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Short communication

An HPLC method for determination of oridonin in rabbits using isopsoralen as an internal standard and its application to pharmacokinetic studies for oridonin-loaded nanoparticles

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ABSTRACT

A simple and sensitive HPLC method has been developed and validated for the determination of oridonin (ORI) in rabbit plasma. A simple liquid–liquid extraction (LLE) method was applied to extract ORI and the internal standard (IS), isopsoralen, from rabbit plasma. Chromatographic separation of ORI and the IS was achieved with a Kromasil C18 5-μm column (250 mm × 4.6 mm) using methanol–water (50:50, v/v) as mobile phase at a flow rate of 1 mL/min. The ultraviolet (UV) detection wavelength was set at 241 nm. The lower limit of quantification (LLOQ) was 0.02 μg/mL. The calibration curves were linear over a concentration range of 0.02–10 μg/mL. The assay accuracy and precision were within the range of 95.1–113.5% and 5.4–8.6%, respectively. This HPLC method was applied successfully to the pharmacokinetic study of ORI-loaded poly(caprolactone)-poly(ethylene oxide)-poly(caprolactone) copolymer nanoparticles (ORI-PCL-PEO-PCL-NP) in rabbits, given as a single intravenous injection at the dose equivalent to 2 mg of ORI/kg, and the pharmacokinetic parameters for ORI were compared with a single intravenous injection of a ORI solution at the same dose.

1. Introduction

The medicinal herb, Donglingcao, with a scientific name of *Rabdosia rubescens*, is native to the Yellow River valley and used as a folk remedy for carcinomas of the heart and the esophagus in China. Oridonin (ORI), a monomeric ingredient of ent-kaurane diterpenoid compounds, is the main active ingredient extracted and isolated from *R. rubescens*. Cell culture experiments showed that oridonin had an inhibitory effect on a variety of human cancer cell lines, such as leukemia (HL60 [1], k562 [2], HPB-ALL [3], NB4 [4], Jurkat [5]); esophageal cancer (CaEs-17 [6]); gastric cancer (MGC80-3 [6]); liver cancer (BEL-7402 [7,8]); lung cancers (SPC-A-1 [9], NCI-H520 [4], NCI-H460 [4], NCI-H1299 [4]) and uterine cervix cancer (Hela [10]). The mechanism underlying the anti-tumor effect of ORI was reported to be related to blocking DNA synthesis and inducing apoptosis of cancer cells, but the definite mechanism of action remains unknown [1,2,7–11].

According to the literature, several HPLC-UV methods [12–15] and one HPLC/ESI-MS method [16] was applied to determine ORI in animal plasma, but only one HPLC-UV method with the lower limit of quantification (LLOQ) being 0.05 μg/mL for determining ORI in rat plasma [12] was fully validated. In this article, a simple and sensitive (LLOQ being 0.02 μg/mL) HPLC-UV method for determining ORI in rabbit plasma was developed, fully validated and successfully applied to the pharmacokinetic study of oridonin-loaded poly(caprolactone)-poly(ethylene oxide)-poly(caprolactone) copolymer nanoparticles (ORI-PCL-PEO-PCL-NP).

2. Materials and methods

2.1. Reagents and chemicals

ORI was purchased from Huike Biotechnology Co. Ltd., Shanxi, China. Isopsoralen was provided by National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China, and used as internal standard (IS). The chemical structures of ORI and isopsoralen are shown in Fig. 1.

ORI-PCL-PEO-PCL-NP was prepared by using the method we reported previously [17].

Methanol (HPLC grade) was obtained from Merck Co. Inc., Germany, and other reagents were of analytical grade.

