Determination of glycyrrhizin in dog plasma by liquid chromatography–mass spectrometry and its application in pharmacokinetic studies

Weichao Ren,† Yang Lu,† Jing Jing,† Jing Zhu,† Di Wan,† Di Zhao,† Jianheng Zheng,† Fang Fang,† Yan He† and Xijing Chen†,‡,*

†Center of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing 210009, China. E-mail: chenxj-lab@hotmail.com
‡School of Pharmacy, University of Queensland, Brisbane, Queensland 4102, Australia

A sensitive liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI-MS) method was established and validated for the determination of glycyrrhizin in dog plasma. After treatment with methanol to precipitate proteins, plasma samples were analyzed on a reversed-phase C18 (ODS) column with a mobile phase of methanol:1% formic acid solution (75:25, v/v). MS determination was performed using negative electrospray ionization (negative ESI) in the selected ion monitoring mode. Glycyrrhizin was monitored at the m/z 821 channel and the internal standard (glucidone) at the m/z 526 channel. The calibration curve was linear over the range from 0.05 µg mL⁻¹ to 10 µg mL⁻¹ with a correlation coefficient above 0.99. This method was successfully applied to the pharmacokinetic studies in beagle dogs. The absolute bioavailability of glycyrrhizin in beagle dogs was 3.24%.

Keywords: glycyrrhizin, LC-MS, beagles, pharmacokinetics, bioavailability

Introduction

Licorice (Glycyrrhiza glabra) is an old herbal drug widely used all over the world. The therapeutic use of licorice dates back to ancient China, Europe and Africa. Licorice possesses many biological activities, such as anti-allergic effects, anti-inflammatory and anti-ulcer effects and has been involved in the treatment of asthma, gastric ulcers, bronchitis, cardiovascular diseases, urinogenital diseases, liver diseases and so forth for thousands of years. Besides its widespread therapeutic use, licorice also plays important roles in the food, cosmetics and tobacco industries as an ingredient.

To date, many components have been identified in licorice. Among these components, glycyrrhizin (chemical structure shown in Figure 1), a triterpene saponin, is among the most active. Glycyrrhizin exhibits wide pharmacological effects, including anti-viral, anti-microbial, anti-inflammatory, hepatoprotective and immunomodulatory effects. Recent studies also demonstrate that it plays protective roles in rats with acute cholestasis and acute vanishing bile duct syndrome. Clinically, it has been widely used in the treatment of patients with chronic hepatitis for a long time. After oral administration, either as the compound alone or as an ingredient in herbal remedy remedies, glycyrrhizin is metabolized to its active metabolite glycyrrhetinic acid, by human intestinal bacteria prior to absorption. This glucuronidation reaction is primarily mediated by UGT1A1, 1A3, 2B4 and 2B7, as described in our previous in vitro study.

Analysis methods by using liquid chromatography–mass spectroscopy (LC-MS) or LC-tandem mass spectroscopy...
Determination of Glycyrrhizin in Dog Plasma have been established for the determination of glycyrrhizin and/or its active metabolite, glycyrrhetinic acid, in human plasma, but some analyses involve extensive sample preparation. Moreover, even with these highly sensitive methods, only glycyrrhetinic acid can be detected in human plasma after oral administration of its precursor compound, glycyrrhizin, when applied to determine pharmacokinetic behavior. Beagle dogs are often used as an animal model for bioavailability studies. It is critical to establish analysis methods to determine glycyrrhizin in beagle plasma for future understanding of its disposition in vivo. At present, a high-performance liquid chromatography (HPLC) method has been reported for the determination of glycyrrhizin in dog plasma. The purpose of this present research is to develop and validate a sensitive and specific Lc-ESI-MS method for the separation and quantification of glycyrrhizin in dog plasma and apply it to a pharmacokinetic study in beagle dogs.

