LC Determination and Pharmacokinetic Study of Hyperoside in Rat Plasma after Intravenous Administration

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A simple and specific high-performance liquid chromatographic (HPLC) method was developed for the pharmacokinetic study of hyperoside (HP, isolated from the leaves of *Crataegus pinnatifida* Bge. var. major) in rats after intravenous administration. The plasma samples were deproteinized with methanol after addition of internal standard (I.S.), baicalin. HPLC analysis was performed on a Diamonsil C18 analytical column, using methanol-0.6% aqueous phosphoric acid (45 : 55, v/v) as the mobile phase with UV detection at 340 nm. The calibration curve was linear over the range of 0.8921–59.7125 μg/ml in rat plasma. The average extraction recovery of HP was 99.33 ± 0.86%, and the relative standard deviations (R.S.D.s) of the intra- and inter-day precisions were no more than 7.8 and 2.5%, respectively. The lower limit of quantification (LLOQ) was 0.8921 μg/ml. The validated method was successfully applied during a pharmacokinetic study in rats after intravenous administration of HP at different doses, and all the results indicated that the pharmacokinetics of HP in rats obeyed nonlinear processes.

Key words—hyperoside; pharmacokinetics; HPLC; rat plasma

INTRODUCTION

The leaves of *Crataegus pinnatifida* Bge. var. major recorded in the Chinese Pharmacopoeia, mainly contain hyperoside (HP), a flavonol glycoside, whose quality is controlled via determining HP using high-performance liquid chromatographic (HPLC) method. Thereby, many researches about HP appeared in literature, such as, the treatment of antidepressant, cancer therapy, strong inhibitor of HBsAg and HBeAg secretion in 2.2.15 cells and DHBV-DNA levels in the HBV-infected duck model, possessing cytoprotective properties against oxidative stress by scavenging intracellular ROS and enhancing antioxidant enzyme activity, protecting against the oxidative damage induced by TBHP, and remarkable wound healing and anti-inflammatory. Otherwise, many methods including capillary zone electrophoresis with electrochemical detection, HPLC, and LC-MS/MS have been reported for quantification of HP in various herbal medicines and biological fluids. However, most of them mainly concentrated on the determination of several active components including HP and pharmacokinetic study of single dose HP in rat plasma, such as Chang Q., et al reported the pharmacokinetic data on HP after intravenous administration, but systematic pharmacokinetic research of HP was no appeared. In addition, the resolutions between HP and other compounds from chromatograms of above some literature were unsatisfactory, and this could produce wrong results. Considering the method investigated using external standard, which could lead to the analytical errors and was unsuitable to the determination of HP in vivo, the method using internal standard was applied in our study.

The aim of this study was to isolate HP from the leaves of *C. pinnatifida* Bge. var. major, subsequently, an HPLC method was developed and validated for the determination of HP in rat plasma. To the best of our knowledge, this is the first report the pharmacokinetic study following intravenous administration of HP at three different doses, and we discussed the pharmacokinetics of HP.

MATERIALS AND METHODS

Materials The internal standard (I.S.), baicalin (Fig. 1(a)) was provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade methanol...
was purchased from Xinxing (Chemical Reagent Plant, Shanghai, China), and the water used in all experiments was purified by a Milli-Q® Biocel Ultrapure Water System (Millipore, Bedford, MA, USA). All other chemicals were of analytical reagent grade purchased from Sinopharm Chemical Reagent Co., Ltd.

Isolation and Identification A sample (5 kg) of the leaves of C. pinnatifida Bge. var. major collected in Shenyang, Liaoning Province, China was extracted twice with 50 l of 60% aqueous phosphoric acid (DMSO-d6, δ ppm: 156.3 (C-2), 133.5 (C-3), 177.5 (C-4), 161.2 (C-5), 97.9 (C-6), 164.1 (C-7), 93.5 (C-8), 156.3 (C-9), 103.9 (C-10), 122.0 (C-1′), 115.2 (C-2′), 144.9 (C-3′), 148.3 (C-4′), 115.9 (C-5′), 121.1 (C-6′), 101.8 (C-1″), 71.2 (C-2″), 73.2(C-3″), 67.9 (C-4″), 75.9 (C-4″), 60.2 (C-6″) compared with the data in literature (3).