2.2. HPLC assay

2.2.1. Chromatographic conditions

The chromatographic separation of ORI and the IS was achieved with a Waters HPLC system in an isocratic mode. This HPLC system

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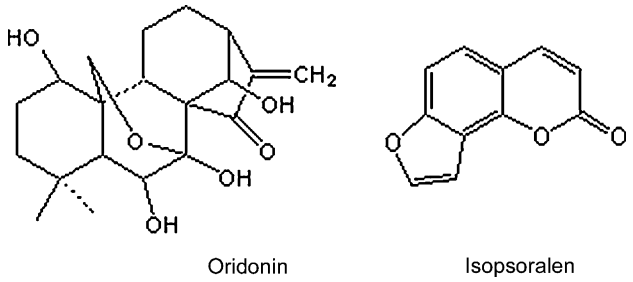

Fig. 1. Chemical structures of oridonin and isopsoralen.

consists of a model 2695 pump, a model 717 Autosampler, a model 2487 UV detector and a Kromasil C_{18} column (250 mm × 4.6 mm, 5 μm, Eka chemicals, Bohus, Sweden) protected with a guard column of the same packing material (7.5 mm × 4.6 mm). The column and autosampler temperatures were kept at 25 and 4 °C, respectively. The mobile phase was composed of methanol and water (50:50, v/v), and the flow rate was 1.0 mL/min. The UV detection wavelength was set at 241 nm. The injection volume was 20 μL.

2.2.2. Quality control samples and calibration standards

Stock solutions of ORI (1 mg/mL) and the IS (1 mg/mL) were prepared in methanol and stored at −20 °C, respectively. The ORI working solutions at concentrations of 0.2, 2.0, 10, 20, 50 and 100 μg/mL were prepared by serial dilution of ORI stock solution with methanol. Similarly, the IS working solution (25 μg/mL) was also prepared by serial dilution of the IS stock solution with methanol.

The ORI calibration standards were prepared daily at concentrations of 0, 0.02, 0.2, 1.0, 2.0, 5.0 and 10 μg/mL by spiking 0.5 mL blank rabbit plasma with 50 μL methanol (for "zero" standard sample) or 50 μL ORI working solutions. In the same way, the QC samples with ORI concentrations at low (0.02 μg/mL), medium (1.0 μg/mL) and high (10 μg/mL) were prepared.

2.2.3. Sample and calibration standard preparation

A simple liquid–liquid extraction method was applied to extract ORI and the IS from rabbit plasma. Briefly, a 0.5 mL blank rabbit plasma, spiked plasma (calibration standard and QC sample) or pharmacokinetic study plasma sample was firstly spiked with 20 μL IS working solution and then extracted with 3 mL ethyl acetate by vortex-mixing for 3 min at room temperature. The upper layer was transferred to a clean tube after centrifugation at 3000 rpm for 10 min. Ethyl acetate was evaporated to dryness under a gentle stream of nitrogen gas at room temperature. The obtained residue was reconstituted in 100 μL methanol and transferred into a sample vial for HPLC assay.

2.3. HPLC method validation

2.3.1. Selectivity

The selectivity of the method was tested by comparing the chromatograms of blank plasma controls from six rabbits with that of plasma spiked with ORI and IS.

2.3.2. Sensitivity

The calibration standard at the lowest concentration, yielding a precision with relative standard deviations (R.S.D.) less than 20% and accuracy within 20% of the nominal concentration, was considered to be the lower limit of quantification.

2.3.3. Calibration curve

Calibration standards at concentrations of 0, 0.02, 0.2, 1.0, 2.0, 5.0 and 10 μg/mL ($n = 2$) were freshly prepared as described above and assayed on the same day. This assay was repeated on five consecutive days with freshly prepared calibration standards. The subsequent five calibration curves ($y = ax + b$), represented by the plots of the peak-area ratios of ORI to IS (y) *versus* the concentration of the calibration standards (x), were generated with weighted ($1/x^2$) linear least-squares regression as the mathematical model.

2.3.4. Accuracy and precision

In order to validate the intra-day accuracy and precision, QC samples (0.02, 1.0 and 10 μg/mL) were freshly prepared and analyzed on the same day ($n = 5$). To validate the inter-day accuracy and precision, the intra-day accuracy and precision assay was repeated on three consecutive days.

Accuracy was calculated by comparing the averaged measured concentration to the nominal concentration, and was expressed in percentage. Precision was evaluated by calculating the R.S.D. of measured concentrations at each QC level with one-way analysis of variance (ANOVA), respectively.

2.3.5. Recovery

The extraction recoveries of ORI from rabbit plasma at QC levels (0.02, 1.0 and 10 μg/mL) were determined by comparing mean peak area ratio of ORI/IS of QC samples to that of unextracted ORI standards in methanol at equivalent ORI level spiked with IS and expressed in percentage, respectively.

