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A fast and sensitive HPLC–MS/MS analysis and preliminary pharmacokinetic characterization of ergone in rats

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ABSTRACT

A fast and sensitive HPLC–APCI-MS/MS method was developed for the determination of ergosta-4,6,8(14),22-tetraen-3-one (ergone) in rat plasma. The plasma sample containing ergone and ergosterol (internal standard) were simply treated with acetone to precipitate and remove proteins and the isolated supernatants were directly injected into the HPLC–APCI-MS/MS system. Chromatographic separation was performed on a 1.8 μm Zorbax SB-C18 column (100 mm × 3.0 mm) with a 97:3 (v/v) mixed solution of methanol and 0.1% aqueous formic acid being used as mobile phase. Quantification was performed by multiple selected reactions monitoring (MRM) of the transitions with $(m/z)^+$ 393–268 for ergone and $(m/z)^+$ 379–69 for the IS. The method was validated in the concentration range of 5–1600 ng/mL for ergone. The precision of the assay (RSD%) was less than 10.5% at all concentrations levels within the tested range and adequate accuracy, and the limit of detection was 1.5 ng/mL. The absolute recoveries of both ergone and ergosterol from the plasma were more than 95%. The developed method has been successfully applied to the pharmacokinetic study of the drug in SD rats.

1. Introduction

Ergosta-4,6,8(14),22-tetraen-3-one (ergone) is one of the best known bioactive steroids, which exists widely in medicinal fungi, lichen and plant such as *Polyporus umbellatus* [1], *Russula cyanoxantha* [2], *Cordyceps sinensis* [3], *Vietnamese xylaria* [4] and *Zopfiella longicaudata* [5]. Ergone, one of the major steroids isolated from the sclerotia of *P. umbellatus*, has been reported to possess diuretic activity [6], anticancer activity [1], inhibitory activity of nitric oxide production [4], and immunosuppressive activity [5]. We have recently reported the diuretic components of *P. umbellatus* by bioactivity-guided method, our study also shows that ergone has diuretic activity [7], which is consistent with the previous report [6].

The pharmacological studies demonstrated that ergone possesses various pharmacological effects and there is an urgent need to study its pharmacokinetics in vivo. Earlier publications have reported methods for analysis of ergone in *P. umbellatus* using HPLC–UV, HPLC-fluorescence detection and HPLC–MS [8–12] and in biological samples using HPLC–UV [6]. The HPLC–UV approach for measuring of ergone in rat plasma has also been validated in our laboratory. However, this method was not sensitive enough for pharmacokinetic studies of ergone. It is an arduous and hard process to choose a suitable internal standard with good peak shape, proper retention time, identical excitation and emission wavelength for HPLC-FLD. To the best of our knowledge, there was no entirely validated HPLC–MS/MS method reported in the literature for quantification of ergone in biological samples, consequently the aim of this study is to develop a fast and sensitive HPLC–APCI-MS/MS method for determination of ergone in plasma samples. The advantages of present method consist in simple sample pretreatment procedure using inexpensive chemicals, and short run time. This method has been comprehensively validated, offering the advantage of simplicity with adequate sensitivity, selectivity, and precision to determine ergone in plasma samples for the first time. The assay was also successfully applied in the pharmacokinetic studies of ergone in male SD rats.

2. Experimental

2.1. Chemicals and reagents

The standard of ergone (Fig. 1A) was isolated by the authors from *P. umbellatus*. The procedure isolation and purification of ergone

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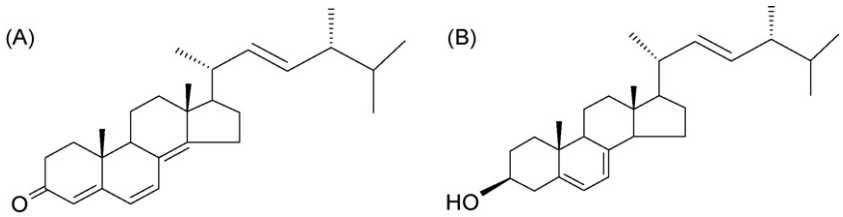

Fig. 1. Chemical structures of ergone (A) and ergosterol (B).

was published in our previous paper [7]. Its structure was characterized by chemical and spectroscopic methods (^{1}H NMR, ^{13}C NMR and MS) and compared with literatures [1]. Ergosterol (Fig. 1B) was used as internal standard (IS) which was one of the main components of *P. umbellatus* purified in our laboratory with 99% purity as determined by HPLC. HPLC-grade methanol was purchased from Baker Company (Baker Inc., USA). Ultrapure water was prepared by a Millipore-Q SAS 67120 MOLSHEIM (France). Other chemicals were of analytical grade.