Chromatographic System and Conditions The analysis was carried out on Shimadzu HPLC system (Kyoto, Japan), consisting of an LC-10AT pump and an SPD-10A VP UV-vis spectrophotometric detector. Data collection and integration were accomplished using an N2000 workstation (Zhejiang University Zhida Information Engineering Co., Ltd., China). The analytes were determined at room temperature. Chromatographic separation of HP and the internal standard was achieved on an analytical column (Diamonsil C18, 4.6 mm×150 mm, i.d., 5 μm) (Dikma Technologies, Beijing, China) protected by a guard column (KR C18, 8.0 mm×35 mm, i.d., 5 μm) (Dalian Create Science and Technology Co., Ltd., China). The mobile phase consisted of a mixture of methanol-0.6% aqueous phosphoric acid (45 : 55, v/v). The mobile phase was passed under vacuum through a 0.45 μm membrane filter and degassed before use. The analysis was carried out at a flow rate of 1 ml/min with the detection wavelength set at 340 nm.

Preparation of Calibration Standards and Quality Control Samples Stock standard solutions of HP and baicalin were prepared with methanol, and the concentrations of them were 31.6 and 40.7 μg/ml, respectively. All solutions were stored at 4°C and were found to be stable for at least 1 month. Six calibrators (0.4, 0.8, 2, 5, 10 and 40 μg/ml) of HP and baicalin (8.14 μg/ml) were prepared by dilution of stock solutions followed by spiking with drug free plasma. Quality control (QC) samples were prepared at low (1 μg/ml), medium (20 μg/ml), and high (35 μg/ml) concentrations in bulk and aliquots were stored at −20°C until analysis.

Plasma Sample Preparation To 200 μl plasma, 20 μl acetic acid, 20 μl baicalin, and 1 ml methanol were added, followed by vortex mixing for 1 min and centrifuged at 890 × g for 15 min. The supernatant
was collected and evaporated to dryness at 50°C under a gentle stream of nitrogen. The dried residue was then reconstituted in 100 μl of mobile phase, and centrifuged at 15 092 × g for 10 min, and an aliquot (50 μl) of the supernatant was injected into the HPLC system.

Method Validation

Selectivity Selectivity was investigated by comparing chromatograms of blank plasma obtained from rats prior to dosing with those of corresponding standard plasma sample spiked with HP and I.S., and plasma sample from rats after intravenous doses of HP and I.S.

Linearity, LOD and LLOQ The linearity was evaluated over the concentration range of 0.8921–59.7125 μg/ml at six levels of HP. The calibration curves for HP in plasma were generated by plotting the peak area ratio of HP to baicalin versus the nominal concentrations in the standard plasma samples. The regression equation was obtained by weighted (1 /c²) least square linear regression. The lower limit of quantification (LLOQ) was defined as the lowest concentration of HP in the calibration curves, giving an acceptable accuracy (R.E.) within ±20% and a precision (R.S.D.) that did not exceed 20%.

Precision and Accuracy The accuracy and precision of the method were evaluated with QC samples at three concentrations and using five replicates on three consecutive days. The intra- and inter-assay precisions were assessed by determining the quality control samples at three concentration levels of HP (1, 20 and 35 μg/ml). For the intra-day validation, five replicates of the QC plasma samples were analyzed on the same day. For the inter-day validation, five replicates of the QC plasma samples were analyzed on three different days. The precision was expressed as the R.S.D. which should be less than 15%, except at the LLOQ where it should not exceed 20%, and the accuracy of the assay was determined by comparing the means of the determined HP concentrations with the nominal concentrations. The mean percentage deviation from the nominal value was expressed as the R.E. which should be within ±15% of the nominal value, except at the lower limit of quantification where it should not exceed ±20%.

Extraction Recovery The extraction recovery was determined by comparing the peak areas of HP obtained for the QC samples (1, 20 and 35 μg/ml, n = 6) that were subjected to the extraction procedure with those obtained from blank plasma extracts that were spiked post-extraction at the corresponding concentrations.

Stability

Short-term Stability Five aliquots of QC samples at low, mid and high concentration unextracted QC samples were stored at −20°C for 4 h in order to determine the short-term stability of HP in rat plasma. Then, the samples were processed and analyzed. The concentrations obtained were compared with the nominal values of QC samples.

Long-term Stability Five aliquots each of low, mid and high concentration unextracted QC samples were stored at −20°C for 3 months. The samples were processed and analyzed, and then concentrations obtained were compared with the nominal values.

Freeze-thaw Stability The stabilities of plasma samples of three freeze and thaw cycles were determined by subjecting five aliquots of QC samples at low, mid and high concentration unextracted QC samples to three freeze-thaw cycles. After completion of the three cycles, the samples were analyzed, and the experimental concentrations were compared with the nominal values.