Experimental
Reagents and materials
Glycyrrhizin and gliquidone (used as an internal standard) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products. Ammonium glycyrrhizinate was supplied by Henan Shuake Pharmaceutical Co. Ltd (Henan, China). Methanol was of HPLC grade (Tedia Co., Inc., USA). All other reagents were of analytical grade.

Instrumentation
Analysis was performed on a Shimadzu 2010 LC-MS system equipped with a SIL-20AC auto-injector, a DGU-20A3 online vacuum degasser, binary CBM-20A pumps, a CTO-20A temperature-controlled column oven, a quadruple mass spectrometer equipped with an electrospray ionization interface (ESI) source and a LC-MS workstation (Shimadzu, Japan). The analytical column was a Shimadzu VP-ODS column (150 mm × 2.0 mm, i.d. 5 µm) (Shimadzu, Japan) kept at 28°C.

LC-ESI-MS conditions for the determination of glycyrrhizin
Samples were determined in the negative electrospray ionization (ESI−) detection mode under following source conditions: gas flow, 1.5 L min⁻¹; detector voltage, 1.6 kV; fixed curve dissolution line (CDL) voltage as in tuning, and CDL temperature of 250°C. Analysis was carried out using selected ion monitoring (SIM) for specific ion [M−H]⁻ and m/z 526 for the internal standard [M−H]⁻. The mobile phase consisted of methanol:1% formic acid solution (75:25, v/v) at a flow rate of 0.2 mL min⁻¹.

Sample preparation
Samples were prepared by adding 10 µL of internal standard (gliquidone in methanol, 75 µg mL⁻¹) to the plasma sample (100 µL) and vortexed, followed by precipitation of proteins with 500 µL of methanol. After vortex and centrifugation (12,000 × g, 3 min), the supernatant was transferred into glass inserts of autosampler vials. An aliquot of 5 µL of each sample was injected into the LC-MS system for analysis.
Preparation of stock solutions and working solutions
Stock solutions of glycyrrhizin and gliclazide were prepared by dissolving the accurately weighed reference compound in methanol to give a final concentration of 2 mg mL$^{-1}$ and 300 µg mL$^{-1}$, respectively. A 75 µg mL$^{-1}$ working solution of the internal standard was prepared by diluting the 300 µg mL$^{-1}$ stock solution of gliclazide with methanol. Working solutions of glycyrrhizin were prepared by diluting the stock solution serially with methanol to concentrations of 0.5 µg mL$^{-1}$, 1 µg mL$^{-1}$, 2 µg mL$^{-1}$, 5 µg mL$^{-1}$, 10 µg mL$^{-1}$, 20 µg mL$^{-1}$, 50 µg mL$^{-1}$, and 100 µg mL$^{-1}$.

Drug administration and sampling
Four healthy male dogs weighing 10.4 ± 0.6 kg (from the Animal Center of Medical College of Southeast University, Nanjing, China) received ammonium glycyrrhizinate at 10 mg kg$^{-1}$ and 1 mg kg$^{-1}$ by intragastric (i.g.) and intravenous (i.v.) administration in two separate sessions according to a randomized cross-over design. Blood samples (1 ml) were then collected by venepuncture at 0 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, 10 h, 24 h, 36 h and 60 h for the i.g. group and 0 h, 0.25 h, 0.5 h, 1 h, 2 h, 4 h, 6 h, 12 h, 24 h, 36 h and 60 h for the i.v. group after glycyrrhizin administration. The blood sample was transferred to a heparinized Eppendorf tube, mixed gently, then centrifuged at 12,000 g for 3 min to obtain 0.1 ml plasma and stored at −20°C until analysis.

