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Pharmacokinetics of ergosterol in rats using rapid resolution liquid chromatography–atmospheric pressure chemical ionization multi-stage tandem mass spectrometry and rapid resolution liquid chromatography/tandem mass spectrometry

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ABSTRACT

Rapid resolution liquid chromatography/tandem multi-stage mass spectrometry (RRLC–MS^n) and rapid resolution liquid chromatography/tandem mass spectrometry (RRLC/MS/MS) methods were developed for the identification and quantification of ergosterol and its metabolites from rat plasma, urine and faeces. Two metabolites (ERG1 and ERG2) were identified by RRLC/MS^n. The concentrations of the ergosterol were determined by RRLC/MS/MS. The separation was performed on an Agilent Zorbax SB-C18 with the mobile phase consisting of methanol and water (containing 0.1% formic acid). The detection was carried out by means of atmospheric pressure chemical ionization mass spectrometry in positive ion mode with multiple reaction monitoring (MRM). Linear calibration curves were obtained in the concentration range of 7–2000, 6–2000 and 8–7500 ng/mL for plasma, urine and faecal homogenate, respectively. The intra- and inter-day precision values (RSD) were below 10%. The method was applied to the pharmacokinetic properties and elimination pathway of ergosterol in rats.

1. Introduction

Ergosterol is best-known as a steroid, found widely in many medicinal fungi such as *Polyporus umbellatus*, *Cordyceps sinensis* and *Hypsizigus marmoreus* [1–3]. The ergosterol from *Polyporus umbellatus* has recently reported diuretic activity in addition to possess cytotoxic activity and anti-inflammatory activity [3–5]. Despite these multiple biological activities, the pharmacokinetic and biochemical aspects of this compound remain unclear. The final effect of the drug *in vivo* might be influenced by many factors, such as body and/or cell-compartment distribution, drug metabolism, lipophilicity, membrane permeability and protein binding. These multiple biological activities of ergosterol need further investigation on pharmacokinetic properties and elimination pathway of ergosterol.

A number of methods have been reported for the quantification of ergosterol in raw materials (*Polyporus umbellatus*, *Brassica napus*, *Cordyceps sinensis*), such as HPLC–UV [1,6–11], HPLC–MS/MS [12] and GC–MS [13]. Current literature shows limited information describing the quantification of ergosterol in biological samples for rat or human plasma, urine and faeces. In addition, the effects on the elimination pathway of ergosterol have not been previously examined. Preclinical research including metabolism and pharmacokinetics of herbal medicine components are of great importance in understanding their biological effects and safety [14,15]. In order to investigate the pharmacokinetic properties and elimination pathway of ergosterol, it is essential to establish a fast and sensitive analytical method that can be used to accurately measure low levels of ergosterol in small volumes of plasma, urine and faeces. The use of UV/vis detection for ergosterol in rat plasma may not be sensitive enough for determination of small tracer amount samples. This method is mainly designed for human biological samples or raw materials with a larger volume, typically 0.5–1.0 mL human plasma [1]. In using a rat model, it is impossible to get a desired amount of biological samples from one animal to perform a pharmacokinetic study, since only one or two blood samples can be taken from each animal for per measurement. Though GC–MS provides high sensitivity required for the bio-analysis of ergosterol, the compound requires to be derivatized before GC separation [13]. In

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addition, thermal decomposition of the compound under GC conditions may create analytical problem. Further reports for a HPLC procedure of ergosterol analysis is based on MS–MS detection using tandem mass spectrometry [12].

Since biological samples usually occur as a complex mixture, long analysis time by HPLC is necessary for complete separation. To accelerate the analysis process, there has been substantial focus on high-speed chromatographic separations. Recently, new technology of rapid resolution liquid chromatography (RRLC) shows promising developments in the area of fast chromatographic separations. RRLC allows satisfactory separation, good resolution and sensitivity, and high-speed detection with complex biological samples [16–20].

LC coupled with ion trap multi-stage MS is becoming a useful technique for drug metabolites detection and identification. LC–MS can effectively produce full-scan mass spectra while still offer high sensitivity and the MS^n capability provides additional information for structural elucidation of the metabolites. Triple quadrupole mass spectrometer method is widely used in quantitative analysis because of its great sensitivity and good selectivity. The objective for this project was to develop (1) rapid resolution liquid chromatography with diode array detection and atmospheric pressure chemical ionization tandem multi-stage mass spectrometry (RRLC–DAD–APCI–MS^n) method to identify ergosterol and its metabolite; (2) rapid resolution liquid chromatography with atmospheric pressure chemical ionization tandem triple quadrupole mass spectrometry (RRLC–APCI–MS/MS) method to quantify ergosterol in the large numbers of low-volume biological matrices (plasma, faeces and urine) generated in pharmacokinetic studies. The total run time of the method per sample was just 3.0 min which is shorter than previously reported [12]. This method was applied to determine the feasibility for preclinical pharmacokinetic studies in the Sprague–Dawley (SD) rat.

2. Experimental

2.1. Chemicals and reagents

The ergosterol standard (Fig. 1A) was isolated and purified by the authors from *P. umbellatus* using the method published previous [21]. The structure was characterized by chemical and spectroscopic methods (^{1}H NMR, ^{13}C NMR and MS) and compared with literatures [22]. Ergosta-4,6,8(14),22-tetraen-3-one (ergone, Fig. 1B) was used as internal standard (IS) which was one of the 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).

