Development and Validation of a Liquid Chromatographic/Mass Spectrometric Method for the Determination of Saikosaponin a in Rat Plasma and its Application to Pharmacokinetic Study

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Abstract

A rapid and sensitive liquid chromatography/mass spectrometry (LC/MS) method was developed and validated for the determination of saikosaponin a in rat plasma. Saikosaponin a was extracted by protein precipitation with acetonitrile and the chromatographic separation was performed on a C18 column. The total analytical run time was relatively short (5.5 min) and the limit of assay quantification (LLOQ) was 10 ng mL\(^{-1}\) using 100 \(\mu\)L of rat plasma. Saikosaponin a and the internal standard (felodipine) were monitored in selected ion monitoring (SIM) mode at \(m/z\) 779.2 and 382.0, respectively. The standard curve was linear over a concentration range from 10 to 5000 ng mL\(^{-1}\) and the correlation coefficients were greater than 0.999. The recoveries of saikosaponin a from plasma were larger than 82% and RSD of inter-day and intra-day assay were below 10%. The method described in this report was sensitive and specific and suitable for pharmacokinetic studies of saikosaponin a in rats.

Keywords: Liquid chromatography–mass spectrometry; Electrospray ionization; Pharmacokinetics; Saikosaponin a; Felodipine

Introduction

Chaihu (Radix bupleuri, Chinese thorowax root) is a favorite medicinal herb in traditional Chinese medicine (TCM) and is used as a key ingredient herb for many Chinese multiherb remedies. There are 40 species of the Bupleurum grown wide in china, while only two of them, the dried roots of Bupleurum Chinese DC., known in commerce as ‘Bei chaihu’ (Northern Chinese Thorowax Root) and the dried roots of Bupleurum scorzonerifolium Wild., known as ‘Nan chaihu’ (Southern Chinese Thorowax Root), are used in therapeutic practice. In China, Radix bupleuri is traditionally used to relieve fever, to calm the liver and to cure drooping and ptosis [1].

Three major oleanane saponins named saikosaponin c, saikosaponin d and many minor saponins have been isolated from Radix bupleuri. Saikosaponins have also been reported to possess antiviral activity against hepatitis B virus, measles virus and herpes simplex virus [2,3]. In addition, saikosaponins have been shown to possess antibacterial, antihepatitis and immunomodulating properties [4,5]. In The Chinese Pharmacopeia (The State Pharmacopoeia Commission of China, 2005), Saikosaponin a is defined as the quality control specification for Radix bupleuri and in many Chinese traditional medicines prescribed with Radix bupleuri, the quantity of Saikosaponin a is also assayed to control the quality of the compound recipes.

To control the quantity of Saikosaponin a in plant materials or recipes, several analytical methods have been developed during the last decade. It generally used LC or LC-ELSD [6-8] determine saikosaponin a. However, the absorption wavelength of saikosaponin a is 205 nm, lying on the terminal of ultraviolet. So it has serious interference in determine saikosaponin a. Zhu et al. [9,10] reported the production of monoclonal antibodies (MAb) against saikosaponin a and development of enzyme-linked immunoabsorbent assay (ELISA) for determination of saikosaponin a in a crude extract of Radix Bupleuri and Kampo medicines using an anti-saikosaponin a MAb. HPLC with a mass spectrometer detector (LC-MS) [11–14] showed superior sensitivity and selectivity compared to HPLC-UV methods.

In this study, a simple and highly sensitive LC-MS method was developed for the determination of saikosaponin a in rat plasma. Study on the Pharmacokinetics of the saikosaponin a in rats makes reference for the absorption and metabolism in vivo.

Figure 1: Chemical structure of saikosaponin a.

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Experimental

Reagents and chemicals

Saikosaponin a and the reference substance of felodipine were purchased from NICPBP (National Institute for the Control of Pharmaceutical and Biological Products, China. batch number: 110777-200405 and 100717-200501). Water was produced by a Millipore (Bedford, MA, USA) Milli-Q water system. Methanol and acetonitrile (Merck, Germany) were of LC-grade.