Data analysis
The pharmacokinetic parameters were calculated using non-compartmental method by 3p87 pharmacokinetic software (version 1.0; Chinese Society of Mathematical Pharmacology, Beijing, China). The values of the peak drug concentration ($C_{max}$) and time to reach the peak maximum ($T_{max}$) were obtained from the values observed. The area under the plasma concentration versus time curves (AUC) was calculated using the trapezoidal rule. Other parameters, such as mean residence time (MRT) and area under the moment curve (AUMC), were obtained from the results of the software. Absolute bioavailability (F) of glycyrrhizin in beagles was calculated using the equation:

$$F = \frac{AUC_{ig} \times D_{ig}}{AUC_{iv} \times D_{iv}} \times 100\%$$

where $AUC_{ig}$ and $AUC_{iv}$ are the AUC values after intragastric and intravenous administration of ammonium glycyrrhizinate, respectively, and $D_{ig}$ and $D_{iv}$ are the doses used for intragastric and intravenous administration, respectively.

Results and discussion
Chromatography and method validation
Sensitivity and specificity
The lower limit of quantification was determined as the minimum concentration that could be accurately and precisely quantified (lowest data point of the standard curve). The specificity of the assay for the analytes versus endogenous substances in the matrix was assessed by comparing the lowest concentration in the calibration curves with reconstitutions prepared with drug-free plasma from five different dogs. The lower limit of quantification was 0.05 µg mL$^{-1}$. No interfering peaks were observed at the retention time of glycyrrhizin, or internal standard in blank dog plasma samples (Figure 2). The retention times of glycyrrhizin and the internal standard were 5.2 min and 8.4 min, respectively.

Calibration curves
Calibration curves were prepared by adding 10 µL glycyrrhizin working solution at serial concentration and 10 µL gliclazide work solution to 90 µL drug-free dog plasma, followed by precipitation, centrifugation and injection into the LC-MS system for analysis. Linearity regression was assessed by plotting the peak-area ratios of glycyrrhizin to the internal standard ($A_{ig}/A_{iv}$) versus known concentrations (C). Good linearity was observed over the range of 0.05–10 µg mL$^{-1}$. The regression equation of the curves was $A_{ig}/A_{iv} = 0.4658 - 0.0034 (r^2 = 1)$.

Accuracy and precision
Accuracy and precision (presented as relative standard deviation, RSD) of the assay were determined using quality control (QC) samples (at 0.2 µg mL$^{-1}$, 2 µg mL$^{-1}$ and 5 µg mL$^{-1}$). Accuracy (%) was determined by the percentage ratio of measured over spiked QC concentration [mean of measured/spiked] × 100%]. Intra-day precision was determined by analyzing replicate aliquots of QCs (n = 5 at each concentration) on the same day. Inter-day precision was determined by repetitive analysis of QC samples (each concentration) on three consecutive days. RSD values for the intra-day assay and inter-day assay were no more than 7% and 15%, respectively.

Recovery
To investigate the relative recovery of glycyrrhizin, plasma samples were spiked with glycyrrhizin at concentrations of 0.2 µg mL$^{-1}$, 2 µg mL$^{-1}$ and 5 µg mL$^{-1}$. The resulting peak-area ratios (analyte : internal standard) were compared with that of the standards prepared in the mobile phase to provide the recovery values. The mean plasma extraction recoveries of glycyrrhizin at concentrations of 0.2 µg mL$^{-1}$, 2 µg mL$^{-1}$ and 5 µg mL$^{-1}$ were 101.1%, 104.6% and 100.8%.

Application of the method in pharmacokinetic study
The method developed was applied to the pharmacokinetic study of glycyrrhizin in dogs. Figures 3 and 4 show the time course of mean glycyrrhizin concentrations in the blood of four normal beagles after oral administration of 10 mg kg$^{-1}$ and intravenous injection of 1 mg kg$^{-1}$ glycyrrhizin, respectively. Pharmacokinetic parameters from non-compartment analysis for intravenous administration were: $t_{1/2} = 14.4$ h, $AUC = 118.1$ µg h mL$^{-1}$ and MRT = 15.9 h (Table 1). Pharmacokinetic parameters for intragastric administration were: $C_{max} = 1.5$ µg mL$^{-1}$, $t_{max} = 4.3$ h, $t_{0.5} = 16.5$ h, $AUC = 32.8$ µg h mL$^{-1}$ and MRT = 17.5 h. The absolute
Figure 3. Mean plasma concentration–time curve of glycyrrhizin in dogs after oral administration of 10 mg kg$^{-1}$ glycyrrhizin.