2.2. Preparation of standards and quality control samples

Primary standard stock solutions of ergosterol (1 μg/mL) and ergone (0.1 μg/mL) were prepared in acetone. Working solutions of ergosterol were prepared using amber glass flasks to avoid photo degradation by appropriate dilution with acetone just before use. The IS working solutions were prepared in the same manner. All solutions were stored in darkness at 4 °C.

Faecal samples were homogenized with acetone 1:10 (g:vol = faeces: acetone) to obtain faecal homogenate. For RRLC determination, the samples for standard calibration curves were prepared by spiking the blank rat plasma, urine and faecal homogenate with 10 μL of the appropriate working solutions to yield the following concentrations: 7, 20, 80, 200, 800, 1600 and 2000 ng/mL for plasma, 6, 20, 80, 200, 800, 1600 and 2000 ng/mL for urine and 8, 20, 200, 800, 2000, 6000 and 7500 ng/mL for faecal homogenate. The quality control (QC) samples were separately prepared in a similar manner as those used for calibration curve. Concentrations of 20, 800 and 1600 ng/mL were used for plasma and urine quality control samples whereas concentrations of 20, 2000 and 6000 ng/mL were used for faecal homogenate corresponding to the low QC, medium QC and high QC, respectively.

2.3. RRLC/MS/MS analysis

The chromatographic separation was performed on an Agilent 1200 Series Rapid Resolution LC system (Agilent Technologies, USA), equipped with a microvacuum degasser, a binary pump, a high performance autosampler, a column compartment, a diode array detector and a quadrupole mass spectrometer. The sample was separated on an Agilent Zorbax SB-C18 (50 mm × 2.1 mm, 1.8 μm) fitted with a C18 guard column (Agilent). An isocratic mobile phase consisted of methanol and water (containing 0.1% formic acid) (98:2, v/v) at a flow rate of 0.4 mL min^{-1}. The column temperature was maintained at 40 °C, and the injection volume was set at 10 μL.

Agilent Technologies 6410 Triple Quad LC/MS equipped with atmospheric pressure chemical ionization (APCI) source was analyzed by Agilent MassHunter B0104. 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^{-1}. Multiple reaction monitoring (MRM) was employed for data acquisition. The optimized MRM fragmentation transitions were m/z 379 → 69 with a fragmenter voltage of 115 V and a collision energy CE of 25 V for ergosterol, and m/z 393 → 69 with a fragmenter voltage of 135 V and a collision energy of 35 V for IS. The dwell time for each transition was 250 ms and 150 ms for ergosterol and IS, respectively.

2.4. Identification of ergosterol metabolite by RRLC–DAD–APCI–MS^n

Qualitative analysis of ergosterol metabolites (ERG1, ergosterol-26-O-β-D-glucopyranoside, Fig. 1C and ERG2, (22*E*,24*R*)-ergosta-5,7-dien-3β-ol-23-O-β-D-glucopyranoside, Fig. 1D) from rat plasma was carried out on Agilent G6320 series LC/MSD Trap Mass Spectrometer system. The chromatographic separation was performed an Agilent 1200 Series Rapid Resolution LC system (Agilent Technologies, USA), equipped with a binary pump, a microvacuum degasser, a high performance autosampler, a column compartment, a diode array detector and a MS detector. The samples were separated on an Agilent Zorbax SB-C18 (50 mm × 2.1 mm, 1.8 μm) with an isocratic mobile phase consisted of 0.1% formic acid solution (A) and methanol (B) at a flow of 0.4 mL min^{-1}. DAD spectra were acquired over a scan range of 190–400 nm. The sample volume injected was 20 μL.

Mass spectrometry experiments were performed with APCI source in positive ion mode. The vaporizer temperature was maintained at 300 °C. The temperature of the drying gas was set at 350 °C. The flow rate of the drying gas and the pressure of the nebulizing gas were set at 6 L min^{-1} and 60 psi, respectively. The capillary voltage was kept at 3.5 × 10^{10} V. The corona current was 4000 nA in APCI–MS^n measurements. Full-scan spectra were acquired over a scan range of m/z 50–600. Optimization of the APCI–MS^n parameters and the qualitative analysis of two metabolites were carried out by using the selected ion monitoring (SIM) mode of the base ion peak at (m/z) 575.6 for ERG1 and (m/z) 577.6 for ERG2. Agilent ChemStation was used to control and process the data the Agilent 6320 Series Ion Trap LC–MS.

