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Quantitative HPLC method and pharmacokinetic studies of ergosta-4,6,8(14),22-tetraen-3-one, a natural product with diuretic activity from *Polyporus umbellatus*

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ABSTRACT: A simple and specific HPLC method with dual wavelength UV detection for the determination of ergosta-4,6,8(14),22-tetraen-3-one (ergone) in rat plasma was developed and proved to be efficient. The method used ergosterol as internal standard (IS). Following a single-step protein precipitation, the analyte and IS were separated on an Inertsil ODS-3 column with a mobile phase containing methanol–water (99:1, v/v) at a flow rate of 1 mL/min. The analytes were detected by using UV detection at wavelength of 350 (ergone) and 283 (IS) nm, respectively. The calibration curve was linear over the range of 0.1–2.0 μg/mL and the lower limit of quantification was 0.1 μg/mL. The intra-day and inter-day precision studies showed good reproducibility with RSD less than 8.5%. The intra-day and inter-day accuracy ranged from 95.6 to 104%. Mean extraction recovery was above 95% at the low, medium and high concentrations. The present HPLC-UV method was simple and reliable. The method described herein had been successfully applied for the pharmacokinetic studies in male SD rats after administration of 20 mg/kg dose of solution of ergone. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: *Polyporus umbellatus*; ergosta-4,6,8(14),22-tetraen-3-one; HPLC-UV; rat plasma; pharmacokinetics

Introduction

In the clinical practice of traditional Chinese medicine (TCM), *Polyporus umbellatus* (Polyporaceae) is often used as anti-cancer and diuretic agent along with other crude drugs over a very long period of time (Jiangsu New Medical College, 1985). Ergosta-4,6,8(14),22-tetraen-3-one (ergone), one of the active components in *Polyporus umbellatus*, has been reported to have a diuretic effect (Yuan *et al.*, 2004; Zhao *et al.*, 2009c), cytotoxic activity (Lee *et al.*, 2005), inhibitory activity of nitric oxide production (Dang and Dang, 2008) and immunosuppressive activity (Fujimoto *et al.*, 2004). The diuretic bioactivity of ergone was validated by pharmacological tests in our laboratory and the study of the mechanism is in progress. Our experimental results have shown that ergone is one of the main and bioactive components of *Polyporus umbellatus* (Zhao *et al.*, 2009a–d). These multiple pharmacological activities of ergone make it worth carrying out a further comprehensive study on the pharmacokinetic properties.

Development of a sensitive method to determine ergone in biological fluids was a prerequisite to the pharmacokinetic evaluation. To the best of our knowledge, there has been no entirely validated HPLC method reported in the literature for quantification of ergone in biological samples, so the aim of our present study was to develop a rapid and sensitive HPLC method for the determination of ergone concentration in plasma and its validation. This method has been comprehensively validated, offering the advantage of simplicity with adequate sensitivity, selectivity, precision and accuracy for the determination of ergone for the first time. The assay was also successfully applied to the pharmacokinetic study of ergone in male Sprague–Dawley rats.

Experimental

Chemicals and Reagents

The standard of ergone (Fig. 1a) was isolated by the authors from *P. umbellatus*. Its structure was characterized by chemical and spectroscopic

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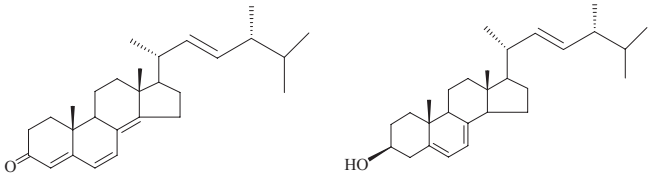
Abbreviations used: TCM, traditional Chinese medicine.



a b

Figure 1. Chemical structures of ergone (a) and ergosterol (b).

methods (^{1}H NMR, ^{13}C NMR and MS) and compared with the data reported in the literature (Lee *et al.*, 2005). Analysis showed that its purity was above 99% (HPLC). Ergosterol (Fig. 1b) was used as the internal standard (IS), which was one of the main components of *P. umbellatus* purified from our laboratory with 99% purity as determined by HPLC. HPLC-grade methanol was purchased from Fisher Company. Ultrapure water was prepared using a Millipore SAS 67120. Other chemicals were of analytical grade.

Chromatographic Conditions

HPLC was performed with a Waters 2695 instrument composed of a quaternary pump, column oven and Waters 2487 dual wavelength absorbance detector, and Empower was used for data collection. The chromatographic separation was performed on an Inertsil ODS-3 analytical column (5 μm, 250 × 4.6 mm i.d., Japan) with the column temperature set at 30°C. An isocratic elution of methanol (A) and water (B) was 99:1 (v/v) in 15 min. The flow rate was 1.0 mL/min with detector wavelength set at 350 nm (maximum absorption wavelength of ergone) and 283 nm (maximum absorption wavelength of ergosterol), and the injection volume was 100 μL.