2.4. Pharmacokinetic study of ORI-PCL-PEO-PCL-NP

2.4.1. Administration and sampling

Rabbits (2.0–2.5 kg, provided by Experimental Animal Center of Shanghai University of Traditional Chinese Medicine) were equally divided into two groups (four for each): group A and group B. All care and handling of animals were performed with the approval of Institutional Authority for Laboratory Animal Care of Shanghai, University of Traditional Chinese Medicine.

Each rabbit in group A was given a single injection of a sterile physiological saline solution containing ORI (2 mg of ORI/kg) through marginal ear vein, respectively. After administration, a volume of 1.5 mL blood samples were taken from the marginal vein of the ear at 0 (prior to dosing), 0.017, 0.083, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0 and 12.0 h, and collected with the heparinized tubes, respectively. Plasma samples were separated immediately by centrifugation at 3000 rpm for 10 min. The plasma sample preparation procedure was immediately applied to each separated plasma sample. Each obtained residue were tightly sealed and stored at −20 °C until analysis. Each rabbit in group B was given a single injection of ORI-PCL-PEO-PCL-NP suspension (obtained from the reconstitution of freeze-dried ORI-PCL-PEO-PCL-NP) at a dose of 2 mg of ORI/kg through marginal ear vein, respectively. A volume of 1.5 mL blood samples were taken from the marginal vein of the ear under the same sampling schedule in group A. The plasma separation and preparation procedure, the residue storage condition were the same as those in group A.

All of the plasma samples were analyzed within a week after separation.

2.4.2. Calculations and statistics

Compartmental and non-compartment pharmacokinetic analysis of plasma ORI concentrations *versus* time data were carried out using BAPP2.0 (Bioavailability program package 2.0, Center for Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing, China) to estimate the pharmacokinetic parameters in

