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A rapid HPLC/ESI-MS method for the quantitative determination of oridonin in rat plasma

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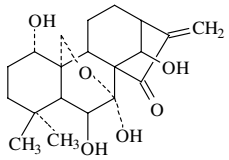
A rapid and accurate method using liquid chromatography with electrospray ionization mass spectrometric detection (HPLC/ESI-MS) was developed and validated for the determination of oridonin in rat plasma. The analytes were extracted with ethyl acetate-n-butyl alcohol (100 : 2, v/v) after spiking the samples with ethyl hydroxybenzoate (internal standard). The separation was carried out on a Diamonsil™ C_{18} column with an isocratic mobile phase consisting of methanol-water (80 : 20, v/v) at a flow rate of 1.0 ml/min. The lower limit of quantification (LLOQ) of the method was 10 ng/ml and the linear range was 10–4000 ng/ml. The intra-day and inter-day accuracy and precision of the assay were less than 9%. This method has been applied successfully to a preliminary pharmacokinetic study involving the intravenous administration of oridonin to rats.

1. Introduction

Rabdosia rubescences (Donglingcao in Chinese), a herbal medicine, is traditionally used in China for the treatment of various diseases such as esophageal carcinoma, liver cancer, lung cancer and tonsillitis (Wang 1984; Wang et al. 1989; Cai et al. 2003; Shang et al. 1995). Oridonin, a diterpenoid extracted from *Rabdosia rubescences*, is the marker compound and a major antitumor component of the plant (Lu et al. 2001; Zuo et al. 2005). Recently, this compound has attracted attention because of its ability to induce apoptosis of a variety of human cancer cells (Liu et al. 2004a; Liu et al. 2004b; Zhang et al. 2004; Hsieh et al. 2005).



Although *Rabdosia rubescences* and oridonin have been applied in clinical treatments for a long time, little information on the pharmacokinetics of oridonin or the determination of this drug in biological fluids has been reported. Zhang et al. (2005) developed a HPLC-UV method to determine oridonin in biological fluids with an LLOQ of 25 ng/ml (using 0.5 ml plasma). The plasma samples were performed by liquid-liquid extraction. However, limited information on the method validation was presented in that paper. For a better understanding of the pharmacokinetics of oridonin, it is essential to have a sensitive and accurate analytical method to determine the concentration of oridonin in biological fluids.

In the present study, we describe a new HPLC/ESI-MS method that is rapid, accurate and suitable for determining oridonin in rat plasma.

2. Investigations and results

2.1. Liquid chromatography and mass spectrometry

The possibility of using electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) sources under positive or negative ion detection mode was evaluated. Higher sensitivity was achieved for oridonin in the negative-ion mode using ESI source. After a series of investigations, a solvent consisting of a mixture of methanol and water was more suitable for the ionization of oridonin compared with an acetonitrile-water mixture. The addition of formic acid (0.05%) to the mobile phase reduced the sensitivity in the negative-ion mode, while aqueous ammonia (0.025%) did not increase the sensitivity.

As shown in Fig. 1, no significant interferences from endogenous substances with analyte or internal standard were detected. Typical retention times for oridonin and the internal standard were 3.35 min and 3.68 min, respectively.

2.2. Assay validation

The standard calibration curve for spiked rat plasma containing oridonin was linear over the range 10 to 4000 ng/ml with a correlation coefficient greater than 0.995. A typical equation for a calibration curve was $Y = 2.30 \times 10^{-4}X + 1.03 \times 10^{-3}$, $r = 0.9962$. The presented analytical method had a lower limit of quantification (LLOQ) of 10 ng/ml for oridonin using 0.1 ml rat plasma with an accuracy of 14.6% and a precision of 13.2% ($n = 5$).

The intra- and inter-day accuracy and precision of oridonin determinations in plasma are summarized in the Table. The intra-day precision was less than 5.1%, and the inter-day precision did not exceed 8.7%. The accuracies ranged from 97.0% to 101.2%.