Preparation of Calibration Standard and Quality Control Samples

Stock solution of ergone was prepared in acetone at the concentration of 125 μg/mL, and was further diluted in acetone to make working standards. Stock solution of IS (125 μg/mL) was prepared in acetone and diluted with acetone to prepare the working solution containing 1 μg/mL of IS. Calibration samples were prepared by addition of working standards of ergone (50 μL) to blank plasma, giving final concentrations of 0.1, 0.25, 0.5, 1.0, 1.25, 1.5 and 2.0 μg/mL. Quality control (QC) samples were separately prepared in a similar manner for the calibration curve, and the range of the calibration curve was 0.1–2.0 μg/mL. Plasma samples of 0.25, 1.0, and 1.5 μg/mL corresponded to the low QC, medium QC, and high QC, respectively.

Sample Pretreatment for Analysis

A 50 μL volume of plasma standard or sample was transferred to a 1.5 mL centrifuge tube, and then 10 μL of IS working solution was spiked and vortex mixed for 1 min. Next 180 μL acetone was added and the sample was vortex mixed for 2 min. After vortex mixing, the mixture was centrifuged at 10,000 rpm for 10 min at a low temperature (4°C). Finally, 100 μL of the supernatant was injected into the HPLC for analysis.

Specificity, Calibration Curves, Linearity, Precision and Accuracy

Specificity of the method was assessed by analyzing five independent sources of blank rat plasma or plasma samples spiked with ergone and IS, to observe the possible interference from plasma with the analyte or the internal standard. Calibration curves for ergone in plasma were generated by plotting the peak area ratios (y) of ergone to IS vs the nominal concentrations (x) in standard plasma by the $1/x^2$ weighted least-square linear regression. The accuracy, intra-day and inter-day precisions of the method were determined for ergone according to FDA guidance for bioanalytical method validation (US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, 2001). Intra-day and inter-day precision and accuracy were determined by replicate analysis of six sets of QC samples at three concentration levels of ergone (0.25, 1.0 and 1.5 μg/mL) within a day or during three consecutive days. The precision was calculated from the ratio of the standard deviation to the mean (RSD). The accuracy of the method was examined by comparing the measured concentrations of QC samples with the theoretical concentrations. Both values were expressed as percentages. The acceptable ranges of intra-day and inter-day accuracy and precision are below 15% bias or RSD.

Extraction Recovery

The recovery of ergone from plasma was determined by injecting the processed QC samples at three concentrations of low QC, medium QC and high QC. Recovery was evaluated by comparing the analyte peak areas obtained from the QC samples (n = 5) after extraction with those obtained from the corresponding unextracted reference standards prepared at the same concentrations.

Stability

The stability of ergone and IS stock solutions was evaluated after storage at room temperature and at 4°C for 30 days. The stability of ergone and IS working solutions was investigated at room temperature for 6 h. QC plasma samples of three concentration levels (0.25, 1.0 and 1.5 μg/mL) were subjected to the conditions below. Short-term stability was assessed by analyzing QC plasma samples kept at room temperature for 6 h, which exceeded the routine preparation time of samples. Long-term stability was determined by assaying QC plasma samples after storage at −20°C for 15 days. Freeze–thaw stability was investigated after three freeze (−20°C)–thaw (room temperature) cycles.

Application to Pharmacokinetics of Ergone

To optimize chromatographic conditions, different method was used to investigate the pharmacokinetics of ergone in rats after oral administration of ergone at a dose of 20 mg/kg, which was determined according to pharmacodynamic experimental results (Zhao *et al.*, 2009c). All procedures involving animals were in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of China. The formulation used for administration was a solution in 2% gum acacia powder. Male SD rats (380 ± 10 g) were purchased from the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China). They were kept in an environmentally controlled breeding room for 7 days before the experiments. Six rats were fasted overnight and had free access to water throughout the experimental period. Blood samples (approximately 0.3 mL) were collected from the carotid vein at control (0 min) and 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 16, 20 and 24 h after administration. The samples were immediately centrifuged at 5000 rpm for 10 min. The plasma was separated into clean tubes and frozen at −20°C until analysis. The plasma concentrations of ergone at different times were expressed as means ± SD and the mean concentration–time curve was plotted. Pharmacokinetic analysis was carried out using a two-compartmental method with the aid of the software DAS 2.0 (issued by the State Food and Drug Administration of China for pharmacokinetic study), and pharmacokinetic parameters were obtained at the same time.