2.2. Instrumentation and analytical conditions

Analysis was performed with an Agilent 1100 series LC system equipped with an Agilent 6410 triple quadrupole mass spectrometer, a quaternary pump, an online degasser, an auto plate-sampler, and a thermostatically controlled column apartment. All the operations, acquisition and analysis of data were controlled by Agilent Masshunter B0104. Chromatographic separations were performed with a 1.8 μm Zorbax SB-C18 column (100 mm × 3.0 mm) maintained at 40.0 °C. The mobile phase was a 97:3 (v/v) mixed solution of methanol and 0.1% aqueous formic acid at the flow rate of 0.5 mL/min. Aliquots of 20 μL were injected into HPLC system for analysis. The system pressure was 200 bar.

An Agilent 6410 triple quadrupole mass spectrometer interfaced to an atmospheric pressure chemical ionization (APCI) source was used for mass analysis and detection. The mass spectrometer was operated in the positive ion detection mode. The current of capillary and chamber of APCI source was set at 84 nA and 3.8 μA, respectively. The temperature was maintained at 325, 100 and 100 °C for gas temperature, MS1 heater and MS2 heater, respectively. The gas flow rate for desolvation was set at 4.0 L/min. Quantification was performed with multiple selected reactions monitoring (MRM) of the transitions with $(m/z)^+$ 393–268 for ergone and $(m/z)^+$ 379–69 for the IS with a scan time of 0.2 s per transition.

2.3. Pretreatment of plasma sample

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 (20 μg/mL) 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 × *g* for 10 min at a low temperature (4 °C). At last, 20 μL of the supernatant was injected into the HPLC for analysis.

2.4. Method validation

2.4.1. Selectivity

Selectivity was investigated by comparing chromatograms of blank plasma obtained from six rats with those of corresponding standard plasma sample spiked with ergone and IS and plasma sample after an oral dose. All six blank plasma samples were extracted to ensure the absence of interfering peaks.

2.4.2. Linearity, lower limit of quantification and preparation of quality control (QC) samples

The stock (125 μg/mL) and working solutions (200 and 2000 ng/mL) of ergone were prepared by dissolving an accurately weighed quantity of ergone in acetone and serial dilution with the same solvent. The stock (125 μg/mL) and working solution (20 μg/mL) of ergosterol were prepared in the same way.

Calibration standards containing 5, 20, 80, 100, 200, 400, 800 and 1600 ng/mL of ergone, and quality control samples containing 5 (low), 400 (medium) and 1600 ng/mL (high) of ergone were prepared with blank rat plasma. To each of 50 μL plasma standards and quality samples, 20 μL of IS working solution prepared in acetone was added. The mixture was then vortex mixed for 2 min and centrifuged at 10,000 × *g* for 10 min. A 20 μL portion of the supernatant was subjected to HPLC–APCI-MS/MS analysis.

The limit of detection (LOD) was defined at a signal-to-noise (S/N) ratio of 3:1; the lower limit of quantification (LLOQ) was defined the lowest quantifiable concentration of analyte with accuracy within 20% and a precision <20%.

2.4.3. Precision and accuracy

The intra-day and inter-day accuracy and precision were evaluated by replicative analysis of five sets of samples spiked with QC samples at three concentration levels of ergone (5, 400, and 1600 ng/mL) within a day or during three consecutive days. The accuracy was expressed as bias, obtained by calculating the percentage of difference between the measured and spiked concentration over that of the spiked value, whereas the precision was denoted by using the relative standard deviation (RSD%).

2.4.4. Extraction recovery

The absolute recovery of the extraction was determined by comparing the peak area obtained from the plasma sample with peak areas obtained by the direct injection of pure ergone standard solutions in the mobile phase at three different concentration levels. The quantification of the chromatogram was performed by using peak area ratios of ergone to IS.