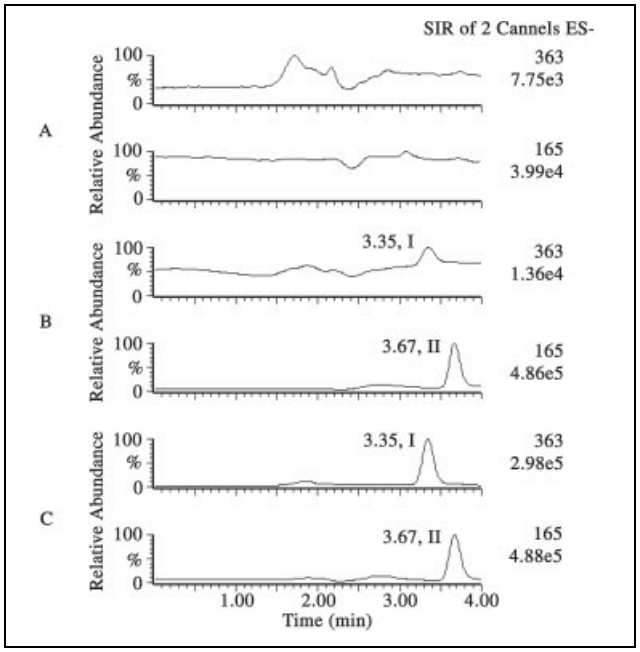


Fig. 1: Representative SIR chromatograms of oridonin plasma sample. (A) a blank rat plasma; (B) a blank rat plasma spiked with oridonin at the LLOQ of 10 ng/ml and internal standard (I.S., 40 ng/ml); (C) rat plasma obtained from a rat 2 h after intravenous administration of oridonin (10 mg/kg). Peak I, oridonin; Peak II, internal standard

Table: Intra-day and inter-day accuracy and precision for the determination of oridonin in rat plasma (3 days, six replicates per day)

Concentration added (ng/ml)	Concentration found (ng/ml)	Accuracy (%)	Precision (%, R.S.D.)	
			Intra-day	Inter-day
20	19.4	97.0	5.1	8.7
200	202.5	101.2	4.6	5.8
2000	2015.1	100.8	2.7	5.2

The extraction recoveries of oridonin under the liquid-liquid extraction conditions were 66.2 ± 3.9, 66.5 ± 4.1 and $63.1 \pm 7.4\%$ at concentrations of 20, 200 and 2000 ng/ml (QC samples), respectively. The recovery of the internal standard was $78.5 \pm 3.4\%$ in rat plasma ($n = 6$).

The analyte was found to be stable in rat plasma for 1 month at $-20\,^{\circ}C$ and in reconstituted mobile phase at room temperature for 24 h (<2% reduction). After storage at 1–4 °C for 2 month, no obvious reduction was found in the stock and working solutions. The analyte was found to be stable after three freeze-thaw cycles with a reduction of less than 8.7%. Oridonin was shown to be stable in rat plasma at room temperature for at least 2 h with a reduction of less than 12.8%.

2.3. Application of the analytical method to pharmacokinetic studies

After intravenous administration of 10 mg/kg oridonin to six Wistar rats, plasma concentrations of oridonin were determined by the above validated HPLC/ESI-MS method. Fig. 2 shows mean plasma concentration-time curve ($n = 6$). Plasma concentrations of oridonin in rats were detectable for at least 48 h after intravenous administration.

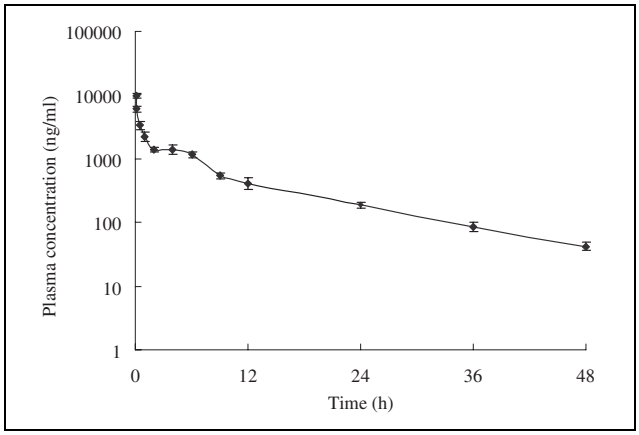


Fig. 2: Mean plasma concentration-time profile of oridonin following an intravenous administration of 10 mg/kg oridonin to six Wistar rats