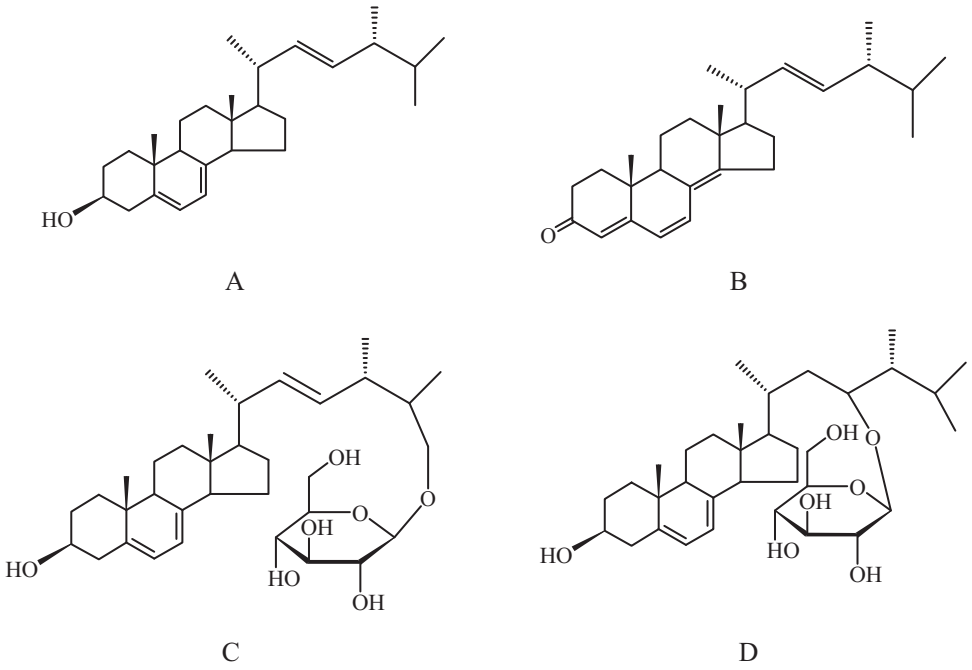

Fig. 1. Chemical structures of ergosterol (A), ergone (IS, B), its metabolites, ERG1 (ergosterol-26-O-β-D-glucopyranoside, (C)) and ERG2 ((22*E*,24*R*)-ergosta-5,7-dien-3β-ol-23-O-β-D-glucopyranoside, (D)) from plasma.

2.5. Extraction procedures for plasma, urine and faeces

2.5.1. Plasma sample

A 50 μL aliquot of plasma standard or sample was transferred to a 1.5 mL centrifuge tube, and then 80 μL of IS working solution (0.1 μg/mL) was spiked and vortex mixed for 1 min. Next 170 μL aliquot of 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 last, 10 μL of the supernatant was injected into the RRLC for analysis.

2.5.2. Urine sample

A 200 μL of urine standard or sample was transferred to a 1.5 mL centrifuge tube, and then 320 μL of IS working solution (0.1 μg/mL) was spiked and vortex mixed for 1 min. Next 480 μL aliquot of 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 last, 10 μL of the supernatant was injected into the RRLC for analysis.

2.5.3. Faeces sample

A 10 μL of faecal homogenate standard or sample was transferred to a 1.5 mL centrifuge tube, and then 40 μL of IS working solution was spiked and vortex mixed for 1 min. 910 μL of acetone was added to the solution and vortex again for 2 min. The mixture was centrifuged at 10,000 × *g* for 10 min. A 10 μL of supernatant of the solution was injected into the RRLC for analysis.

2.6. Assay validation

The method was validated for selectivity, linearity, precision, accuracy, extraction recovery and stability according to the FDA guideline for validation of bioanalytical methods [23].

2.6.1. Selectivity

Selectivity was studied by comparing chromatograms of five different batches of blank plasma, urine and faecal homogenate obtained from five rats with those of corresponding standard plasma, urine and faecal homogenate samples spiked with ergosterol and IS and plasma sample after oral doses of ergosterol.

2.6.2. Linearity and lower limit of quantification (LLOQ)

Calibration curves were prepared by assaying standard plasma, urine and faecal homogenate samples at seven concentrations of ergosterol ranging 7–2000, 6–2000 and 8–7500 ng/mL, respectively. The linearity of each calibration curve was determined by plotting the peak area ratio (*Y*) of ergosterol to IS versus the nominal concentration (*X*) of ergosterol. The calibration curves were constructed by weighted (1/*x*) least square linear regression. The lowest standard on the calibration curve should be accepted as LLOQ if the following two conditions are met: the analyte response at the LLOQ should be at least 5 times the response compared to blank response, and the analyte peak (response) should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80–120%.

2.6.3. Precision and accuracy

Intra-day accuracy and precision evaluations were performed by repeated analysis of ergosterol in rat plasma, urine and faecal homogenate on the same day. The run consisted of five replicates of each low, medium and high QC samples. Inter-day accuracy and precision were assessed by analysis of samples consisting of five replicates of three concentrations samples for ergosterol on three consecutive days.

Accuracy was calculated as the percentage of the concentration of drug measured from calibration curve to the theoretical concentration value of drug added to the blank plasma, urine and faecal homogenate. Precision was expressed as the relative standard deviation, RSD (%), of measured concentrations for each QC samples. The values within ±15% for accuracy and precision were considered acceptable.

2.6.4. Recovery and matrix effect

Recovery of ergosterol was evaluated at three concentrations levels of QC samples (20, 800 and 1600 ng/mL for plasma and urine, 20, 2000 and 6000 ng/mL for faecal homogenate) on the same

day. Recovery data were determined by comparing the peak area ($n = 6$ at each concentration) obtained from plasma, urine and faecal homogenate samples spiked with analyte before extraction with those from the corresponding standards diluted with acetone. The matrix effect was determined by the ratio of the amounts of ergosterol dissolved with blank matrix extract against those dissolved with acetone. The procedure was repeated three times.

2.6.5. Stability

To ensure the reliability of the results in relation to handling and storing of biological samples and stock standard solutions, stability studies were carried out at three different concentration levels (low, medium, high). The stability of ergosterol and IS stock solutions were evaluated after storage at room temperature and at 4 °C for 30 days. The stability of ergosterol and IS working solutions were investigated at room temperature for 24 h. QC plasma, urine and faecal homogenate samples of three concentration levels were subjected to the following conditions. Short-term stability was assessed by analyzing QC plasma, urine and faecal homogenate samples kept at room temperature for 6 h. This time was chosen since it exceeded the routine preparation time of samples. Long-term stability was determined by assaying QC plasma, urine and faecal homogenate samples after storage at −20 °C for 30 days. Freeze–thaw stability was investigated after three freeze (−20 °C)–thaw (room temperature) cycles.

