Simultaneous Determination of Oleanolic and Ursolic Acids in Rat Plasma by HPLC–MS: Application to a Pharmacokinetic Study After Oral Administration of Different Combinations of QingGanSanJie Decoction Extracts

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Received 5 July 2014; revised 31 October 2014

A liquid chromatography–mass spectrometry method has been developed and validated for rapid simultaneous determination of the oleanolic and ursolic acid contents in rat plasma with betulinic acid as the internal standard (IS). The plasma samples were prepared by a liquid–liquid extraction procedure. Chromatographic separation was performed with a Chromasil-C18 column (250 mm × 4.6 mm, i.d. 5 μm) with methanol–water as mobile phase at 1 mL/min. The detection was accomplished under selected-ion-monitoring mode with a negative electrospray ionization interface. Linear calibration curves were obtained between the range of 0.86–421.2 and 0.94–462.0 ng/mL for oleanolic and ursolic acids, with lower limits of quantification at 0.43 and 0.47 ng/mL, respectively. The extraction recovery exceeded 70% in plasma. The intra- and interday precision values were <9.8% with the accuracy as −7.0 to 9.9% at three different QC levels in both cases. The pharmacokinetic behaviors of oral dosage of QingGanSanJie decoctions were then studied in rats following the developed approach. The \( t_{1/2} \) values of the oleanolic and ursolic acids after oral administration of the monarch medicine extract were significantly different (\( P < 0.05 \)) from other prescription extracts containing different herb pieces with different compatibilities. Bimodal phenomena appeared in every concentration–time curve for the oleanolic and ursolic acids at 3–8 h after administration. The minister, assistant and guide medicines in the formula could prolong the metabolism of the oleanolic and ursolic acids in vivo, providing an experimental basis for the slow onset and long action of the Traditional Chinese Medicine compound.

Introduction

The QingGanSanJie (QGSJ) decoction, a Chinese herbal compound prescription, consists of 14 types of traditional herb pieces and has been studied during long-term clinical applications against tumors in our hospital. QGSJ is primarily used to treat hepatocellular carcinoma (HCC) and to prevent its recurrence after liver resection. The herb pieces and dosage of this formula are adjusted based on different symptoms presented in the patients. Numerous clinical observations revealed (1) that QGSJ could improve liver function, mitigate symptoms and inhibit liver cancer growth, tumor metastasis and recurrence, leading to the improvement of patient survival rates.

The theory of Traditional Chinese Medicine classifies different herbal components into four groups: monarch, minister, assistant and guide medicines. In the QGSJ formula, Actinidia valvata Dunn, Salvia chinensis Benth, Herba Hedyotidis Diffusa and Portulaca grandiflora Hook are the monarch medicines, which have the effects of antipyretic and detoxification and play a major role in the treatment of HCC. Rhizoma Atractylodis Macrocephalae, Ganoderma lucidum and Poria are the minister medicines, which strengthen vital qi to eliminate pathogenic factors; Akebia quinata Decne, Ardisia japonica Blume, Bupleurum chinense DC, Radix Scutellariae Baicalensis, Artemisia capillaris Thunb and Herba Sedi Sarmentosi are the assistant medicines, which disperse stagnated liver qi to promote bile flow; and Radix Glycyrrhizae is the guide medicine, which is commonly used to coordinate the drug actions in Chinese herbal formulas. Some research groups reported that the monarch herbs contain a pair of isomers: oleanolic and ursolic acids (2–5). Pharmacological studies have shown that both isomers have low toxicity and various biological activities, including good inhibitory effect on HCC (6–10). Our previous research pointed out that oleanolic and ursolic acids primarily deposit in A. valvata Dunn, S. chinensis Benth and Herba Hedyotidis Diffusa in monarch medicines (11). Oleanolic and ursolic acids have low oral bioavailability because they are strongly fat soluble (12, 13). Furthermore, methyl substitutions at different positions reveal that oleanolic acid could not be metabolized by using human liver microsomes (14), and it was very useful to study their pharmacokinetics in vivo after multicomponent administration. Although oleanolic and ursolic acids were separated in baseline by using reversed-phase columns in some laboratories, their retention times were more than 20 min because of their high lipophilicity (15–17). Pre-column derivatization can make the retention time shorter, but it increases the complexity of the sample processing (18, 19). Due to the large number of in vivo biological samples being analyzed, it is considered better to have the running time of each sample as short as possible. In addition, the biological matrix effects make the baseline separation of oleanolic and ursolic acids very difficult. Several analytical methods have been discussed to quantify the oleanolic or ursolic acids, which include high-performance liquid chromatography–mass spectrometry (HPLC–MS) (20, 21) and high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) (22–24). However, there are still no published simple and rapid analytical methods for the...
simultaneous determination of oleanolic and ursolic acid contents in vivo.