3. Discussion

In analysis of biological samples, internal standards are usually needed to rectify the probable error in sample processing and determination. Though in the method provided by Zhang et al. (2005) no internal standard was used, an internal standard is necessary to get high accuracy when HPLC is equipped with MS as the detector. A number of compounds have been evaluated as internal standard including ponicidin, diazepam, acetophenetidin, bifendate, ethyl hydroxybenzoate, and propyl hydroxybenzoate. Ponicidin, a compound with a structure similar to oridonin, showed retention behavior different from oridonin and severely interfered by endogenous material. Acetophenetidin, bifendate and ethyl hydroxybenzoate showed similar retention behavior with oridonin, but under the optimal MS condition, acetophenetidin and bifendate had low sensitivity and poor repeatability. Finally, ethyl hydroxybenzoate was adopted because of its similarity in retention behavior, ionization and less endogenous interference though the structural and chemical properties of these two compounds are different.

The HPLC-UV method established by Zhang et al. applied to the pharmacokinetics of oridonin in rabbits had a LLOQ of 25 ng/ml using 0.5 ml plasma. Under that LLOQ, the concentration of oridonin in rabbit plasma was detectable for 24 h after intravenous administration. The present HPLC/ESI-MS method has a LLOQ of 10 ng/ml using 0.1 ml plasma and the concentration of oridonin in rat plasma was detectable for at least 48 h after intravenous administration. The $T_{1/2}\beta$ of oridonin in rats is about 10 h, so the present method is sensitive enough to investigate the pharmacokinetics behavior of oridonin in rats.

In conclusion, a rapid and accurate HPLC/ESI-MS method was developed for the analysis of oridonin in rat plasma. The method was validated and used successfully for a preliminary pharmacokinetic study of oridonin in rats following intravenous administration.

4. Experimental

4.1. Materials

Oridonin (98.9%) was extracted from aerial parts of *Rabdosia rubescences* and refined in our laboratory (identified by ^{1}H NMR, UV and MS). The internal standard, ethyl hydroxybenzoate (99.5%), was supplied by Shenyang Dongxing Reagent Factory (Shenyang, China). HPLC-grade methanol was purchased from Concord Tech. Co. (Tianjin, China) while HPLC-grade ethyl acetate and *n*-butanol were from Tianjin Kermel Chemical

Reagents Development Centre (Tianjin, China). All other reagents were of analytical grade. Distilled water, prepared from deionized water, was used throughout the study.

4.2. HPLC/MS conditions

HPLC was performed using a Waters 1525 Binary pump, which was controlled by Masslynx 4.0 Software (Waters Corp.). The mobile phase consisted of methanol-water (80 : 20, v/v) at a flow rate of 1.0 ml/min and the injection volume was 20 µl. The analytical column used was a Diamonsil^{TM} C_{18} column (200 mm × 4.6 mm i.d., 5 µm) from Dikma Tech. (Beijing, China) at a column temperature of 25 °C. A T-junction was placed between the LC column and the ESI/MS system. The splitting ratio was set at 25% (0.25 ml/min of effluent was introduced into the ESI-MS system).
A ZQ2000 micromass spectrometer (Waters Corp.) fitted with a Z-Spray ion interface was used for all analyses. Ionization was achieved using electrospray in the negative mode. The following parameters were optimized for the analysis of oridonin: capillary voltage, 3.0 kV; cone voltage, 25 V; source temperature, 105 °C; and desolvation gas (nitrogen) heated to 350 °C and delivered at a flow rate of 350 L/h. Quantification was performed using selected ion recording (SIR) of m/z 363 for oridonin and m/z 165 for ethyl hydroxybenzoate.

