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Research Paper

Biodistribution evaluation of icaritin in rats by ultra-performance liquid chromatography–tandem mass spectrometry



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ABSTRACT

Ethnopharmacological relevance: Icaritin (ICT) is a major bioactive prenylflavonoid derivative contained in the *Epimedium* which is a widely used herbal medicine for the treatment of infertility, impotence, cardiovascular and skeletal diseases listed in the Chinese Pharmacopoeia. The aim of this study is to investigate the tissue distribution of ICT in rats by ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS)

Materials and methods: ICT was intraperitoneally administrated to rats for 7 consecutive days at dose levels of 20, 40 and 60 mg/kg/day, respectively. Various tissue homogenates were pretreated by protein precipitation with acetonitrile. ICT and internal standard coumestrol were separated on a BEH C₁₈ column with a gradient mobile phase and detected using precursor-product ion transitions of m/z 367.1 → 297.1 for ICT and 267.0 → 211.1 for coumestrol at the negative ionization mode, respectively.

Results: ICT was widely distributed in rat's various tissues and its concentrations in tissues increased with elevated doses. A sensitive and reliable UPLC–MS/MS method was firstly established to quantify ICT in rat tissues. The lower limit of quantification was 0.5 ng/mL based on 100 μ L of tissue homogenates. The intra- and inter-day accuracy at all levels fell in the ranges of 90.8–103.4% and 91.6–100.3%, and the intra- and inter-day precision (RSD) were in the ranges of 2.9–10.5% and 2.6–9.1%, respectively.

Conclusions: The UPLC–MS/MS showed good accuracy, precision and recovery and was suitable for the quantification of ICT in rat tissues. Wide distribution of ICT could helpfully elucidate systemic effects and various functions of ICT.

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1. Introduction

The *Epimedium* has been used as a tonic, aphrodisiac and antirheumatic for the treatment of infertility, impotence, cardiovascular and skeletal diseases in China, Japan and Korea for more than 2000 years (Ma et al., 2011). Icaritin (ICT, Fig. 1A) is recognized as a major bioactive prenylflavonoid derivatives contained in plants of the *Epimedium* (Ma et al., 2011). ICT has been demonstrated to possess several pharmacological activities. These include the osteoprotective effect as seen by its ability to increase the formation of new osteoblast and reducing osteoclastogenesis (Huang et al., 2007a), increasing the differentiation of mesenchymal stem cells (Sheng et al., 2013), the reduction of incidence in steroid-associated osteonecrosis in rabbits (Zhang et al., 2009), as well as the prevention of osteoporosis in ovariectomised rats (Peng et al., 2013). Its anti-tumorigenesis function in regards to breast

(Tiong et al., 2012), prostate (Chen et al., 2010, Huang et al., 2007b), endometrial (Tong et al., 2011), chronic myeloid leukemia (Li et al., 2013a) and renal cancer cells (Li et al., 2013b) has been demonstrated. Its cardiovascular improvements, via initiating cardiomyocyte differentiation from rat embryonic stem cells have also been exhibited (Zhu and Lou, 2005; Wo et al., 2008). As well as, neurological protection from beta-amyloid induced neurotoxicity in an Alzheimer model has been shown (Wang et al., 2007). A phase I clinical trial of ICT for the treatment of advanced breast cancer showed that ICT exhibited good safety and tolerance, and a phase II clinical trial of ICT for the treatment of advanced hepatocellular carcinoma is ongoing (ClinicalTrials.Gov, 2014). As such, accurate and simple methods to measure ICT concentration in tissues and plasma are critical for the development of ICT as a pharmaceutical-quality health intervention.

There have been three publications for the quantification of ICT from the consumption of crude extracts from the *Epimedium* species or as a pure compound in plasma, urine and bile (Shen et al., 2007; Shen et al., 2009; Chang et al., 2012). However, there have been various shortcomings for those methods. Shen et al.

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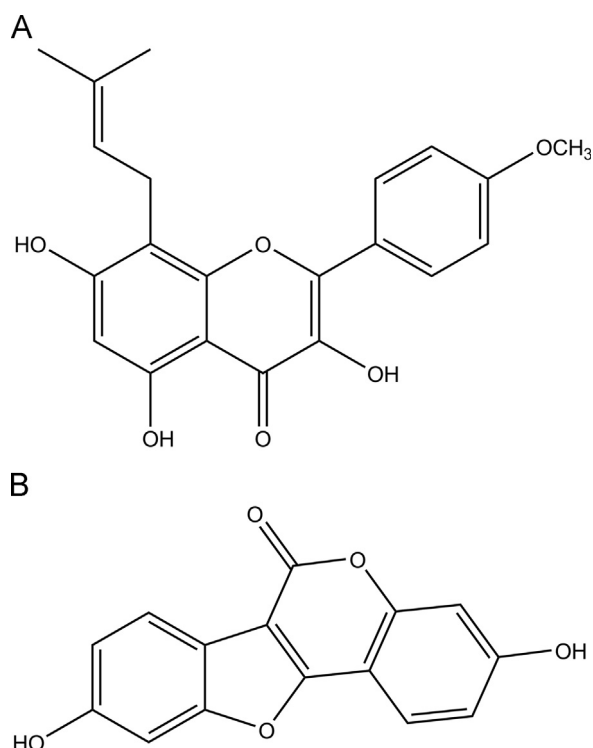