Our study develops a simple, rapid and reliable LC–MS method to quantify oleanolic and ursolic acid contents in rat plasma simultaneously and to compare their pharmacokinetic behaviors after single oral treatment of rats with different combinations of medicinal herb extracts from QGSJ. This analytical method greatly facilitates the efficiency and safety evaluation of QGSJ in the treatment of HCC.

Experimental

Chemicals and reagents

*Actinidia valvata* Dunn, *S. chinensis* Benth, *A. quinata* Decne, *A. japonica* Blume, *Herba Hedysartis Diffusa*, *P. grandiflora* Hook, *B. chinense* DC, *Radix Scutellariae Baicalensis*, *Rhabroma Atractylodis* Macrocephalae, *A. capillaris* Thunb, *Herba Sedi Sarmentosi*, *G. lucidum*, *Radix Glycyrrhizae* and *Poria* were purchased from Shanghai Qingpu Chinese Medicine Decotion Pieces Company (Shanghai, China) and authenticated by Prof. Sun from the Department of Pharmacognosy at the Second Military Medical University (Shanghai, China). Analytical standards for oleanolic, ursolic and betulinic acids as internal standard (IS) (all >98% purity) were purchased from China’s National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade methanol was acquired from Burdick & Jackson (Ulsan, Korea), and HPLC grade formic acid was procured from Anaqua Chemical Supply Company (Houston, TX, USA). Ultrapure water was used in the experiments. All other reagents were of analytical grade.

Instrumentation and conditions

Agilent 1100 HPLC system (Santa Clara, CA, USA) with a G1322A degasser, a G1311A quaternary pump, a G1367A well-plate autosampler, a G1316A thermostated column compartment and a G1315B DAD detector was used. An Agilent G1946D single quadrupole mass spectrometer (Santa Clara, CA, USA) equipped with an electrospray source was connected to the LC system. Data acquisition and analysis were processed by Agilent ChemStation (version B.02.01).

Chromatographic separation was achieved using the Chromasil-C18 column (250 mm × 4.6 mm, i.d. 5 μm) with Agilent SB C18 guard column (12.5 mm × 4.6 mm, i.d. 5 μm). The sample was isocratically eluted with solvent A (methanol)–solvent B (0.1% aqueous formic acid) = 86:14 for 14 min with the flow rate of 1 mL/min at 25°C, and the injection volume was 40 μL. The flow outlet was connected to the mass spectrometer by a three-way joint with 1:4 post-column split ratio.

The electrospray ionization conditions were optimized as follows; 3,500 V capillary, 40 psi nebulizer, 10 L/min drying gas, 350°C gas temperature and 180 V fragmentor. Select ion-monitoring (SIM) in the negative ion mode was used. Oleanolic acid, ursolic acid and betulinic acid ions were all recorded as m/z 455.4 ([M–H]−) for quantification.

Preparation of solution

Following the amount ratio of herbal medicines in QGSJ, 440 g of herbs (two prescription doses) were crushed to pieces. The mixture was subsequently extracted twice with six volume equivalents of 70% ethanol by refluxing. The extracts were combined and concentrated to 40 mL under vacuum to yield herbal medicine solution (named as extract IV) for rats. Subsequently, extracts I (monarch herbs), II (monarch and minister herbs) and III (monarch, minister and guide herbs) were similarly prepared with the same proportions.

