD of the matrix effect, recovery, or process efficiency was less than 4.7%, which indicated good reproducibility. The detailed data are summarized in Table 2.

Stability: All samples were examined by comparing them with freshly prepared QC samples. Geniposide was stable in rat plasma with acceptable accuracies (88.67-110.59%) and precisions (< 5.87%) after 24 h at room temperature, 14 days at −70 °C, 24 h at the autosampler, and 3 freeze/thaw cycles. Therefore, the present method was considered applicable for routine analysis.

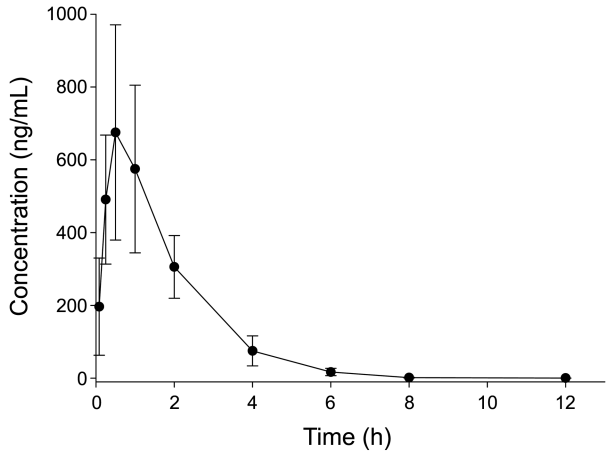

Figure 3. Mean plasma concentration-time profile of geniposide after oral administration of GF (50 mg/kg of geniposide) to rats (n = 4).

Table 3. Stability data for geniposide in rat plasma (n = 3)

Stability (% remained)	Spiked concentration (ng/mL)	
	10	500
Short-term stability (room temperature for 4 h)	97.9 ± 2.9	101.7 ± 1.7
Long-term stability (−20 °C for 14 days)	95.2 ± 5.5	100.7 ± 3.6
Freeze-thaw stability (−20 °C, 3 cycles)	104.9 ± 4.5	89.1 ± 2.9
Autosampler stability (4 °C for 24 h)	108.2 ± 2.0	103.8 ± 2.5

Table 4. Pharmacokinetic parameters for geniposide after oral administration of GF to rats (n = 4)

Parameter	Geniposide (50 mg/kg)
C_{max} (μg/mL)	0.68 ± 0.29
T_{max} (h)	0.44 ± 0.13
AUC (μg·h/mL)	1.46 ± 0.37
$T_{\lambda 1/2}$ (h)	0.94 ± 0.05

Pharmacokinetic Study of Geniposide in Rat Plasma. We used the developed method for pharmacokinetic analysis of geniposide after oral administration of GF to rats. The mean plasma concentration versus time curve of geniposide is shown in Figure 3, and the pharmacokinetic parameters are presented in Table 4. After the oral administration of GF (as 50 mg/kg of geniposide), the mean C_{max} of geniposide was 0.68 μg/mL at 0.44 h and the mean AUC was 1.46 μg·h/mL. The mean apparent $t_{1/2}$ was estimated to be 0.94 h.

Conclusion

We developed and validated the LC-MS/MS method for quantitative analysis of geniposide, the bioactive compound of gardenia fruits, in rat plasma. The developed method was sensitive, accurate, and reproducible and was successfully used for pharmacokinetic analysis of geniposide after oral administration in rats. The method would be useful for pharmacokinetic studies of geniposide present in herbal medicines or food supplements.

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