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Rapid resolution liquid chromatography–mass spectrometry and high-performance liquid chromatography-fluorescence detection for metabolism and pharmacokinetic studies of ergosta-4,6,8(14),22-tetraen-3-one

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ABSTRACT

Ergosta-4,6,8(14),22-tetraen-3-one (ergone) from many medicinal plants has been demonstrated to possess a variety of pharmacological activities *in vivo* and *in vitro*, including cytotoxic, diuretic and immunosuppressive activity. Metabolism and pharmacokinetic studies on rat were conducted for ergone. Rapid resolution liquid chromatography with atmospheric pressure chemical ionization tandem multi-stage mass spectrometry (RRLC-APCI-MS^n) and high-performance liquid chromatography with fluorescence detection (HPLC-FLD) methods were applied for the identification and quantification of ergone and its metabolite from rat plasma, faeces and urine. A metabolite was identified by RRLC-DAD-APCI-MS^n: 22,23-epoxy-ergosta-4,6,8(14)-triaen-3-one (epoxyergone). The concentrations of the analyte with its metabolites were determined by HPLC-FLD at excitation wavelength of 370 nm and emission wavelength of 485 nm. The samples were deproteinized with methanol after addition of camptothecin as internal standard (IS). The analysis was performed on a Diamonsil C18 column (150 mm × 4.6 mm × 5 μm) with a mobile phase gradient consisting of methanol and water at a flow rate of 1 mL min^{-1}. The assay was linear over the concentration range of 42–1500, 36–7500 and 42–1500 ng mL^{-1} for plasma, faecal homogenate and urine respectively. The absolute recoveries were found to be 97.0 ± 1.2%, 98.1 ± 0.7% and 96.6 ± 1.8% for plasma, faecal homogenate and urine respectively. The intra-day and inter-day relative standard deviations (RSD) were less than 10%. The previous HPLC–MS/MS method is not affordable for most laboratories because of the specialty requirement and high equipment cost. However, the HPLC-FLD method is economic and operating simply for quantitative determination of ergone and its metabolite in rat plasma, faeces and urine. In addition, liquid chromatography coupled with ion trap multi-stage mass spectrometry is becoming a useful technique for ergone metabolite identification.

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 has been reported to possess cytotoxic activity [1], diuretic activity [6], inhibitory activity of nitric oxide production [4] and immunosuppressive activity [5]. We have recently reported the ergone also has diuretic activity [7], which is consistent with a previous report [6]. Ergone has been studied in several tumor cell lines showing high cytotoxic effect *in vitro* [1]. The cytotoxic bioactivity was also validated by *in vitro* tests in our previous research and the study of the mechanism is in progress. However, 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 pharmacological activities of ergone make it worth carrying out further comprehensive studies on pharmacokinetic properties and elimination pathway of ergone.

Several analytical methods have been published for the quantification of ergone in raw materials, such as HPLC with UV detection [6,8–10], fluorescence detection (FLD) [11], and mass spectrometry (MS) [12]. Previously, our laboratory developed a simple HPLC–MS/MS method to measure ergone in rat plasma [13]. The

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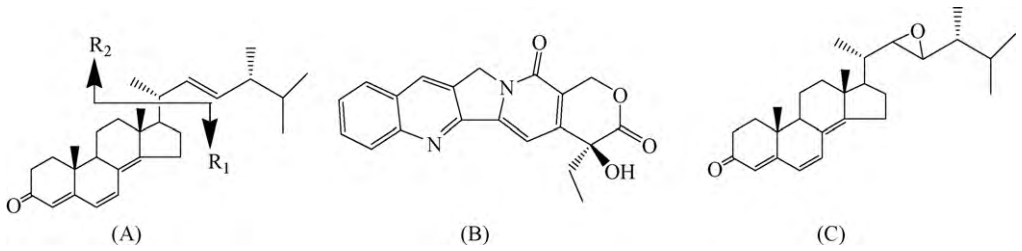

Fig. 1. Chemical structures of ergone (A), camptothecin (B) and epoxyergone (C).