Animals and Pharmacokinetic Study All animal studies were performed according to the Guidelines for the Care and Use of Laboratory Animals that were approved by the Committee of Ethics of Animal Experimentation of Liaoning University of Traditional Chinese Medicine. Male Wistar rats, weighing 250–300 g, were obtained from the Laboratory Animal Center of Liaoning University of Traditional Chinese Medicine (Shenyang, China). They were kept in an environmentally controlled breeding room for 1 week before the experiments and fed with standard laboratory food and water ad libitum and fasted overnight before the experiment. Three groups (five rats/group) were randomly assigned to receive HP solution via a tail vein injection at doses of 5, 10 and 20 mg/kg, respectively. Blood samples (0.4 ml) were collected into heparinized tubes from the vena orbitalis at times of 0.05, 0.08, 0.17, 0.25, 0.33, 0.50, 0.83, 1.33, 2.00, 3.00 and 4.00 h after intravenous administration and then centrifuged at 890 × g for 15 min. The obtained plasma was stored at −20°C until analysis. Physiological saline (0.5 ml) was administered to compensate for the blood loss after each blood withdrawal.
**RESULTS**

**Method Validation**

**Selectivity** To determine the selectivity of this method, blank rat plasma, plasma spiked with known amounts of HP and baicalin and plasma samples from rats after intravenous doses of HP were, respectively, analyzed (Fig. 2). The chromatograms showed that there were no interfering peaks in the region of the peaks of the analyte and I.S. The retention times of HP and I.S. were approximately 9.5 min and 19.3 min, respectively. A complete baseline resolution of HP and I.S. with adjacent peaks was obtained from the chromatogram. The total run time was 30.0 min.

**Linearity, LOD and LLOQ** The evaluation of the linearity was performed with a six-point calibration curve over the concentration range of 0.8921–59.7125 µg/ml. The slope and intercept of the calibration graphs were calculated by weighted (1/c^2) least squares linear regression. The regression equation of the calibration curves was typically: $y = 1.4824x + 0.1776$, and $r$ was 0.9957, where $y$ is the peak area ratio of HP to I.S., and $x$ is the plasma concentration of HP.

The limit of detection (LOD) was 0.2766 µg/ml, which was determined by a signal-to-noise ratio ($S/N$) of 3. The lower limit of quantification defined as the lowest concentration on the calibration curve, was 0.8921 µg/ml, with the precision and accuracy within 15% verified by repeated analysis.

**Precision and Accuracy** The results are shown in Table 1. The intra- and inter-day precisions were satisfactory with R.S.D.s less than 7.8 and 2.5%, respectively. The R.E.s of intra- and inter-day accuracy were within 1.6 to 2.4%. The precision (R.S.D.) determined at each concentration level is required not exceeding 15% and accuracy (R.E.) within ±15% of the actual value which conform to the criteria for the analysis of biological sample according to the guidance of USFDA.14

**Extraction Recovery** The extraction recoveries of HP at three concentrations (1, 20 and 35 µg/ml) were no less than 98.40±2.32% (Table 2), and that of I.S. was 97.90±4.5%, suggesting that there was negligible loss during extraction. The high recovery could be attributed to the high solubility of HP in methanol and the one-step protein precipitation used in the sample preparation.

**Stability** The short-term stability, long-term stability and freeze-thaw stability of HP in plasma were listed in Table 3, which indicated that no significant degradation occurred during chromatography.

![Fig. 2. Mean Plasma Concentration-time Curves of Hyperoside in Rats after an Intravenous Administration of Doses of 5, 10 and 20 mg/kg](image)

**Table 1. Precision and Accuracy of Hyperoside Determination in Rat Plasma**

<table>
<thead>
<tr>
<th>Added conc. (µg/ml)</th>
<th>Intra-day</th>
<th>Inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean detected conc. (µg/ml)</td>
<td>R.S.D. (%)</td>
</tr>
<tr>
<td>1</td>
<td>$1.024 ± 0.0980$</td>
<td>7.8</td>
</tr>
<tr>
<td>20</td>
<td>$20.47 ± 0.782$</td>
<td>3.4</td>
</tr>
<tr>
<td>35</td>
<td>$35.68 ± 1.04$</td>
<td>1.8</td>
</tr>
</tbody>
</table>

*Intra-day: n=5, inter-day: n=3 days with 5 replicates per day.*

**Table 2. Recovery of Hyperoside in Rat Plasma**

<table>
<thead>
<tr>
<th>Added conc. (µg/ml)</th>
<th>Recovery (%)</th>
<th>R.S.D. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>99.50±2.16</td>
<td>1.2</td>
</tr>
<tr>
<td>20</td>
<td>100.1±3.56</td>
<td>2.1</td>
</tr>
<tr>
<td>35</td>
<td>98.40±2.32</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*µ=6.*
Table 3. Stabilities of Hyperoside in Rat Plasma at Three QC Levels

<table>
<thead>
<tr>
<th>Stability</th>
<th>Conc. found (mean±S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 μg/ml</td>
</tr>
<tr>
<td>Short-term stability (room temp)</td>
<td>0.9700±0.067</td>
</tr>
<tr>
<td>Long-term stability (−20°C)</td>
<td>1.053±0.032</td>
</tr>
<tr>
<td>Freeze-thaw stability</td>
<td>1.040±0.057</td>
</tr>
</tbody>
</table>