2.7. Application to pharmacokinetics of ergosterol

To validate the method, a trial with real samples was undertaken to determine ergosterol in the plasma and excretion samples of healthy rats, administered a single oral dose of ergosterol. The study was conducted in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of People's Republic of China. All procedures and the care of the rats were in accordance with institutional guidelines for animal use in research. Rats were housed in individual metabolic cages on standard laboratory in a temperature controlled room (22 ± 2 °C) with a 12 h light/dark cycle.

Male SD rats, 200 ± 10 g, fasted overnight with free access to water for at least 12 h, were dosed orally by gavages with 100 mg/kg body weight of ergosterol dissolved in plant oil as a vehicle. Rats were divided into three groups ($n = 6$) based on the time of blood sampling with two animals each. The control groups received the vehicle only. The blood samples were collected from retro-orbital puncture with ether and transferred into heparinized tubes at control and 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 24 and 36 h after oral administration. Samples were immediately centrifuged at 5000 × *g* for 10 min and the plasma was frozen at −20 °C and stored until analysis. The estimation of ergosterol in all the samples was undertaken within 36 h of blood collection by the method described above.

Excretion was studied in another four groups ($n = 6$) based on the time of urine and faeces sampling with three animals each. The control group received the vehicle only. Rats were housed with food and water *ad libitum* in individual metabolic cages, except for the final 12 h before a single oral administration of 100 mg/kg of ergosterol. Faeces and urine were collected after administration different periods (0–2, 2–4, 4–6, 6–8, 8–10, 10–12, 12–14, 14–16, 16–18, 18–24 and 24–36 h). The amount of faeces and urine collected over each period was recorded, respectively, and then urine and faeces was stored at −20 °C until analysis.

Pharmacokinetics analysis was carried out by noncompartmental method with the aid of the software DAS 2.0 (the State Food and Drug Administration of China for pharmacokinetic study) and pharmaceutical parameters were obtained.

The percentage of ergosterol eliminated in faeces was calculated with the accumulated ergosterol eliminated in all periods divided by administration amount (ergosterol eliminated over each period was calculated using the amount of faeces multiplied by the concentration of ergosterol in it).

3. Results and discussion

3.1. MRM transition optimization

The optimum collision voltages were defined as generating the highest signal for the main fragment ion. The results of the optimized conditions for each MRM transition in the RRLC/MS/MS are summarized as follows. First, optimization for performance and sensitivity was conducted using the fragmenter voltage selected to produce the highest signal intensity for the precursor ion. The collision energy was then adjusted to produce the highest intensity for the main fragment. The fragmenter voltage flow injections of ergosterol and IS standard solution were performed by varying the fragmentor voltages from 75 to 155 V in steps of 10 V. From the total ion chromatogram, the corresponding $[M+H-H_2O]^+$ for ergosterol and $[M+H]^+$ for IS were used to obtain the extracted ion chromatogram for the different fragmenter voltages; the optimum fragmenter voltages were then selected. With the optimized fragmenter voltage, an additional flow injection experiment was performed to optimize collision energy. Each time segment of the analysis was performed with increasing collision energy in steps of 5 V between 10 and 50 V. To improve detection sensitivity, the acquisition time was also divided into 0.5–2.5 min and 2.5–3.0 min for m/z 379 → 69 and m/z 393 → 69, respectively.

The positive ionization mode was selected for the quantification of ergosterol by the presence of a hydroxyl group. APCI was chosen for better intensity response to the analyte than did electrospray ionization. The product-ion spectra of these compounds are shown in Fig. 2. Preliminary scan showed the most abundant ions were protonated molecules $[M+H-H_2O]^+$ m/z 379 and $[M+H]^+$ m/z 393 for ergosterol and IS, respectively (data not shown). Parameters such as desolvation temperature, APCI source temperature and flow rate of desolvation gas were optimized to obtained highest intensity of protonated molecules of ergosterol and IS. The production scan spectra showed high abundance fragment ions at m/z 69 and 69 for ergosterol and IS, respectively. The ion transitions of m/z 379 → 69 for ergosterol and m/z 393 → 69 for IS were chosen for MRM. Chemical structures with fragmentation and product ion spectrum of ergosterol and IS are summarized in Fig. S1 (supporting information).

3.2. Optimization of chromatographic conditions

RRLC–APCI–MS/MS operation parameters were carefully optimized for the determination of ergosterol based on previous studies [24,25]. Reversed-phase chromatographic media with a 1.7 or 1.8 μm particle size offer significant advantages in resolution, speed, and sensitivity for analytical determinations. In our study, 1.8 μm particle size columns were selected. The separation and ionization of ergosterol and IS were affected by the composition of mobile phase. The presence of a small amount of formic acid in the mobile phase improved the detection of the analytes in positive ion mode of the RRLC/MS/MS and, consequently, improved the sensitivity. The methanol–water system was performed on RRLC column to increase the efficiency of analysis and provide a better peak shape.