2.4.5. Stability

The stability of ergone and IS stock solutions was evaluated after storage at room temperature and at 4 °C for 30 days. Short-term stability was assessed by analyzing QC plasma samples kept at room temperature for 6 h that exceeded the routine preparation time of samples. Long-term stability was determined by assaying QC plasma samples (5, 400 and 1600 ng/mL) after storage at −20 °C for 30 days. Freeze–thaw stability was investigated after three freeze (−20 °C)–thaw (room temperature) cycles.

2.5. Application to pharmacokinetics of ergone

To assess the applicability of the present work, this method was used to investigate the pharmacokinetics of ergone in rats after oral administration of ergone at a dose of 20 mg/kg. All proce-

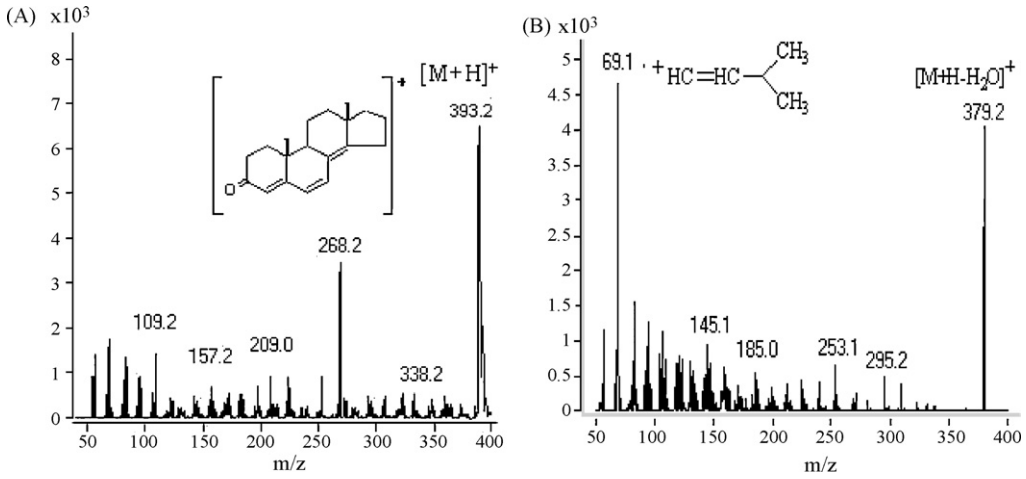

Fig. 2. Positive APCI mass spectra of ergone (A) and IS (B).

dures involving animals were in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of People's Republic 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 starting 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 carotid vein at control (0 min) and 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10 and 12 h after the 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 mean ± S.D. and the mean concentration–time curve was plotted. The pharmacokinetic parameters for ergone were evaluated by analyzing the data of plasma concentration–time profiles, which was calculated by the pharmacokinetic software, DAS 2.0 (issued by the State Food and Drug Administration of China for pharmacokinetic study) with two-compartment analysis. The following two-compartmental pharmacokinetic parameters were calculated based on the moment method: half-life ($t_{1/2}$), clearance (CLz/F, L/h/kg), volume of distribution (Vz/F, L/kg), and area under the concentration–time curve (AUC).

3. Results and discussion

3.1. Isolation and purification of ergone

The purity of ergone was above 99% as determined by HPLC in our previous paper [9]. The characteristics of ergone are: light yellow needles from light petroleum, mp 113–115 °C; ^{1}H NMR ($CDCl_3$, 500 MHz) 5.73 (1H, s, H-4), 6.03 (1H, d, *J* = 9.5 Hz, H-6), 6.61 (1H, d, *J* = 9.5 Hz, H-7), 0.96 (3H, s, H-18), 1.00 (3H, s, H-19), 1.06 (3H, d, *J* = 6.7 Hz, H-21), 5.20 (1H, dd, *J* = 15.3, 7.3 Hz, H-22), 5.46 (1H, dd, *J* = 15.3, 7.3 Hz, H-23), 0.83 (3H, d, *J* = 6.7 Hz, H-26), 0.85 (3H, d, *J* = 7.0 Hz, H-27), 0.93 (3H, d, *J* = 7.0 Hz, H-28); ^{13}C NMR ($CDCl_3$, 125 MHz) 34.3 (C-1), 34.4 (C-2), 199.4 (C-3), 123.3 (C-4), 164.4 (C-5), 124.7 (C-6), 134.1 (C-7), 124.7 (C-8), 44.7 (C-9), 37.0 (C-10), 19.2 (C-11), 35.9 (C-12), 44.2 (C-13), 156.1 (C-14), 25.6 (C-15), 27.8 (C-16), 56.0 (C-17), 19.2 (C-18), 16.8 (C-19), 39.4 (C-20), 21.4 (C-21), 135.2 (C-22), 132.8 (C-23), 43.1 (C-24), 33.3 (C-25), 19.8 (C-26), 20.1 (C-27), 17.8 (C-28). These data were consistent with the previous report [1]. The data of mass spectrum was published in our previous paper [12].