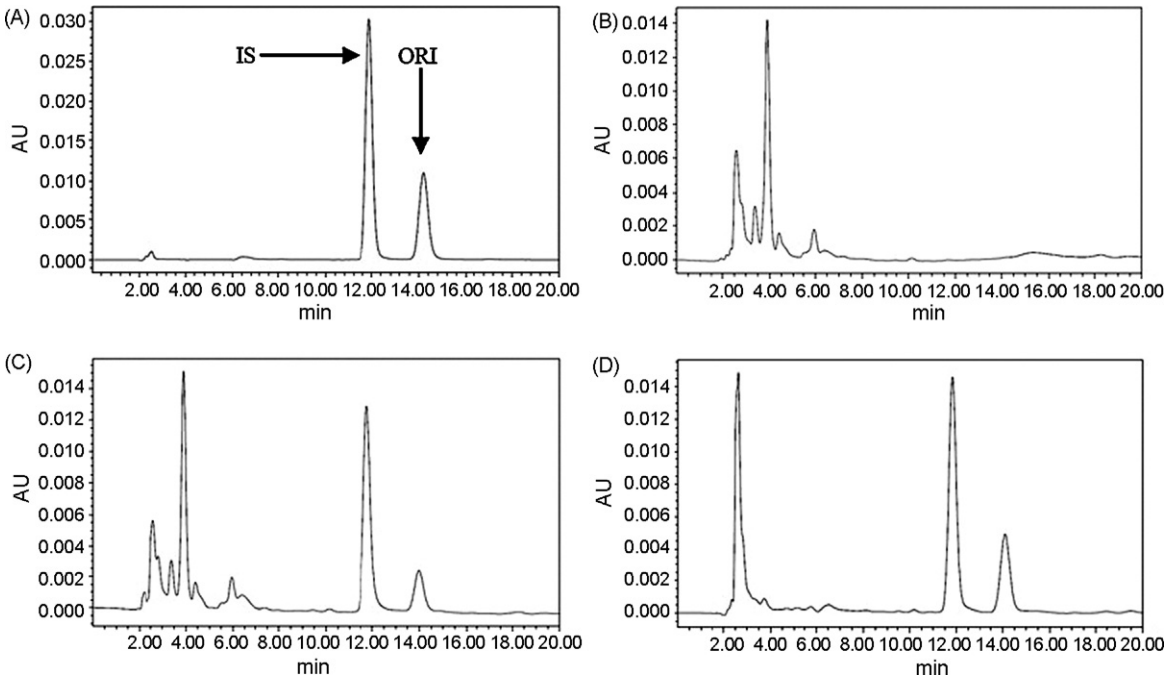

Fig. 2. Representative chromatograms of: (A) ORI and isopsoralen (IS) standards; (B) blank rabbit plasma; (C) blank rabbit plasma spiked with ORI (1 μg/mL) and IS (1 μg/mL); (D) rabbit plasma sample at 0.083 h after a single intravenous injection of ORI-loaded poly(caprolactone)-poly(ethylene oxide)-poly(caprolactone) copolymer nanoparticles (ORI-PCL-PEO-PCL-NP) and spiked with IS.

groups A and B, respectively. Non-linear regression analysis showed that the model fitting experimental data in group A, as well as data in group B, was a two-compartment open model. The CL, $\ln(t_{1/2\alpha})$, $\ln(t_{1/2\beta})$, $\ln(V_\beta)$, ln(AUC) and ln(MRT) were compared by using independent-samples *t*-test, respectively. A value of $p < 0.05$ was considered to be significant.

3. Results and discussion

3.1. HPLC method development

Several mobile phases were used to determine ORI in formulations or in biological matrixes by HPLC. In the latest study, acetonitrile/0.01 M ammonium acetate (0.5% acetic acid) (30:70, v/v) [12] was chosen as mobile phases to determine ORI in rat plasma. In this assay, methanol/water (50:50, v/v) was selected as the mobile phase for the determination of ORI in rabbit plasma, because of its very simple composition and optimal separation of ORI and IS.

Several substances were tested as IS, and isopsoralen was chosen as the most appropriate one in the present assay, because it was stable and did not exist endogenously in plasma. Moreover, it did not interfere with the matrix of rabbit plasma sample and it was well separated from ORI.

Liquid–liquid extraction method was used for sample preparation. In order to extract ORI from rabbit plasma, ethyl acetate, diethyl ether, chloroform and acetone were all tested, and finally ethyl acetate was adopted due to its high extraction efficiency and less interference.

3.2. HPLC method validation

3.2.1. Selectivity

No interference of endogenous and extraneous peaks with ORI or IS at their respective retention times (RT_{IS} = 12.0 min; RT_{ORI} = 14.6 min) in blank rabbit plasma was observed, as shown in Fig. 2.

3.2.2. Sensitivity

The LLOQ of ORI in 0.5 mL rabbit plasma was observed to be 0.02 μg/mL.

The limit of detection (LOD) of ORI was 0.01 μg/mL, based on a signal to noise ratio >3.

3.2.3. Linearity of calibration curve

The method has a good linearity over the range of 0.02–10 μg/mL. The mean regression equation from five replicate calibration curves was $y = 0.037681(\pm 0.000535)x - 0.00211(\pm 0.001481)$. The square of mean correlation coefficient (r^2) was 0.9967.

3.2.4. Precision and accuracy

Table 1 is a summary of the intra- and inter-day precision and accuracy validation with QC samples as described above. In the range of 0.02–10 μg/mL, intra- and inter-day accuracy ranged from 95.1% to 112.3% and 96.2% to 113.5%, respectively. The intra- and inter-day assay precision (R.S.D.) ranged from 6.3% to 7.8% and 5.4% to 8.6%, respectively. These results indicated that this method had good accuracy and precision.

3.2.5. Recovery

The mean extraction recoveries of ORI were found to be 52.7 ± 3.4 for 0.02 μg/mL, 53.3 ± 2.6 for 1.0 μg/mL and 55.6 ± 1.7% for 10 μg/mL, respectively.