HPLC–MS/MS method provided good specificity and accuracy for determining ergone in rat plasma. However, there is no information describing the identification and quantification of ergone and its metabolite in rat plasma, faeces and urine. In addition, the effects on the elimination pathway of ergone have not previously examined. In general, 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 ergone, it is essential to establish a highly sensitive and reliable analytical method that can be used to accurately measure low levels of ergone in small volumes of plasma, faeces and urine. The use of UV/vis detection for ergone in rat plasma may not be sensitive enough for determination of trace samples although this approach has been validated in our laboratory. Also, this method is mainly designed for human biological samples or raw materials in a relatively large volume, typically 0.5–1.0 mL human plasma. In the case of rat studies, it is impossible to get a desired set of biological samples from one small animal to perform a pharmacokinetic study, for only one or two blood samples can be taken from each animal for measurement by HPLC method.

LC coupled with ion trap multi-stage MS is becoming a useful technique for drug metabolites detection and identification. This is because the ion trap can effectively produce full-scan mass spectra while still offering high sensitivity and the MS^n capability of ion trap MS also provides additional information for structural elucidation of the metabolites. At the same time, FLD is widely used in quantitative analysis because of its great sensitivity and good selectivity as well as its relatively low cost with respect to MS. To best of our knowledge, none of the published methods have utilized fluorescence and MS^n as the means of detection for the rat plasma, faeces and urine identification and quantification of ergone and its metabolite. Camptothecin was used in the study as the internal standard (IS). The objective of the current effort 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 ergone and its metabolite and (2) HPLC-FLD method to quantify ergone and its metabolite in the large numbers of low-volume biological matrices (plasma, faeces and urine) generated in pharmacokinetic studies after oral administration of 20 mg ergone per kg of body weight. The applicability of this method was determined for preclinical pharmacokinetic studies in the Sprague–Dawley (SD) rat.

2. Materials and methods

2.1. Chemicals and reagents

The standard of ergone was isolated by the authors from *P. umbellatus* and also synthesized by chemical method (Fig. 1A). The procedure of isolation, purification and synthesis of ergone was published previously [7]. Its structure was characterized by chemical and spectroscopic methods (^{1}H NMR, ^{13}C NMR and MS) and compared with those found in the literature [1]. Camptothecin (batch No.: 100532-200401) of purity of 99% was used as an internal standard (Fig. 1B). It was a gift from professor Rui-Chao Lin (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China). HPLC-grade methanol was purchased from Baker Company (Baker Inc., USA). Ultra high purity water (UHP) was prepared by a Millipore-Q SAS 67120 MOLSHEIM (France). Other chemicals were of analytical grade and their purity was above 99.5%.

2.2. Preparation of calibration standards and quality control samples

Standard stock solutions of ergone (100 μg mL^{-1}) and camptothecin (200 ng mL^{-1}) were prepared by dissolving suitable amounts of pure substance in methanol and were stored in darkness at 4 °C.

Faecal samples were homogenized with methanol in the ratio 1:20 (g:vol = faeces:methanol) to obtain faecal homogenate. For HPLC determination, calibration standards of ergone at concentration levels of 42, 120, 250, 500, 1000, 1200 and 1500 ng mL^{-1} were prepared by spiking appropriate amount of the standard solutions in blank plasma and urine obtained from healthy rats. Calibration standards of ergone at concentrations of 36, 120, 500, 1000, 2000, 6000 and 7500 ng mL^{-1} were also prepared by spiking appropriate amount of the standard solutions in faecal homogenate obtained from healthy rats. The quality control (QC) samples were separately prepared in a similar manner as those used for calibration curve. Concentrations of 120, 500 and 1200 ng mL^{-1} were used for plasma and urine calibration standards whereas concentrations of 120, 2000 and 6000 ng mL^{-1} were used for faecal homogenate corresponding to the low QC, medium QC and high QC respectively.

2.3. Chromatographic conditions

HPLC was performed with a Dionex HPLC instrument (Dionex Corporation, California, USA) composed of a P680 pump, a thermostatted column compartment TCC-100, an RF-2000 fluorescence detector set at λ_{ex} = 370 nm, λ_{em} = 485 nm, and Chromeleon was used for data collections. The chromatographic separation was performed on a reversed-phase Diamonsil C18 column (150 mm × 4.6 mm × 5 μm, Beijing, China) with the column temperature set at 30 °C. The mobile phase consisted of methanol (A) and water (B) using a gradient elution of 80% A at 0–4.5 min, 80–100% A at 4.5–5.5 min, 100% A at 5.5–18 min, and then decreased to 80% A and equilibrated for 10 min before the injection of the next sample. The flow rate was 1.0 mL min^{-1}. The injection volume was 20 μL.