3.2. Optimization of the chromatographic conditions

To develop an accurate, valid and optimal chromatographic condition, the different HPLC parameters including mobile phase (methanol–0.1% formic acid aqueous solution, methanol–water or acetonitrile–0.1% formic acid aqueous solution), category of column (Agilent Zorbax SB-C18 column, 100 mm × 3.0 mm, 1.8 μm, Agilent Zorbax SB-C18 column, 150 mm × 2.1 mm, 5 μm, Agilent Zorbax Eclipse XDB-C18 column, 150 mm × 2.1 mm, 5 μm, or Waters symmetry C18 column, 50 mm × 2.1 mm, 5 μm), column temperature (30, 40, 45 or 50 °C) and flow rate of mobile phase (0.4, 0.5 or 0.6 mL/min) were all examined and compared. Finally, plasma samples were separated by HPLC on a 1.8 μm Zorbax SB-C18 column (100 mm × 3.0 mm) using a solvent system consisting of methanol and 0.1% formic acid aqueous solution at 40 °C. The system provides higher resolution, greater baseline stability and higher ionization efficiency.

Ergosterol was chosen as IS for its similarity with the analytes in structure (Fig. 1B) and chromatographic behavior (Figs. 2 and 3).

Fig. 3 shows a rat plasma sample 2 h after oral administration of ergone at a dose of 20 mg/kg. The ergone and IS of the retention times were 6.3 and 5.2 min, respectively, with a total

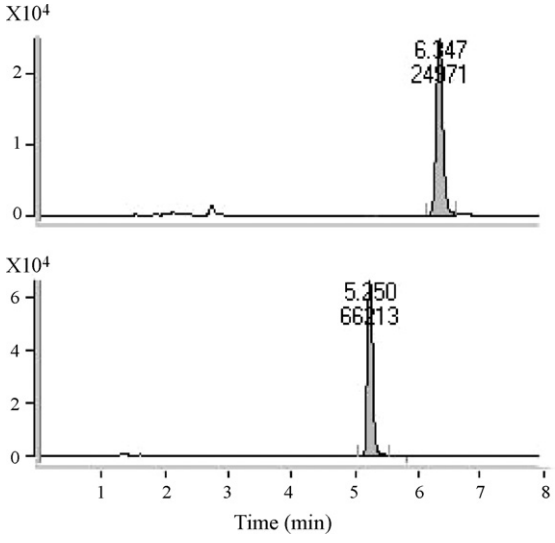

Fig. 3. Representative chromatogram of plasma sample from a rat 2 h after administration of 20 mg/kg of ergone, the concentration of ergone was found to be 932 ng/mL.

Table 1
The recovery, precision and accuracy ($n = 5$) of the assay method.

Concentration (ng/mL)	Absolute recovery		Intra-day		Inter-day	
	Mean (%)	RSD (%)	Precision (RSD%)	Accuracy (bias%)	Precision (RSD%)	Accuracy (bias%)
5	96.4	4.8	5.9	4.6	10.5	−8.3
400	97.1	2.3	7.6	7.2	8.7	−5.6
1600	95.8	3.6	3.7	6.1	9.4	4.5

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

Experimental conditions	Parameter	QC concentration (ng/mL)		
		5	400	1600
Short-term stability	Calculated concentration (ng/mL)	4.8	385	1550
	RSD (%)	2.9	3.5	3.0
Long-term stability	Calculated concentration (ng/mL)	5.2	415	1640
	RSD (%)	6.5	3.2	2.8
Freeze–thraw stability	Calculated concentration (ng/mL)	4.7	380	1546
	RSD (%)	4.2	2.9	3.2

run time of less than 8 min. System suitability parameters for the method were as follows: theoretical plates for ergone and IS were 5400 and 4300, respectively. Tailing factor was less than 1.1 for both ergone and IS and resolution between ergone and IS was 1.5.