In order to avoid the contamination of earlier eluted endogenous components of the sample matrix onto the MS system a switch technique was developed. The first 0.5 min eluent was switched

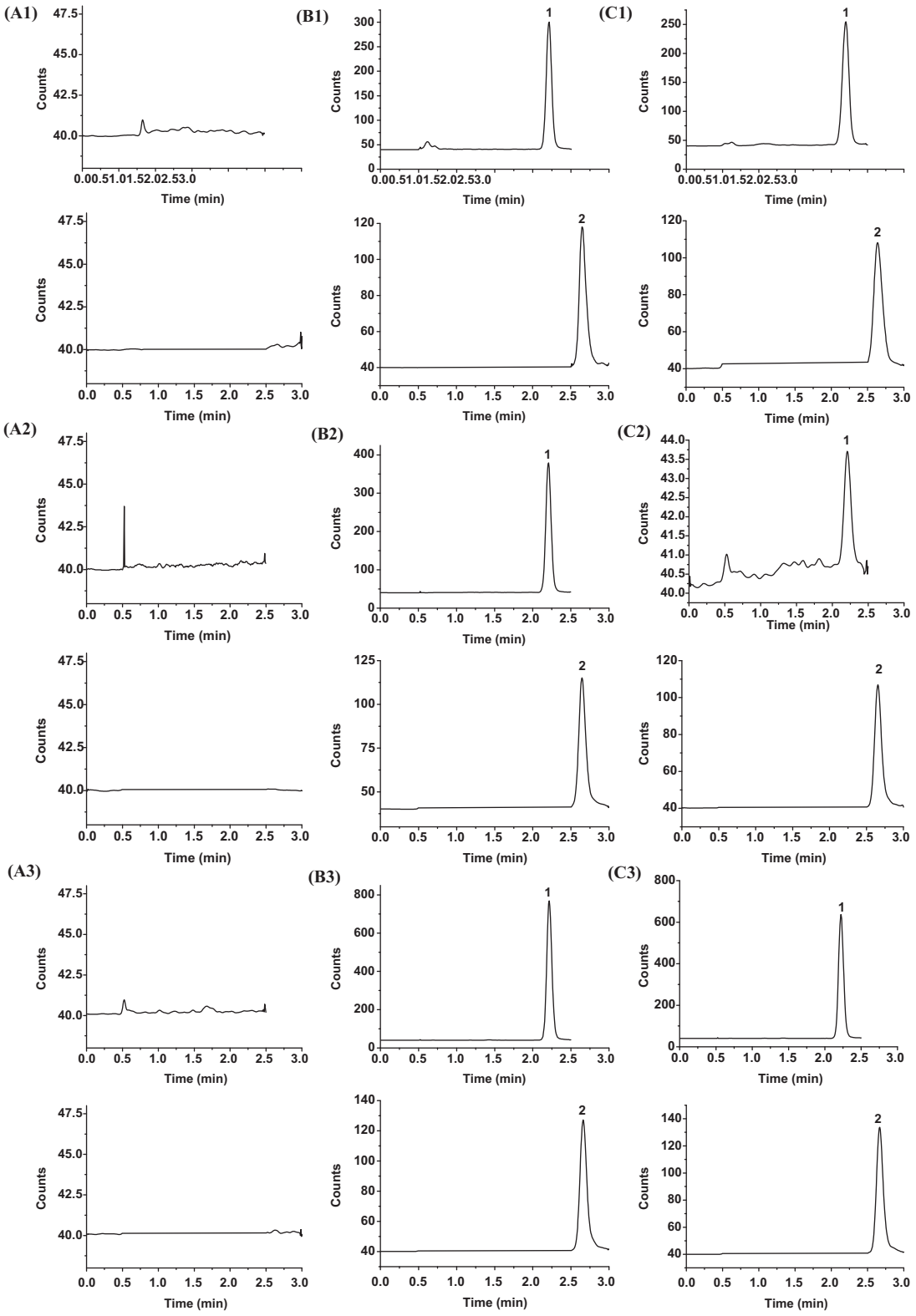

Fig. 2. Chromatograms of ergosterol and IS in rat plasma, urine and faecal homogenate: (A1) blank rat plasma, (A2) blank urine, (A3) blank faecal homogenate, (B1) blank plasma spiked with ergosterol (200 ng/mL) and IS, (B2) blank urine spiked with ergosterol (200 ng/mL) and IS, (B3) blank faecal homogenate spiked with ergosterol (200 ng/mL) and IS, (C1) a rat plasma sample after oral administration, (C2) a urine sample after oral administration, (C3) a faecal homogenate sample after oral administration (1, IS; 2, ergosterol).

from the MS detector, and the eluent from 0.5 to 3.0 min was allowed to enter into the MS system and recorded. Two channels were used for recording, channel 1 for ergosterol with a retention time of 2.2 min, and channel 2 for the IS with a retention time of 2.6 min. Under these chromatographic conditions, both ergosterol and IS were rapidly eluted and the total run time was 3.0 min per sample, less than previously reported [24]. Fig. 2 shows both ergosterol and IS well separated with excellent peak shapes,