Animals

The experimental animals were Sprague-Dawley rats from Shanghai SIPPR/BK experimental animal Co., Ltd, Ltd, weighing 230±20 g. The study was approved by the Animal Ethics Committee of the Nanjing University of Traditional Chinese Medicine. The animals were cared for according to the regulations of the Animal Committee.

Instruments and LC-MS conditions

An Alliance 2695 LC system (Waters, Milford, MA, USA) coupled with a triple-quadrupole tandem Waters Quattro Micro mass spectrometer was used. The Mass Lynx 4.1 software was used for instrumental control and for acquisition and processing of the data. AMS detector with an electrospray ionization (ESI) interface in negative ion mode (ESI −) was used for quantitative analysis, with acquisition in SIM mode. The m/z ratios [M-H]− for saikosaponin a and [M-2H]− for felodipine were recorded simultaneously.

LC separation was performed on an Agilent Zorbax SB-C18 (150 mm×2.1 mm, I.D.5 μm) column (Agilent Technologies, Wilmington, DE, USA) with a Security Guard (12.5 mm×2.1 mm, I.D.5 μm) column (Agilent Zorbax SB-C18, DE, USA), kept at 30°C. The sensitivity of MS detection is directly dependent on the number of ions produced from the mobile phase in the interface. It is expected that mobile phase additives will affect the sensitivity of LC–MS. Use of neutral buffer (ammonium acetate or ammonium formate) or weak acid (acetic acid) led to good response compared with use of alkaline solution. On the basis of these results, the mobile phase, consisting of methanol and deionized water containing 0.1% ammonium acetate (84:16, v/v), a flow rate of 0.2 mL/min was selected as mobile phase additive for detection of saikosaponin a.

The optimized electrospray conditions were: detect voltage: 3.5 kV; cone voltage: 57 V; source temperature: 120°C; desolvation temperature: 350°C; desolvation gas flow (nitrogen): 500 L h−1. On the basis of these results to achieve high sensitivity, symmetrical peaks and good separation of the analyte.

Preparation of standard and quality control (QC) samples

Stock solutions of saikosaponin a (478 μg mL−1) and felodipine as an internal standard (408 ng mL−1) were prepared in methanol. The stock solutions were protected from light and kept at 4°C until used. The stock solutions were successively diluted with methanol to prepare working solutions just prior to use. These solutions were spiked into drug-free rat plasma samples to give final concentrations of 10, 25, 50, 100, 250, 500,2500,5000 ng mL−1. Quality control (QC) samples were prepared to the target concentrations of approximately 40, 400, 4000 ng mL−1 in the same way as the plasma samples for calibration. Standard calibration samples and QC samples were stored at -20°C until use.

Sample preparation

To each of disposable plastic tubes with 100 μL plasma samples (calibration standards, QC samples, pharmacokinetics plasma samples), 10 μL of 1.5(felodipine, 408 ng mL−1) was added except the blank plasma sample. After vortexed for 30s, 300 μL of acetonitrile was added to each of these plasma samples. The obtained solution was vortexed for 3 min at room temperature. The mixture was centrifuged at 12,000 rpm for 10 min. An aliquot of 320 μL of the upper organic layers were transferred to polypropylene tubes, and evaporated to dryness in the centrifugal thickener (Centrivap console, Labconco Co, USA) at 50°C for 120 min. The residue, dissolved into 100 μL mobile phase, was vortexed for 2min and then centrifuged for 10min at 12000g. 10 μL supernatant was injected into the LC/MS/MS system for analysis.