3.3. Method validation

3.3.1. Selectivity

Assay selectivity was evaluated by analyzing blank plasma samples obtained from six rats. All samples were found to be free of interferences with the ergone and IS. The mass spectra of the ergone and IS are shown in Fig. 2. $(m/z)^+$ 393–268 for ergone and $(m/z)^+$ 379–69 for the IS were chosen for quantification due to their high stability and intensity. Representative MRM chromatograms are shown in Fig. 3.

3.3.2. Linearity, precision, accuracy and recovery

The standard curves showed good linearity over the concentration range 5–1600 ng/mL for ergone in rat plasma. A $1/x$ weighted least-square linear regression equation of $Y = 0.0029X - 0.0189$ was observed with a correlation coefficient of 0.9998. Under the optimized conditions, the limit of detection of (S/N = 3) and the limit of quantification (S/N = 10) observed to be 1.5 and 5 ng/mL, respectively.

The intra-day and inter-day precision (RSD%, $n = 5$) for the ergone spiked control samples at 5, 400, and 1600 ng/mL levels varied between 3.7 and 10.5. The corresponding intra-day and inter-day accuracy (bias%, $n = 5$) ranged between −8.3 and 7.2 (Table 1).

The absolute recoveries of both ergone and ergosterol from the plasma were more than 95%, indicating that most of ergone in the plasma sample was extracted with no obvious interferences in the chromatogram.

3.3.3. Stability of ergone in plasma

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.

3.4. Results of pharmacokinetic study

After oral administration of ergone at a dose of 20 mg/kg, plasma concentration of ergone was determined by the described HPLC–APCI-MS/MS. The plasma concentration–time profiles are represented in Fig. 4. Pharmacokinetic parameters are listed in Table 3. Our results indicate that the maximum plasma concentration was 1.29 μg/mL at a concentration peak time of 3.81 h (Fig. 4) (Table 3). Yuan et al. determined the content of ergone in bile. The results demonstrate that ergone is absorbed by oral route [6]. Ergone possible undergoes hepatic uptake and biliary excretion of enteropepatic circulation, which is mainly in feces via bile.

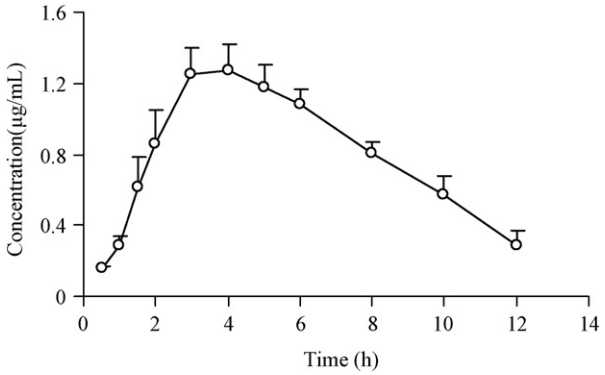

Fig. 4. Mean (±S.D.) plasma concentration–time profile of ergone in the plasma of healthy rats ($n = 6$), which were administered a single oral dose of 20 mg/kg.

Table 3
The pharmacokinetic parameters (mean ± S.D.) of ergone in rats following oral administration at dose of 20 mg/kg ($n = 6$).

Parameters	Mean ± S.D.
C_{Max} (μg/mL)	1.29 ± 0.15
T_{Max} (h)	3.81 ± 0.72
$t_{1/2}$ (h)	3.19 ± 0.92
AUC_{0-t} (μg h/mL)	9.52 ± 0.68
$AUC_{0-\infty}$ (μg h/mL)	11.3 ± 1.1
CLz/F (L/h/kg)	1.79 ± 0.18
Vz/F (L/kg)	8.73 ± 1.79

4. Conclusion

We have demonstrated that ergone presented in plasma samples at low ppb levels can be reliably assayed with coupled HPLC–APCI-MS/MS system by using a direct sample injection

technique after the plasma protein was simply removed by acetone precipitation. The quantification of ergone has been validated in the concentration range from 5 to 1600 ng/mL, which displayed a highly reliable precision and accuracy; no signal interferences from endogenous compounds have been observed. This method has been successfully applied to the pharmacokinetics study of ergone in SD rats. The further development of this method would facilitate the adaptability of ergone assay in other biological samples such as urine, feces and so on.

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