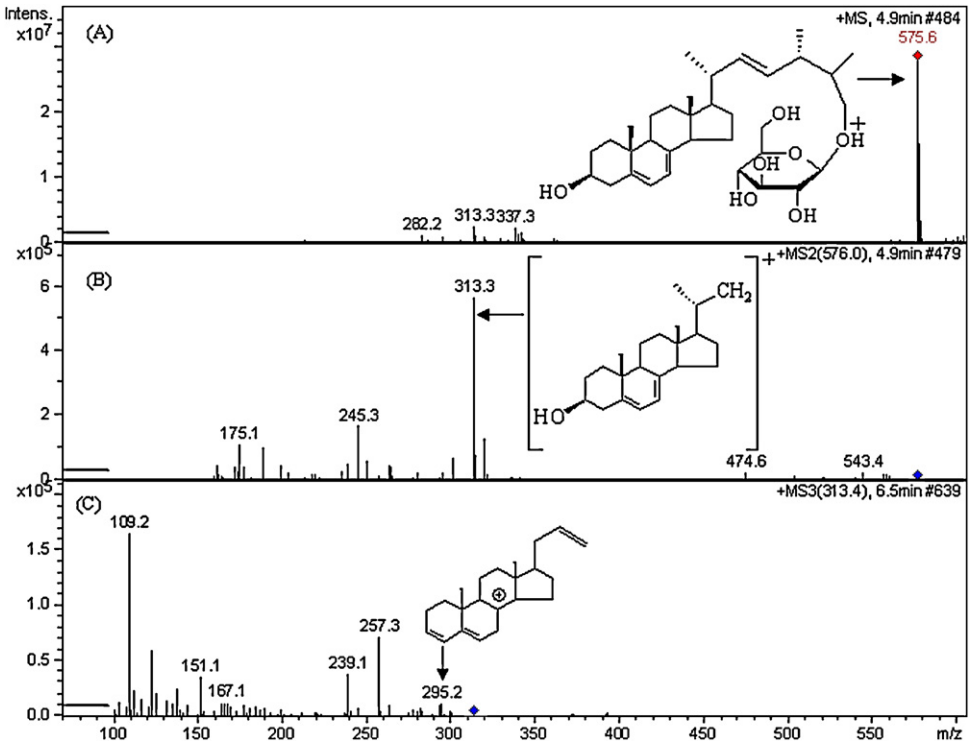

Fig. 3. MS1 (A), MS2 (B) and MS3 (C) mass spectrum of the ion at of *m/z* 575.6 for ERG1, showing the suggested structures of the parent ion and the main fragment ion.

and no interfering peaks in the blank samples and in all biological samples (plasma, urine and faecal homogenate). The narrow chromatographic peaks, produced by RRLC resulted in an increase in the chromatographic efficiency and sensitivity. Both high sample throughput and high sensitivity met the requirement for data collection in a pharmacokinetic study.

The ideal internal standard in RRLC/MS/MS analysis is compound that has similar structure, chromatographic and mass spectrometric behavior to the analyte. Ergone was chosen as the internal standard not only by virtue of its similarity in chromatographic behavior and mass spectrometric response but also for its relative stability. In the present work, we obtained ergone a very good chromatographic separation between ergosterol and ergone.

3.3. Results of ergosterol metabolite (ERG1 and ERG2) identification

The mass spectra of ERG1 and ERG2 are shown in Figs. 3A–C and 4A–C. Peaks at *m/z* 575.6 and 577.6 corresponded to protonated ERG1 and ERG2. The analytes formed were predominantly protonated molecular ion $[M+H]^+$. Peaks at *m/z* 575.6 and 577.6 corresponded to protonated ERG1 and ERG2 (Figs. 3A and 4A).

The peak at *m/z* 575.6 for ERG1 and *m/z* 577.6 for ERG2 was selected as a precursor ion for APCI–MS^n studies and MS2 for ERG1 (Fig. 3B) and ERG2 (Fig. 4B) were performed. The most abundant ion originating from precursor ions at *m/z* 575.6 is a characteristic MS2 fragment ion $[M+H-262]^+$ at *m/z* 313.3 (Fig. 3B). The most abundant ion originating from precursor ions at *m/z* 577.6 is a characteristic MS2 fragment ion $[M+H-264]^+$ at *m/z* 313.3 (Fig. 4B). The daughter ion at *m/z* 313.3 was isolated and further fragmented (Figs. 3C and 4C), resulted in ions at *m/z* 295.2, 257.3, 239.1 and 109.1, which were consistent with a previous report [12].

The UV spectra of ergosterol and its two metabolites (ERG1, ERG2) are similar and present an UV absorption wavelength maximum (λ_{max}) at 272 and 282 nm. UV spectra of ergosterol, its metabolites, ERG1and ERG2 are summarized in Fig. S2 (supporting information). Comparison of the MS3 spectra of ergosterol and strongly suggested that in metabolites the core structure of the steroidal-membered remained intact (Figs. 3C and 4C). We concluded that side-chain was oxidized and became β-D-glucopyranoside structure (Fig. 1C and D). The results further demonstrated that the ring systems of ergosterol and do not show any major difference.

3.4. Assay validation

3.4.1. Selectivity

Selectivity of plasma, urine and faeces was demonstrated by comparing the chromatograms of five independent biological samples (plasma, urine and faeces) from blank rats, for both a blank and a spiked sample. No significant interferences of ergosterol and IS in plasma, urine and faeces samples were detected (Fig. 2). The ergosterol and IS of the retention times were 2.2 and 2.6 min, respectively.

3.4.2. Linearity

Calibration curves of ergosterol were linear over the concentration range of 7–2000 ng/mL for plasma, 6–2000 ng/mL for urine and 8–7500 ng/mL for faecal homogenate. The typical equation was $Y = 0.0013\ X + 0.0123$ ($r = 0.9998$) for plasma, $Y = 0.0015X + 0.0053$ ($r = 0.9994$) for urine and $Y = 0.0012X + 0.0104$ ($r = 0.9996$) for faecal homogenate. During routine analysis each analytical run included a set of calibration samples, a set of QC samples and the unknowns. The LLOQ of ergosterol in rat plasma, urine and faecal homogenate were 7, 6 and 8 ng/mL, respectively.