4.3. Preparation of stock and working solutions

The stock solution of oridonin (1 mg/ml) was prepared in methanol and was further diluted with mobile phase to give a series of working solutions with concentrations of 20, 40, 80, 400, 800, 4000 and 8000 ng/ml. Aqueous solutions of 40, 400 and 4000 ng/ml were used for stability testing by diluting the stock solutions with methanol-water (5 : 95, v/v). A solution containing ethyl hydroxybenzoate (80 ng/ml) was also prepared using mobile phase. All stock solutions and working solutions were stored at 1–4 °C.

4.4. Preparation of calibration standards and quality control (QC) samples

Calibration standards and QC samples of oridonin were prepared by adding 50 µl of the working solutions and 50 µl ethyl hydroxybenzoate solution to 100 µl of drug-free rat plasma. Calibration standards were prepared at concentrations of 10, 20, 40, 200, 400, 2000 and 4000 ng/ml of oridonin in plasma, while QC samples were prepared at concentrations of 20, 200 and 2000 ng/ml.

4.5. Sample preparation

To 100 µl of plasma in glass centrifuge tubes were added 50 µl ethyl hydroxybenzoate (80 ng/ml) and 50 µl mobile phase. Samples were then vortex-mixed for 30 s and extracted with 3 ml ethyl acetate-*n*-butyl alcohol (100 : 2, v/v). After vortex-mixing for 1 min and shaking for 10 min, the organic and aqueous phases were separated by centrifugation at 2000 × g for 10 min, then the upper organic layer was transferred to another tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 µl mobile phase followed by vortex-mixing and centrifugation at 2000 × g for 10 min. Then, 20 µl of an aliquot of supernatant was injected onto the HPLC/ESI-MS system. For samples containing oridonin at a concentration higher than the upper limit of the range in the standard curve, an aliquot of the sample was first diluted with blank rat plasma and then 0.1 ml of the diluted sample was treated as described.

4.6. Assay validation

Standard curves ranging from 10 to 4000 ng/ml oridonin were run on three separate days. Calibration curves were constructed using a $1/x^2$ weighted linear regression of the peak-area ratios of the analyte to internal standard versus the plasma concentration of the analyte.

Six replicates of QC samples at 20, 200 and 2000 ng/ml oridonin were included in each run to determine the intra-day and inter-day precision of the assay. The accuracy was determined as the percentage difference between the mean detected concentrations and the nominal concentrations. The lower limit of quantification (LLOQ) is defined as the lowest concentration of standard that can be measured with an acceptable accuracy and precision (≤20% for both parameters).
The extraction recoveries of oridonin at three QC levels were determined by comparing peak areas obtained from plasma samples with those found by direct injection of a standard solution of the same concentration.
The stability of oridonin in plasma was assessed by analyzing triplicate QC samples with oridonin concentrations of 20, 200 and 2000 ng/ml stored for 2 h at ambient temperatures, three cycles of freezing at −20 °C and thawing, reconstituted extract at room temperature and stored for 1 month at −20 °C, respectively. Concentrations following storage were compared with freshly prepared samples of the same concentrations.

4.7. Application of the method

Male Wistar rats, weighing approximately 230–250 g, were obtained from the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China). The animal experimentation was approved by the Animal Ethics Committee of Shenyang Pharmaceutical University (Shenyang, China). Rats (n = 6) received an intravenous injection of 10 mg/kg oridonin in the thigh vein (2 ml/kg). The drug was dissolved into ethanol-water (3 : 7, v/v) and adjusted to be isotonic using sodium chloride. Blood samples (250 µl) were collected in heparinized tubes from each rat at 0.083, 0.167, 0.5, 1, 2, 4, 6, 9, 12, 24, 36 and 48 h after administration. Blood samples were immediately centrifuged and stored at −20 °C until analysis. Plasma collected from 6 vehicle-injected rats served as blank.

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