Figure 2. MS chromatogram of glycyrrhizin in beagle plasma. (a) Blank beagle plasma. (b) Blank beagle plasma containing glycyrrhizin and internal standard. (c) A plasma sample from beagle after a single dose oral administration of glycyrrhizin. The internal standard is also shown.

Figure 4. Mean plasma concentration–time profile of glycyrrhizin in beagle plasma after intravenous administration of mono ammonium glycyrrhizin at a single dose of 1 mg kg$^{-1}$.
Various physiological factors contribute to a reduction in availability of drugs for entry into the systemic circulation. In terms of glycyrrhizin, the low bioavailability may be attributed to enzymatic hydrolyzation both in the stomach and the intestine after oral administration.

### Conclusion

This report describes a sensitive and specific HPLC-ESI-MS method for the determination of glycyrrhizin in dog plasma. The preparation of the sample is very simple and only needs the addition of methanol followed by centrifugation. The lower limit of quantification is as low as 0.05 µg mL\(^{-1}\). The detection time is less than 10 min. It is shown that the method developed is reliable and can easily be applied to the pharmacokinetic study of glycyrrhizin in beagle dogs.

### Acknowledgements

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### References


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Table 1. Pharmacokinetic parameters of glycyrrhizin in plasma from four beagle dogs after intravenous injection of monoammonium glycyrrhizin.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sample number*</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>( t_{\text{0}} (\text{h}) )</td>
<td>12.5</td>
<td>15.5</td>
<td>15.5</td>
</tr>
<tr>
<td>AUC (µg.h·mL(^{-1}))</td>
<td>124.7</td>
<td>130.5</td>
<td>94.1</td>
</tr>
<tr>
<td>AUMC (µg.h(^2)·mL(^{-1}))</td>
<td>1700.1</td>
<td>2284.2</td>
<td>1525.4</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>13.6</td>
<td>17.5</td>
<td>16.2</td>
</tr>
</tbody>
</table>

*Results are given for each dog, numbers 1 to 4.

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Table 2. Major pharmacokinetic parameters obtained from four beagle dogs after oral administration of glycyrrhizin.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sample number*</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>( C_{\text{max}} (\text{µg mL}^{-1}) )</td>
<td>0.9</td>
<td>1.6</td>
<td>2.2</td>
</tr>
<tr>
<td>( T_{\text{max}} (\text{h}) )</td>
<td>4</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>( t_{\text{0}} (\text{h}) )</td>
<td>10.9</td>
<td>13.3</td>
<td>20.9</td>
</tr>
<tr>
<td>AUC (µg.h·mL(^{-1}))</td>
<td>16.3</td>
<td>37.9</td>
<td>47.8</td>
</tr>
<tr>
<td>AUMC (µg.h(^2)·mL(^{-1}))</td>
<td>211.7</td>
<td>661.2</td>
<td>973.5</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>13.0</td>
<td>17.5</td>
<td>20.4</td>
</tr>
<tr>
<td>Absolute bioavailability (%)</td>
<td>1.11</td>
<td>3.06</td>
<td>5.85</td>
</tr>
</tbody>
</table>

*Results are given for each dog, numbers 1 to 4.

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Bioavailability was 3.24% (Table 2). Various physiological factors contribute to a reduction in availability of drugs for entry into the systemic circulation. In terms of glycyrrhizin, the low bioavailability may be attributed to enzymatic hydrolyzation both in the stomach and the intestine after oral administration.\(^{22}\)
Determination of Glycyrrhizin in Dog Plasma