The oleanolic and ursolic acid contents in the four extracts were quantitatively analyzed following a previously established HPLC–DAD method (11). The concentrations of oleanolic and ursolic acids in extract I were 348.4 and 761.8 μg/mL, while they were 392.5 and 846.7 μg/mL in extract II, 374.9 and 818.9 μg/mL in extract III and 382.4 and 772.6 μg/mL in extract IV.

Preparation of calibration, quality control and ISs

The standard stock solutions for oleanolic (2.106 mg/mL) and ursolic acids (2.31 mg/mL) were prepared separately in methanol. Working standard solutions of oleanolic and ursolic acids were prepared by combining a known proportion of each primary stock solution and diluting them with methanol in volumetric flasks. The assay standard samples containing oleanolic (421.2, 168.5, 67.39, 26.96, 10.78, 4.31 and 0.86 ng/mL) and ursolic acids (462.0, 184.8, 73.92, 29.57, 11.83, 4.7 and 0.95 ng/mL) were prepared by spiking 20 μL of the working standard solution into 200 μL of blank rat plasma. Quality control (QC) samples at low, medium and high concentration gradients were prepared independently in the same manner. The assay standard samples and QC samples were prepared during each analytical batch. The IS working solution was prepared by diluting the betulinic acid stock solution (717.0 μg/mL) to 1.79 μg/mL with methanol. All the solutions were stored at 4°C and brought to room temperature before use.

Sample preparation

To each 200 μL aliquot of the plasma sample, 20 μL of IS solution and 1 mL of mixed organic solvent (ethyl acetate−n-hexane = 4:1) were added and vortexed for 1 min in a 1.5-mL polypropylene tube; the mixture was centrifuged at 1,500 × g for 5 min. Subsequently, 800 μL of the supernatant was transferred into a new 1.5-mL polypropylene tube and evaporated to dryness in a speedVac concentrator. The residues were re-dissolved in 50 μL of methanol; the mixture was vortexed for 1 min before being centrifuged at 9,600 × g for 5 min. Finally, 40 μL of the supernatant was injected for the LC–MS analyses.

Method validation

Assay specificity

The selectivity of the assay was assessed by comparing the chromatograms from six batches of blank plasma obtained from different rats with those from the corresponding standard plasma samples spiked with the analytes and the IS.

Linearity of the calibration curves, lower limit of detection and lower limit of quantification

The calibration curves for the two compounds were constructed by plotting the peak area ratios of the analyte to the IS against the concentration of analyte in the range of 0.16× to 4×. The linearity of the calibration curves, lower limit of detection and lower limit of quantification were determined by plotting the peak area ratios of the analyte to the IS against the concentration of analyte.
plasma concentrations using a 1/x weighted linear least-squares regression model. The linearity of the two compounds determined in spiked rats’ plasma was obtained by seven calibration standards in five independent runs; the calibration curves had a correlation coefficient (r) of 0.995 or higher.

The lower limit of detection (LLOD) was the lowest concentration with a signal-to-noise ratio of at least 3. The lower limit of quantification (LLOQ) was the concentration with a signal-to-noise ratio of at least 5. An acceptable accuracy is < 20% deviation, and the precision is between 80 and 120%.

**Precision and accuracy**

The intraday precision and accuracy were determined at three different concentration levels (4.313, 26.96 and 168.5 ng/mL for oleanolic acid, and 4.731, 29.57 and 184.8 ng/mL for ursolic acid) for five QC sample replicates on the same day. The interday precision and accuracy were determined at three different concentration levels for five QC sample replicates from three independent batches. The precision was expressed as the relative standard deviation (RSD), and the accuracy was calculated as the percentage bias from the nominal concentration (relative error).

**Recovery and matrix effects**

The extraction recoveries of the oleanolic and ursolic acids were determined by comparing the response ratio of extracted plasma QC samples (Set1) with those of extracted blank plasma spiked with the corresponding concentrations (Set2). The extraction recovery = Set1 × 1.25/Set2. The extraction recovery of the IS was measured at the same concentration with different QC levels. The matrix effect was determined at three different QC concentrations with five replicates. The absolute matrix effect was evaluated by comparing the peak areas of the analytes added to the extracted blank plasma (Set2) with those of the methanol dissolution (Set3). The relative matrix effect was expressed as the RSD of the mean peak areas of the analytes in the extracted blank plasma. When the peak area ratio of the analytes fell between 85 and 115%, the matrix effect was negligible.