3.4.3. Accuracy and precision

The intra- and inter-day precision and accuracy for ergosterol from biological matrices QC samples were measured below 5.7% and 9.5%, respectively, with relative recovery from 96.0% to 105.0%

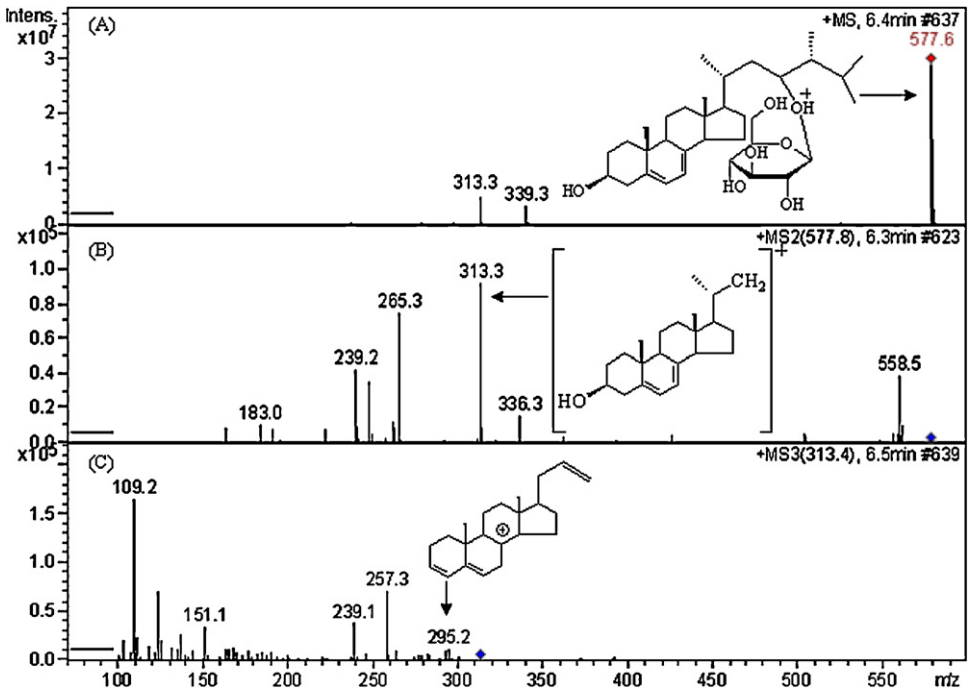

Fig. 4. MS1 (A), MS2 (B) and MS3 (C) mass spectrum of the ion at of *m/z* 577.6 for ERG2, showing the suggested structures of the parent ion and the main fragment ion.

(Table 1). The results indicate the method showed good precision and accuracy.

3.4.4. Extraction recovery

Recovery data are shown in Table S1 (supporting information). The mean extraction recovery of ergosterol from plasma, urine and faecal homogenate was 90.2 ± 1.4%, 91.6 ± 1.3% and 91.6 ± 0.9%, respectively. The mean relative recovery for IS was 94.8 ± 2.9% (n = 5). The results indicate the method showed high recovery. To evaluate the effect of the sample matrix on MS response, we compared the instrument response for quality control by injecting samples into the mobile phase with responses for the same amount of ergosterol with samples that were not extracted and extracted samples. All the ratios were between 85% and 115%. No significant matrix effect for ergosterol was observed.

3.4.5. Stability

The results of short-term, long-term, and freeze–thaw stability of ergosterol in plasma, urine and faeces were found to be stable. The results of stability of ergosterol in plasma, urine and faeces are summarized in Table S2 (supporting information). The relative standard deviations were within ±15%. Long-term stability studies of ergosterol in different biological matrices showed appreciable stability over 30 days. Furthermore ergosterol was found to be stable at room temperature for a period of 6 h. All the results well met the criterion for stability measurements.

Table 1
Summary of precision and accuracy of ergosterol in rat plasma, urine and faeces (n = 3 assays, 5 replicates per assay).

Added con. (ng/mL)	Found con. (mean ± SD, ng/mL)	Recovery (%)	Intra-day (RSD %)	Inter-day (RSD %)
Plasma				
20	21.0 ± 1.3	105.0	5.5	7.1
800	794.5 ± 26.0	99.3	3.1	3.7
1600	1617.2 ± 49.0	101.0	2.7	3.6
Urine				
20	20.5 ± 1.4	102.5	5.3	8.5
800	824.9 ± 34.8	103.1	4.8	3.3
1600	1576.1 ± 56.4	98.5	4.1	2.7
Faeces				
20	19.2 ± 1.5	96.0	5.7	9.5
2000	1996.9 ± 41.1	99.8	2.1	2.6
6000	6000.8 ± 80.8	100.0	1.1	1.2

3.5. Application of the method

The diuretic activity of ergosterol was reported previously [4]. Pharmacokinetic studies of this drug are important for further biological and biochemical research and for future clinical trials. After a single oral administration of ergosterol (100 mg/kg) to SD rats, the concentrations of ergosterol in plasma, urine, and faecal homogenate were determined by RRLC/MS/MS. The mean plasma concentration–time curves of ergosterol after administration in rats are shown in Fig. 5. Pharmacokinetic parameters are listed in Table 2.