Results and Discussion

Optimization of Chromatographic Conditions and Sample Preparation

To develop an accurate, valid and optimal chromatographic conditions, the different HPLC parameters including mobile phase

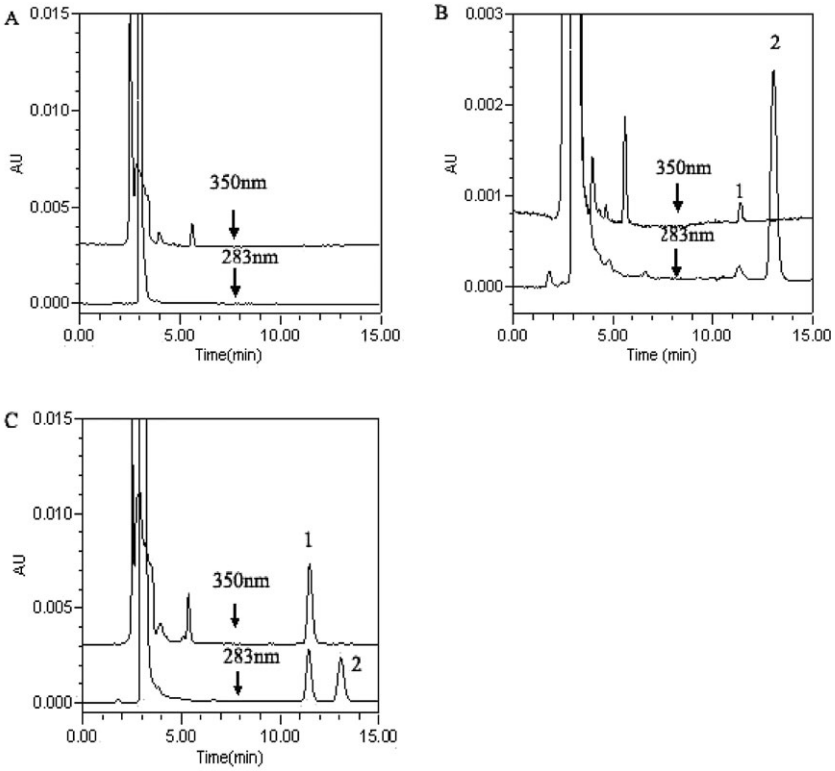


Figure 2. Chromatograms of (A) a blank plasma sample, (B) LLOQ of blank plasmas spiked with ergone and IS, (C) a plasma sample from a rat 2 h (0.858 μg/mL) after oral administration of ergone. Peak 1, ergone; peak 2, IS.

(methanol–water or acetonitrile–water), category of column (Inertsil ODS-3 column, 5 μm, 250 × 4.6 mm; Kromasil C_{18} column, 5 μm, 250 × 4.6 mm, or Diamosil C_{18} column, 5 μm, 250 × 4.6 mm), column temperature (30, 35 or 40°C) and flow rate of mobile phase (0.8, 1.0 or 1.2 mL/min) were all tested to provide sufficient resolution among biomatrix components, ergone and IS. Finally, the optimal HPLC condition was determined as mentioned previously. The retention times for ergone and IS were 11.4 and 13.1 min, respectively ($n = 6$).

Extraction procedures including protein precipitation and liquid–liquid extraction were studied. Acetone, *n*-hexane and chloroform were used to extract ergone and the IS from the plasma. After the extraction, the organic layer was transferred to a 1.5 mL centrifuge tube and evaporated to dryness under a stream of nitrogen in a water bath at 40°C. The residues were reconstituted in the proper volume of chloroform. We found that the use of liquid–liquid extraction technique resulted in a relatively low recovery for ergone and IS. The process of the sample concentration was time-consuming, so protein precipitate method was considered. Methanol, ethanol, acetone and acetonitrile were used to precipitate the plasma protein, and then the upper layer was directly injected for analysis. The extraction efficiency of acetone was higher than that of methanol, ethanol and acetonitrile. Finally, we chose acetone as the reagent for plasma sample pretreatment. Extraction time and volume of acetone were tested, and the results indicated that extraction recoveries were not improved when the shaking time was longer than 2 min and volumes larger than 180 μL. This method facilitated the collection of the blood in the period of pharmacokinetics study and resulted in a less consumption of extraction solvents and shorter sample pretreatment time.

Specificity, Linearity, Limit of Detection, Lower Limit of Quantitation, Precision and Accuracy

Under current HPLC-UV conditions, ergone and the IS were clearly separated chromatographically with retention times of 11.4 and 13.1 min, respectively. Results of HPLC-UV analysis of five different randomly selected drug-free rat plasma samples showed no interfering peaks exhibited at the retention times of either the target or the IS (Fig. 2A), when compared with the peaks of lower limit of quantitation (LLOQ; Fig. 2B). Typical chromatograms of a plasma sample from a rat 2 h after dosing are shown in Fig. 2(C).