**Stability**

Three aliquots of the low, medium and high concentration QC samples (4.31, 26.96 and 168.5 ng/mL for oleanolic acid; 4.73, 29.57 and 184.8 ng/mL for ursolic acid) were used to test the stability of the analytes. The stability of the analytes in the rat plasma was studied under different storage conditions: short-term stability at room temperature for 4 h, long-term stability at −40°C for 1 month, post-preparative stability at room temperature for 24 h and three freeze–thaw cycles.

**Pharmacokinetic study**

The experimental protocol was approved by the Animal Ethics Committee of the Second Military Medical University. Male Sprague–Dawley rats weighing 200–220 g were supplied by Sino-British Sippr/BK Laboratory Animals Ltd (Shanghai, China). The rats were maintained in air-conditioned animal quarters at 22 ± 2°C and 50 ± 10% relative humidity. Water and food (Laboratory Rodent Chow, Shanghai, China) were provided ad libitum. The animals were acclimatized to the facilities for 5 days and fasted with free access to water for 12 h before each experiment. The rats were randomly placed into four groups of five rats: groups A, B, C and D were orally administered with extracts I, II, III and IV, respectively. Blood samples (0.4 mL) were collected into heparinized tubes before administration, as well as 0.167, 0.333, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 h after dosing. After centrifugation at 1,500 × g for 5 min, the plasma samples were obtained and frozen at −40°C until analysis.

**Results**

**LC–MS optimization**

The direct determination of oleanolic and ursolic acid contents from biological samples required a highly sensitive and selective analytical method. In the beginning of this study, we attempted to use LC–MS/MS to obtain good LOD, but oleanolic and ursolic acids did not produce fragment or daughter ions with high collision energy due to their stable characters, resulting in absence of suitable fragmentation channel. Finally, LC–MS was used to develop the analytical method. Because the full-scan mass spectra showed better ionization signal of oleanolic and ursolic acids in negative ionization mode, we selected the [M–H]− ion (m/z 455.4) for MS detection. The mass spectra for oleanolic acid, ursolic acid and IS are presented in Figure 1. The main electro-spray ionization parameters, including the nebulizer pressure, the drying gas flow rate and fragmentor voltage in the SIM, were optimized by flow injection analysis with oleanolic acid standard. The analytes in rat plasma were identified by the retention time and the molecular weight.

The structural similarity of ursolic and oleanolic acids is a big obstacle for chromatographic separation. In addition, the analysis time for each sample should be short due to the large number of samples. We screened columns with different filler materials, diameters and lengths, indicating that column particles coated with larger number of carbons could better separate ursolic and oleanolic acids. Increasing the diameter and length of the column could increase the stationary phase capacity, thus improve chromatographic resolution while lengthen the analysis time. Although the two analytes could be separated by columns filled with smaller particles, the separation was susceptible to the influences from the plasma matrix. We selected the Chromasil-C18 (250 mm × 4.6 mm, i.d. 5 μm) column due to the fact that it provided a resolution > 2.0 for ursolic and oleanolic acids.

The representative LC–MS chromatogram of the IS and two analytes in an entire time run was shown in Figure 2A. Because only one peak (9.5 min in Figure 2B) existed in the beginning of the chromatogram, the analysis cycles could be cut into two 14-min segments; the retention times of betulinic, oleanolic and ursolic acids were ~80, 110 and 130 min, respectively, in the second segment, while the 9.5-min peak in the first segment did not affect the analysis. Therefore, the next sample injection was set at 14 min after the first sample injection, during the second half of the analysis. The extract ion chromatogram (EIC) of the second half overlapped with the first half of the next sample, but without any interference. The entire analysis time was cut in half without interference, and the whole running time can be shortened by half, which was very helpful in running numerous biological samples.

**Optimization of the extraction procedure**

We compared liquid–liquid extraction and protein precipitation techniques during sample preparation. The recovery from liquid–liquid extraction was higher for ursolic and oleanolic acids. The stability of the analytes in the rat plasma was obtained by seven calibration standards in five independent runs; the calibration curves had a correlation coefficient (r) of 0.995 or higher.
Figure 1. Deprotonated molecular ions in the mass spectra for oleanolic acid (A), ursolic acid (B) and betulinic acid (C).