Fig. 1. Chemical structures of (A) ICT and (B) coumestrol.

reported gas chromatography–mass spectrometry (GC–MS) (Shen et al., 2007) and liquid chromatography–tandem mass spectrometry (Shen et al., 2009) (LC–MS/MS) methods for assay of ICT in human and rat serum, respectively, in which the former involved laborious precolumn derivatization and the latter employed a time-consuming multiple-step liquid–liquid extraction and large amount of rat plasma (0.5 mL). In addition, ICT was detected by GC–MS and LC–MS/MS after administration of *Epimedium* crude extract containing icariin, icariside and epimedin, which were finally metabolized to ICT resulting in increasing actual ICT concentration in plasma, compared to pure ICT (Shen et al., 2009, 2007). Therefore, elevation of actual ICT concentration due to the biotransformation with administration of *Epimedium* extract led to faulty calculation of pharmacokinetic parameters compared to pure ICT, unfortunately, no attention was paid to that. Chang et al. described high performance liquid chromatography with a ultraviolet detection method for analysis of ICT in rat plasma, bile and urine, which suffered from low quantification limit of 20 ng/mL resulting in failure detection of parent ICT in biological matrix (Chang et al., 2012).

To our knowledge, no analytical method has been published to quantify ICT in rat tissues (liver, spleen, kidney, heart, lung, muscle, thymus and brain). Therefore, we have developed a robust, fast and sensitive ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method employing a simple one-step protein precipitation for measurement of ICT in rat tissues. Moreover, this method has also been successfully applied to the quantification of pure ICT in rat tissues after intraperitoneal administration of ICT at various dose levels.

2. Materials and methods

2.1. Materials

ICT was provided by Ctech Scientific Pte Ltd. (Singapore). Coumestrol (Fig. 1B) as an internal standard (IS), formic acid,

ammonium formate and Cremophor EL were purchased from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile was obtained from Merck (Darmstadt, Germany). HPLC grade water was produced with Sartorius Arium 611VF water purification system (Milan, Italy). All other reagents were commercially available and of analytical grade.

2.2. LC–MS/MS instrumentation

A Waters Acquity TQD UPLC–MS/MS system consisted of a binary solvent manager, a sample manager, a column oven and a TQD triple quadrupole mass spectrometer with an orthogonal electrospray ionization source Z-spray. All data were acquired and processed using MassLynx version 4.1 software.

2.3. Chromatographic conditions

The chromatographic analysis was carried out utilizing a Waters UPLC BEH C18 analytical column (2.1 mm × 50 mm, 1.7 μm particle size) protected by a Waters VanGuard guard column (2.1 mm × 5 mm, 1.7 μm particle size) of the same packing material. Column temperature was maintained at 40 °C. The mobile phase consisted of two eluents, namely, elution A (95:5 acetonitrile/water containing 2 mM ammonium formate and 0.05% formic acid) and elution B (water with 2 mM ammonium formate and 0.05% formic acid, pH3.0), delivered at a constant flow rate of 0.3 mL/min. The gradient elution was: 0–1.5 min, 10% A; 1.5–2.2 min linear from 10% to 90% A; 2.2–5.0 min, 90% A; 5.0–5.5 min linear from 90% to 10% A, returned to initial conditions; 5.5–6.5 min 10% A, equilibration of the column. Total run time per sample was 6.5 min and all injection volumes were 4 μL at a mode of partial loop with needle overflow. Between injections, the sampling needle was washed for 2 min each with a weak wash of 1200 μL (90% water, 10% acetonitrile) and a strong wash of 600 μL (acetonitrile).

2.4. Mass spectrometric conditions

The Acquity UPLC was coupled to a TQD mass spectrometer operated in negative electrospray ionization mode. Quantification was performed using multiple reaction monitoring (MRM) mode to monitor precursor–product ion transitions of m/z 367.1 → 297.1 for ICT, 267.0 → 211.1 for the IS at negative ionization mode. The optimal instrument operating conditions were as follows: capillary, 3.0 kV; cone, 60 V; source temperature, 150 °C; and desolvation temperature, 300 °C. Nitrogen gas flow was 600 L/h and 50 L/h for desolvation and cone, respectively. Argon was employed as a collision gas with a flow rate of 0.11 mL/min. The collision voltage values of ICT and the IS were 27 and 29 V, respectively.

2.5. Preparations of stock and standard working solutions

Stock solutions of ICT (500 μg/mL) and coumestrol (100 μg/mL) were prepared in acetonitrile and stored at –20 °C. Working standard solutions of ICT were prepared in acetonitrile at concentrations from 5 to 200 ng/mL. Working IS was 1 μg/mL by diluting the stock solution of coumestrol.

2.6. Sample preparation

Rat tissue homogenates were homogenized using a high-speed homogenizer by adding purified water to liver, spleen, kidney, heart, lung, muscle, thymus and brain tissues at a ratio of 5: 1. The calibration standards samples for ICT were prepared by spiking 90 μL of blank tissue homogenates with 10 μL of ICT working solution. The ultimate concentrations of ICT in rat tissue homogenates were 0.5–20 ng/mL.

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