Linear calibration curves were obtained over the concentration range 0.1–2.0 μg/mL for ergone in rat plasma. A typical calibration plot equation was $y = 1.1516x - 0.0015$ with a correlation coefficient of 0.9997, where y is the mean peak area ratio of the analyte to the IS and x is the concentration of the analyte. The limit of detection (LOD) for ergone was found to be 0.04 μg/mL based on a signal-to-noise ratio (S/N) ratio of 3:1 and the LLOQ was 0.1 μg/mL with acceptable precision and accuracy using 50 μL plasma sample.

The results of precision and accuracy are presented in Table 1. All the values of precision and accuracy including LLOQ were within the specified ranges and therefore acceptable.

Extraction Recovery

The results showed that extraction recoveries of ergone from rat plasma were 95.8 ± 4.9, 96.2 ± 3.4, and 96.9 ± 2.9% at concentrations levels of 0.25, 1.0 and 1.5 μg/mL, respectively. The mean recovery was 96.3 ± 3.9%. Recovery of the IS was 94.8 ± 4.7% at the concentration used (1 μg/mL).

Table 2. Stability of ergone in rat plasma at three QC levels ($n = 5$)

Experimental conditions	Parameter	QC concentration (μg/mL)		
		0.25	1.0	1.5
Short-term stability	Calculated concentration (μg/mL)	0.239	1.05	1.51
	RSD (%)	4.1	2.9	2.1
Long-term stability	Calculated concentration (μg/mL)	0.241	1.02	1.49
	RSD (%)	6.8	2.8	3.1
Freeze–thaw stability	Calculated concentration (μg/mL)	0.245	1.07	1.5
	RSD (%)	3.5	3.6	4.7

Table 1. Precision and accuracy for the determination of ergone at three concentration levels in rat plasma (intra-day, $n = 6$; inter-day, $n = 6$ series per day, 3 days)

Nominal concentration (μg/mL)	Calculated concentration (μg/mL)	Accuracy (%)	Precision, RSD (%)
Intra-day			
0.25	0.239	95.6	8.5
1.0	1.02	102	2.0
1.5	1.47	98.0	2.0
Inter-day			
0.25	0.241	96.4	5.9
1.0	1.04	104	4.0
1.5	1.48	98.6	1.3

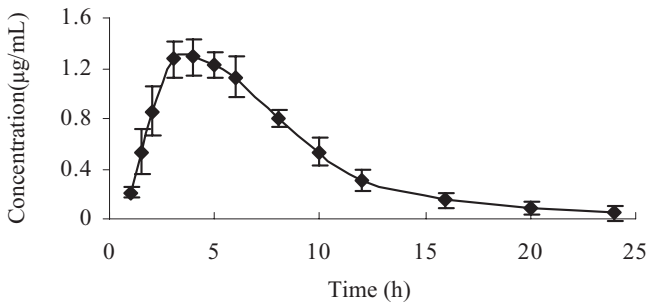


Figure 3. Mean (± SD) plasma concentration–time profile of ergone in the plasma of healthy rats ($n = 6$), that were administered a single oral dose of 20 mg/kg of ergone.

Stability

Table 2 summarizes the results of short-term, long-term, and freeze–thaw stability of ergone in plasma. All the results well met the criterion for stability measurements.

Application to Pharmacokinetic Study

After administration, the plasma concentrations of ergone were determined by the described method. The ergone plasma concentration–time data were analyzed. The concentration–time curve is shown in Fig. 3. The pharmacokinetic parameters are summarized in Table 3. Our results indicate that the maximum plasma concentration was 1.36 μg/mL at a concentration peak time of 3.52 h (Fig. 3 and Table 3). Yuan *et al.* (2004) determined the content of ergone in bile, which is mainly in feces via bile. The results demonstrate that ergone is absorbed by the oral route.

Table 3. Pharmacokinetic characteristics of ergone in the plasma of healthy rats ($n = 6$) that were administered a single oral dose of 20 mg of ergone per kg of body weight

Ergone pharmacokinetic parameter values	
AUC_{0-24h} (μg h/mL)	13.5
C_{max} (μg/mL)	1.36
$t_{1/2}$ (h)	3.43
T_{max} (h)	3.52

Conclusions

In the present study, the objective proposed in the introduction was achieved. A simple and specific HPLC method with dual wavelength UV detection has been developed for quantitative determination of ergone in rat plasma. The method was comprehensively validated over a concentration range of 0.1–2.0 μg/mL ($r > 0.9997$) and it offered good accuracy and precision. A simple sample pretreatment with protein precipitation was applied compared with multiple extraction and concentration steps of the previously published methods. The pharmacokinetic results are useful for potential clinical applications of ergone.

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