Method validation

The method validation assays were carried out according to the currently accepted U.S. Food and Drug Administration (FDA) bioanalytical method validation guidance [15]. The method was fully validated for its specificity, linearity, lower limits of quantification (LLOQ), accuracy and precision. To evaluate assay specificity, six independent lots of rat blank plasma were analyzed for excluding any endogenous co-eluting interference by comparing them with the assay of plasma spiked with analytes. The precision was calculated as the relative standard deviation (RSD) and the accuracy was evaluated as analytical recovery. For validation of the bioanalytical method, accuracy and precision should be determined using a minimum of five determinations per concentration level (excluding blank samples). The mean value should be within 15% of the theoretical value, except at LLOQ, where it should not deviate by more than 20%. The precision around the mean value should not exceed 15% of the CV, except for LLOQ. Intra-day precision and accuracy were evaluated at three different plasma concentrations (40, 400, 4000 ng mL−1) by replicate analytes of five spiked plasma samples on the same day. The inter-day precision and accuracy determinations were carried out on three different days. Recovery experiments were performed by comparing the analytical results of extracted samples at three concentrations with pure standards without extraction. The LLOQ was considered as the concentration that produced a signal-to-noise (S/N) ratio greater than 10. The sample solution stability was assessed at three concentration levels (40, 400, 4000 ng mL−1). The freeze and thaw stability study samples at three concentrations were stored at -20°C and subjected to two freeze–thaw cycles. The short-term stability of saikosaponin a during storage in the autosampler at 4°C, was performed by repeated injection every 2 h for a period of 10 h. The long-term stability of saikosaponin a in plasma was assessed in three concentration levels after storage at -20°C for 4 weeks.

Application for pharmacokinetic study

Plasma samples were collected at 0.5, 1.0, 1.5, 2, 3, 4, 6, 8, 10, 12 h before and after single intragastric administration of saikosaponin a at 50 mg kg−1 to six Sprague-Dawley rats under fasted conditions. Other preparations were made immediately after drug administration and six rats were given a dosage of saikosaponin a at 5 mg kg−1 by intravenous injection. Plasma samples were collected at times of 5, 10, 20, 30, 50, 70, 100, 160, 240, 360 and 540 min after dosing, 400 μL of blood samples were collected in the heparinized tubes. Then centrifuged for 10 min at 3000 rpm; the supernatant was transferred to labeled plastic vials at -20°C until analysis was carried out.

Results and Discussion

Chromatography and mass spectrometry conditions

The analyte and IS both formed predominantly deprotonated molecules [M-H] in the full scan spectra, with m/z at 779.2 for saikosaponin a and 382.0 for IS (Figure 2). Under the current optimized LC and MS conditions, the retention times for saikosaponin a and felodipine (IS) were 3.96 and 4.41 min, respectively. Figure 3 shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with saikosaponin a and IS and a plasma sample collected after injection of saikosaponin a. No significant interference from endogenous substances with anlyte or IS was detected. The overall chromatographic run time was finished within 5.5 min.

Linearity of calibration curves and lower limits of quantification

The calibration curves were constructed by calculating the peak area ratios (Y) of saikosaponin a to IS against saikosaponin a concentrations (X). The regression equation of the standard curve was: \[ Y = (0.00491 \pm 0.00015)X - (0.04093 \pm 0.02563) \] (n = 5), with a correlation coefficient over 0.999. It showed good linear relationships between the peak area ratios and the concentrations. The assay was linear over a concentration range from 10 to 5000 ng mL\(^{-1}\). The lower limit of quantification (LLOQ) obtained in this method was 10 ng/mL. At the LLOQ level, the intra-day precision was 4.3% and the accuracy was 96.9%. The LLOQ was considered as the concentration that produced a signal-to-noise (S/N) over 10. With the present LLOQ, the concentrations of saikosaponin a in rats could be determined 540 min after intravenous injection of 5 mg kg\(^{-1}\) saikosaponin a.