Table 1
The validation of intra- and inter-day accuracy and precision with QC samples ($n = 5$)

Concentration (μg/mL)	Precision (%R.S.D.)		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
0.02	7.8	8.6	95.1	96.2
1.0	7.7	7.9	99.1	98.6
10	6.3	5.4	112.3	113.5

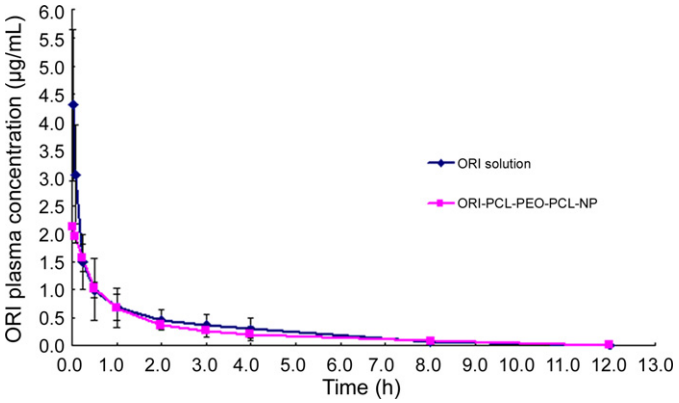

Fig. 3. Mean plasma concentration–time profiles of ORI in rabbits ($n = 4$) after a single intravenous injection of a sterile physiological saline solution containing ORI (2 mg of ORI/kg) and ORI-loaded nanoparticles suspension at the dose of 2 mg of ORI/kg, respectively.

Table 2
Pharmacokinetic parameters (mean ± S.D., $n = 4$) of ORI after intravenous administration of ORI solution (group A) and ORI-PCL-PEO-PCL-NP (group B) at a single dose equivalent to 2 mg of ORI/kg

Parameters	Group A	Group B
	ORI solution	ORI-PCL-PEO-PCL-NP
$t_{1/2\alpha}$ (h)	0.11 ± 0.05	0.44 ± 0.31
$t_{1/2\beta}$ (h)	2.12 ± 0.87	3.58 ± 0.43[a]
V_β (L/kg)	1.72 ± 0.16	3.49 ± 0.17[a]
CL (L/h)	1.44 ± 0.61	1.53 ± 0.20
$AUC_{0-\infty}$ (h μg/mL)	3.53 ± 1.31	2.97 ± 0.37
MRT (h)	2.41 ± 1.07	3.01 ± 0.47

Note: A represents $p < 0.05$ compared with group A. $t_{1/2\alpha}$: Distribution half-life; $t_{1/2\beta}$: elimination half-life; V_β: apparent volume of distribution; CL: clearance; AUC: the area under the plasma concentration–time curve; MRT: mean residence time.

3.2.6. Other validation parameters

Dilution effect of blank rabbit plasma was evaluated with dilution samples (25 and 50 μg/mL) prepared following the same procedure of QC sample preparation and further diluted with blank rabbit plasma. No unacceptable dilution effect was founded.

Stability of solution was evaluated. Stock solutions of ORI (1 mg/mL) and IS were both observed to be over 99% of the nominal concentrations after storage at −20 °C for 30 days, compared with freshly prepared solutions, respectively. Similarly, working solutions of ORI and IS were over 99% of the nominal concentrations after storage at 4 °C for 7 days, respectively.

Freeze thaw cycle procedure was not applied to the QC samples in our study, considering that ORI was reported to be not stable in rat plasma after stored at −20 °C for 12 h [12]. Instead, ORI was immediately extracted after each pharmacokinetic study blood sample was taken and plasma sample immediately separated by centrifugation.

3.3. Pharmacokinetic study of ORI-PCL-PEO-PCL-NP

The mean plasma concentration–time profiles of ORI are shown in Fig. 3.

The mean calculated pharmacokinetic parameters are presented in Table 2. As compared with a single intravenous injection of a sterile physiological saline solution containing ORI (2 mg of ORI/kg), there were no significant differences in the main pharmacokinetic parameters, such as AUC, MRT and CL, but V_β was significantly increased, indicating the change in tissue distribution behavior of ORI following the administration of ORI-PCL-PEO-PCL-NP.

4. Conclusion

A simple, sensitive, accurate and precise HPLC-UV method was developed and validated for the quantitative determination of ORI in rabbit plasma. The LLOQ of the method was 0.02 μg/mL and the sensitivity was relatively high compared with those previously reported HPLC-UV methods. The proposed method was successfully applied to a pharmacokinetic study of ORI-loaded nanoparticles in rabbits.

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