Figure 2. Representative EIC chromatograms of the analytes in a whole time run (A), blank plasma (B), spiked standard solution in blank plasma (C) and rat plasma sample after oral administration of QGSJ extract (D). The peaks marked in chromatograms were: (1) betulinic acid (IS), (2) oleanolic acid and (3) ursolic acid, respectively.
acids. We then investigated different extraction solvents, including diethyl ether, ethyl acetate and mixtures of ethyl acetate and n-hexane. Ether evaporated easily, but emulsification occurred, thus hindering separation and recovery. With ethyl acetate, the organic phase and the aqueous phase separated clearly, but an endogenous component interfered with the chromatographic peak assigned to oleanolic acid. By adding appropriate amount of n-hexane, the disturbance was overcome. Finally, we decided to use ethyl acetate–n-hexane in the ratio of 4:1.

**Method validation**

**Selectivity**

The representative chromatograms in SIM for oleanolic, ursolic and betulinic acids are shown in Figure 2. The retention times for betulinic, oleanolic and ursolic acids were ~8.0, 11.0 and 13.0 min, respectively; there is no interference from endogenous substances observed at the retention times of the analytes or IS.

**Linearity of the calibration curves, LLOD and LLOQ**

The slope values of the calibration curves were $y = 372.28x - 0.0078$ ($r = 0.9998$) for oleanolic acid and $y = 339.2x + 0.117$ ($r = 0.9996$) for ursolic acid. The LLOQs in plasma were 0.86 ng/mL for oleanolic acid and 0.95 ng/mL for ursolic acid, respectively. The signal-to-noise ratios were $>5$ with deviation $<20\%$ ($n = 5$). The LLODs of the two analytes were obtained after diluting the samples to 0.43 and 0.47 ng/mL, respectively.

**Precision and accuracy**

Table I summarizes the intra- and interday precisions and accuracies for the oleanolic and ursolic acids in the QC samples. The precisions were reported as the RSD and ranged from 4.8 to 9.3% and from 3.6 to 9.8% for the intra- and interday determinations, respectively. The accuracies ranged from $-7.0$ to 60.0% and from $-6.7$ to 9.9% for the intra- and interday determinations, respectively. All of the intra- and interday precision and accuracy values were acceptable.

**Extraction recovery and matrix effect**

The extraction recoveries determined for oleanolic acid, ursolic acid and betulinic acid are displayed in Table II. At the three concentration levels of the two analytes, the absolute extraction recoveries ranged from 70.8 to 84.5%. The extraction recovery of the IS was $>83.1\%$. Although the matrix effects caused ion suppression in the analytes, there were no severe variations at any QC concentration level (RSD $<15\%$); the matrix effects were consistent and concentration-independent. Therefore, although absolute matrix effects were observed, the analytical method was stable and reliable.

**Sample stability**

The stability of the analytes was assessed under various conditions. The results presented in Table III indicate that these analytes were stable in plasma after three freeze–thaw cycles, at room temperature for 4 h, and at $-40^\circ$C for 1 month. The post-preparative stability of the analytes showed no significant degradation from the extracted samples kept in the autosampler at room temperature for 24 h.

**Applications to a pharmacokinetic study**

The mean concentration–time curves of oleanolic and ursolic acids from four types of single oral solution dosages are shown in Figure 3. The pharmacokinetic parameters were calculated via a non-compartment model with the Kinetica 4.4 software (Table IV).

The $t_{1/2}$ values for oleanolic and ursolic acids after orally administering the monarch medicine solution were significantly different ($P < 0.05$) from the oral administration of the other solutions. There were no significant differences between the other
three groups. Compared with the oral administration of the monarch medicine solution, the $t_{1/2}$ value of oleanolic acid increased from 8 h to $\approx 16$ h after oral treatment of the monarch medicines with the other components in QGSJ; the $t_{1/2}$ value of ursolic acid also increased from 4 to 8 h. In both cases, the half-lives approximately doubled, indicating that the combination of the sovereign medicines with other components significantly prolonged the $t_{1/2}$ of oleanolic and ursolic acids in rats, generating a longer-lasting effect. Other pharmacokinetic parameters, including $C_{\text{max}}$, $\text{AUC}_{(0-24)}$, $\text{AUC}_{(0-\infty)}$, and $T_{\text{max}}$, remained unchanged.