\( n=5 \)

Table 4. Pharmacokinetic Parameters of Hyperoside in Rats after Intravenous Administration of Doses of 5, 10 and 20 mg/kg

<table>
<thead>
<tr>
<th>Parameter</th>
<th>5 (mg/kg)</th>
<th>10 (mg/kg)</th>
<th>20 (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( T_{1/2} ) (h)</td>
<td>0.2081±0.126</td>
<td>0.4958±0.0614</td>
<td>1.154±0.0688</td>
</tr>
<tr>
<td>Vc (l/kg)</td>
<td>0.2479±0.150</td>
<td>0.07679±0.0097</td>
<td>0.06874±0.00480</td>
</tr>
<tr>
<td>CL (l/h/kg)</td>
<td>0.7683±0.0778</td>
<td>0.5309±0.0170</td>
<td>0.5207±0.00554</td>
</tr>
<tr>
<td>AUC_{0→t} (mg・l/h)</td>
<td>6.560±0.628</td>
<td>9.429±0.363</td>
<td>9.603±0.102</td>
</tr>
</tbody>
</table>

mean±S.D., \( n=5 \).

**DISCUSSION**

**Chromatography** To obtain suitable retention times and good separation for the analysis, many mixed solutions were used as mobile phase, such as methanol-water \((44:56=53:47)\), acetonitrile-water \((25:75=27:73)\). Additionally, to improve the peak shape, 0.1-0.7% phosphoric acid was added in, however, only when 0.6% phosphoric acid was applied to the mobile phase, a good peak shape and good separation were obtained. Finally, the mobile consisting of methanol-water containing 0.6% phosphoric acid \((45:55, v/v)\) was chosen in our study.

The UV absorption spectrum of HP has two maximum absorptions at 255 and 355 nm, and I.S. at 275 nm. The interferences from endogenous substances in the plasma were observed when the wavelength was...
set at 255 nm, and the peak area of I.S. was weak at 355 nm, which is unfavorable to the determination of HP. Considering the above factors, 340 nm was chosen as the detection wavelength and proved to be suitable for the analysis of HP and I.S.

To determine HP of rat plasma, the wavelength was set at 340 nm, the LOD of this method was 0.2776 μg/ml which was lower compared with 0.3 μg/ml and 0.86 μg/ml determined at 360 nm[9] and 270 nm,[13] respectively. Therefore, the method with lower LOD was sensitive enough to describe the whole concentration-time curves after three intravenous doses of HP. To improve the sensitivity of both analytes and I.S. simultaneously, the wavelength of 340 nm instead of 360 nm was chosen in our method which using the internal standard was preferable to the analysis of HP in vivo though LOD slightly increased compared that of the in vitro method[15] using the external standard.

According to the features of HP and I.S., the sample was deproteinized with methanol which was not only advantageous to the protein precipitation but also to the extract of HP and I.S. To get high extraction efficiency, we have tried adding 0, 10, 20, 30 μl of acetic acid in the plasma because HP was easily dissociated in weak acid. Eventually, 20 μl of acetic acid was added to the plasma.

Selection of I.S. It is necessary to select a suitable internal standard which is structurally or chemically similar to the analyte, HP. Hence, hesperidin and isoquercitrin have been considered as the internal standards. However, the retention time and resolution of them comparing with HP were unsatisfactory. Baicalin, having similar chromatographic behaviors to the analyte, finally was chosen as the internal standard for the assay.

Pharmacokinetic Study To obtain more pharmacokinetic information of HP, the pharmacokinetic study following intravenous administration at three different doses was developed, thus the some pharmacokinetic parameters can be elucidated via multiple dosing. The weight of 1/c² was chosen by comparing with the goodnesses of fit of three doses. According to F test and AIC, a three-compartment open model (Weight=1/c²) gave the best fit to the plasma concentration-time curves obtained in rats. After administered with 1 mg/kg, 20 mg/kg and 35 mg/kg of HP, there were significant dose-dependent increasing in t½, and decreasing in systemic clearance (CL) with increasing doses (p<0.05). Area under the plasma concentration-time curve from 0 h to the time of last measurable concentration (AUC₀→) values versus dose was no linear over the administered dose range. In a word, the HP investigation of dose proportionality indicated that there was no good correlation between AUC and dose. In addition, the CL presented significant difference at three dose levels, suggesting that the pharmacokinetics of HP in rats obeyed nonlinear processes as the metabolic enzyme of drug or carrier of drug membrane-permeable process was saturated in high concentration.

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REFERENCES