Recovery and matrix effect

Different solvents for the extraction of saikosaponin a from rats plasma have been reported. During the method development, we tried using less toxic solvents such as ether, methanol and acetonitrile for the extraction of saikosaponin a, but did not obtain a good recovery of saikosaponin a until the centrifugal thickener was employed. In this paper, centrifugal was developed compared with a simple and efficient liquid-liquid extraction. The recoveries of saikosaponin a determined at 40, 400, 4000 ng mL\(^{-1}\) were 82.6±7.6%, 87.1±6.1% and 81.8±3.4%, respectively, while the recovery of the IS determined at 408 ng mL\(^{-1}\) was 83.0±4.6% (Table 1). These results were indicative of good recovery.

The matrix effect of blank plasma in the six batches spiked after the sample preparation with 40, 400, 4000 ng mL\(^{-1}\) of saikosaponin

<table>
<thead>
<tr>
<th>Sample</th>
<th>Spiked conc. (ng mL(^{-1}))</th>
<th>Recovery (%)</th>
<th>Matrix effect (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>saikosaponin a</td>
<td>40</td>
<td>83±8</td>
<td>92±9</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>87±6</td>
<td>93±7</td>
</tr>
<tr>
<td></td>
<td>4000</td>
<td>82±3</td>
<td>89±8</td>
</tr>
<tr>
<td>felodipine</td>
<td>408</td>
<td>93±5</td>
<td>96±6</td>
</tr>
</tbody>
</table>

Table 1: Recovery of saikosaponin a (mean±standard deviation (SD) and felodipine (internal standard).
a were found to be within the acceptable range (Table 1). The same evaluation was performed on the IS and no significant peak area differences were observed (Table 1). Thus, we concluded that ion suppression or enhancement from plasma matrix was negligible for this assay.

**Accuracy and precision**

QC samples at three concentrations were analyzed in five replicates for determining the accuracy and precision of this method. The results were shown in Table 2. The intra-batch accuracy for saikosaponin a ranged from 95.2 to 103.7% at the tested concentrations with the precision (RSD) between 3.0 and 4.8%. The inter-batch accuracy for saikosaponin a ranged from 95.1 to 100.0% at three different concentrations with the precision (RSD) between 3.0 and 4.8%. The same results were found to be within the acceptable range (Table 1). The same evaluation was performed on the IS and no significant peak area differences were observed (Table 1). Thus, we concluded that ion suppression or enhancement from plasma matrix was negligible for this assay.

**Stability**

Results of the short-term stability, freeze/thaw stability, autosampler stability and long-term stability are shown in Table 3. The mean percentages of deviation of calculated versus theoretical concentrations were less than or equal to 10.8% for short-term stability, less than or equal to 10.5% for autosampler stability and less than or equal to 13.6% for long-term stability. The good stability of saikosaponin a simplified the precautions needed for laboratory manipulations during the analytical procedures.

**Pharmacokinetic study**

The well-validated method was successfully applied to determine the plasma concentrations of saikosaponin a in rats following a single caudal vein administration of saikosaponin a (5 mg kg\(^{-1}\)). Pharmacokinetic parameters were calculated using the DAS pharmacokinetic software package (Beijing Bosom Science & Technology Co. Ltd). The time-plasma concentration profile of asiaticoside were 1907±342 ng mL\(^{-1}\) and 5 min and \(t_{\text{max}}\) was 100.6±9.60 min. However, by the intragastric administration groups, the absorbed amount of drug leads to very low concentrations in the circulation, lower than a few ng mL\(^{-1}\).

**Conclusion**

A LC-MS assay for the simultaneous saikosaponin a and felodipine (IS) plasma concentration was developed. The main advantages of this method are: (1) small amount of sample required (100 μL of plasma); (2) processing is simple and rapid; (3) excellent recovery; and (4) short analytical time. The new method was successfully applied to the characterization of pharmacokinetics of saikosaponin a in rats after intravenous injection. While there are only few pharmacokinetics data published on saikosaponin a, this simple, accurate, precise and robust analytical method could be useful for such studies.

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