The ergosterol was detected in rat plasma, urine and faeces samples collected from 0 to 36 h after oral administration. The results indicate ergosterol levels in faeces are higher than in plasma and urine in rat. Almost 62.5% of administered dose is cumulative in the

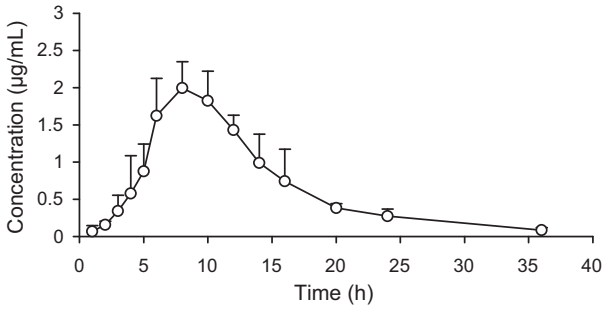

Fig. 5. Mean (±SD) plasma concentration–time profile of ergosterol in the plasma of healthy rats (n = 6), that were administered a single oral dose of 100 mg/kg of ergosterol.

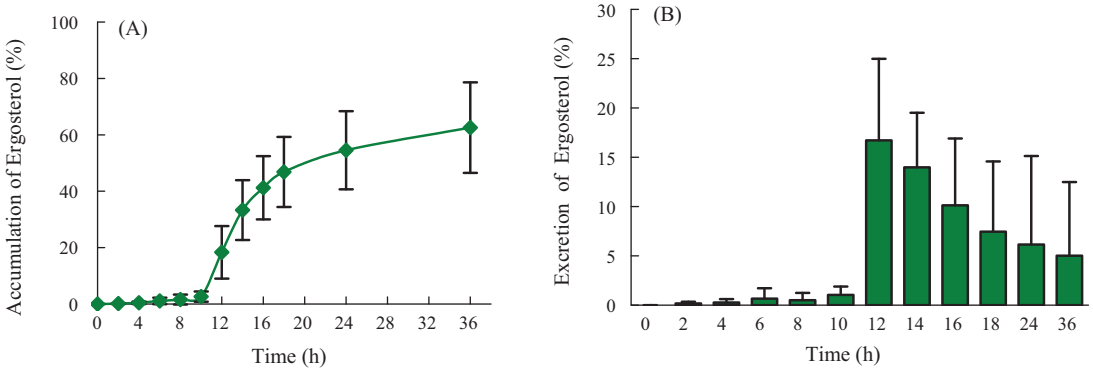

Fig. 6. Accumulation (A) or excretion (B) of ergosterol in faeces of rats after a single 100 mg/kg oral dose.

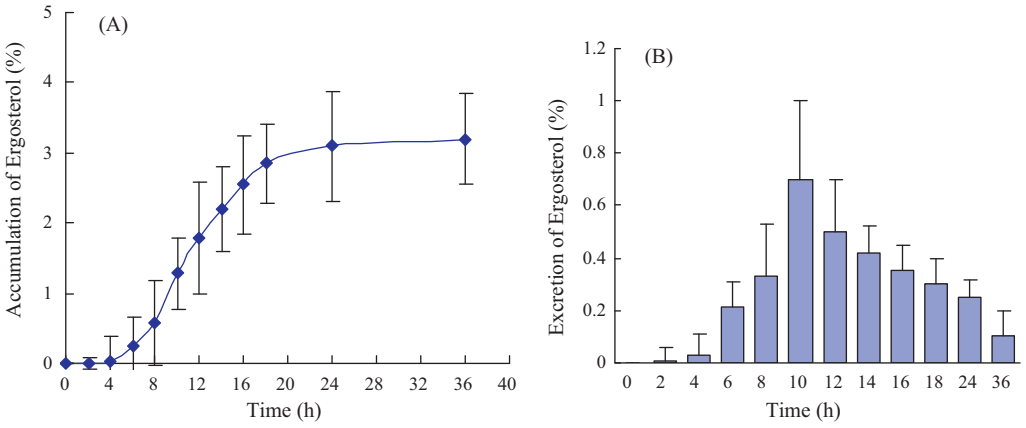

Fig. 7. Accumulation (A) or excretion (B) of ergosterol in urine of rats after a single 100 mg/kg oral dose.

Table 2
Pharmacokinetic parameters obtained after administration of ergosterol in SD rats ($n = 6$).

Pharmacokinetic parameters	Mean ± SD
$AUC_{0-36\,h}$ (μg h mL^{-1})	22.29 ± 5.08
C_{Max} (μg/mL)	2.27 ± 0.19
$t_{1/2}$ (h)	5.90 ± 1.41
T_{Max}(h)	8.00 ± 1.18

faeces within 36 h after oral administration of ergosterol at a dosage of 100 mg/kg (Fig. 6A), but only 3.2% of loading dose was found in urine (Fig. 7). The data also indicate the time to reach maximum excretion quantity of ergosterol was between 12 and 14 h after dose (Fig. 6B).

4. Conclusion

To determine the pharmacokinetic parameters of ergosterol metabolism *in vivo* in SD rats a novel method was developed using RRLC–DAD–APCI–MS^n. The data show the major elimination pathway of ergosterol is through the faeces, which is essential for understanding the safety and efficacy for the potential use as a therapeutic drug. RRLC–APCI–MS/MS method was developed and validated for the determination of ergosterol in rat plasma, urine and faeces, which showed great advantages of the simple sample preparation but also selectivity and short analysis time of 3.0 min. The small amount of biological matrices required (0.2 mL per determination) made this method suitable for routine analysis in preclinical pharmacokinetic studies, and the method was helpful in clinical pharmacokinetic studies. The method RRLC–APCI–MS/MS can also be used as a reference for therapeutic drug monitoring.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.05.025.

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