**Discussion**

We developed a simple pre-treatment method to extract oleanolic and ursolic acids in rat plasma and then analyzed them by regular C18 column within 14 min. The method could be easily reproduced and was fully validated and successfully applied to the pharmacokinetics study in rats after oral administration of different combinations of herb medicines composed to QGSJ decoction.

Most studies in the literature about pharmacokinetics of oleanolic acid or ursolic acid in rat plasma were investigated by single
compound administration. In this study, we obtained the pharmacokinetic parameters of oleanolic and ursolic acids after oral dosage of QGSJ decoction extracts with different combinations and analyzed them together. The $t_{1/2}$ of ursolic acids in the group of monarch medicine solution treatment was ~4 h in rat plasma, which was comparable with its single administration (20). The half-life of oleanolic acid was extended to 8 h than the reported results (24) due to its low hepatic metabolism rate. Multi-component dosing may decrease its total clearance. $T_{\text{max}}$ values of oleanolic and ursolic acids were not changed, but a bimodal phenomenon appeared in all concentration–time curves for the oleanolic and ursolic acids starting from 3 to 8 h after administration, as shown in Figure 3. The relatively non-polar oleanolic and ursolic acids were absorbed primarily by facilitated diffusion, which was delayed by the competition among other components in the formula. A secondary absorption of oleanolic and ursolic acids occurred after other components were absorbed. This phenomenon was also observed after oleanolic acids soft capsule intragastric administration in rats (25).

The fifth rat died in 4 h after QGSJ was orally administered. The concentration of oleanolic acid in plasma was detected at a high level. The blood concentration of ursolic acid rose to and remained at 314.11 ng/mL at the time of death; this value was much higher than in the other rats and assumed to be the cause of death. Therefore, the toxicity of oleanolic and ursolic acids should be taken seriously.

**Conclusion**

In this study, a sensitive and reliable LC–MS method for rapid simultaneous determination of oleanolic and ursolic acids in rat plasma with facile sample pre-treatment was developed and validated. The LC–MS assay was successfully applied to a comparative pharmacokinetic study of oleanolic and ursolic acids in rats after orally administering different herb combinations from QGSJ. The data indicated that the $t_{1/2}$ values of oleanolic and ursolic acids were significantly extended when the monarch medicines were orally administered with other components of QGSJ. The herbal medicines acting as the ministers, assistants and guide in the QGSJ could prolong the metabolism of oleanolic and ursolic acids in vitro, providing an experimental basis for the slow onset and long activity of these Chinese medicinal compounds. The toxicity of oleanolic and ursolic acids caused by the changes in the metabolic process should also be taken seriously.

**Acknowledgments**

The authors thank Prof. Lian-na Sun in the Department of Pharmacognosy of Second Military Medical University for authenticating the herb pieces in the study.

**Funding**

This research was supported by the National Natural Science Foundation of China (no. 81303300) and Shanghai Science and Technology Support Project (134101900107).

**References**

4. Xu, M.J., Yong, Y.L., Shi, J.C.; Study on the chemical constituents of *Salvia chinensis* Benth; *Zhong Cao Yao* (1987); 18: 46.
5. Ly, H.C., He, J.; Studies on the chemical constituents in herb of *Hedyotis diffusa*; *Natural Product Research and Development*, (1996); 8: 34–37.
17. Liu, G.L., Zhang, X.L., You, J.M., Song, C.H., Sun, Z.W., Xia, L., et al.; Highly sensitive and selective pre-column derivatization high-performance liquid chromatography approach for rapid determination of triterpenes oleanolic and ursolic acids and application to *Sverutta* species; optimization of triterpenic acids extraction and pre-
column derivatization using response surface methodology; *Analytica Chimica Acta*, (2011); 688: 208–218.


