



Original article

Simultaneous determination and pharmacokinetics of sixteen *Angelicae dahurica* coumarins *in vivo* by LC–ESI–MS/MS following oral delivery in rats

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ABSTRACT

Background: The roots of *Angelica dahurica* cv. Qibaizhi is frequently used in clinical practice as a traditional Chinese medicine. However, a comprehensive study of the pharmacokinetics of this medicine has not been carried out.

Method: A sensitive and specific liquid chromatographic–tandem mass (LC–MS/MS) spectrometric method was established to investigate pharmacokinetics of sixteen coumarins of *Angelicae dahuricae Radix* (ADR) in rat plasma, including xanthotoxol (1), oxypeucedanin hydrate (2), 5-hydroxy-8-methoxypsoralen (3), (–)-marmesin (4), byakangelicin (5), columbianetin (6), psoralen (7), xanthotoxin (8), neobyakangelicol (9), isoimipinellin (10), bergapten (11), heraclenin (12), oxypeucedanin ethanolate (13), imperatorin (14), phellopterin (15), isoimperatorin (16). Detection was performed on a triple quadrupole mass spectrometer in multiple-reaction-mode (MRM).

Results: The method established in this assay was successfully applied to the pharmacokinetic study of the selected coumarins in rat plasma after oral administration of the extract of ADR, and the pharmacokinetic characteristics of sixteen coumarins were clearly elucidated.

Conclusion: This pharmacokinetic identification of multiple coumarins of ADR in rats provides a significant basis for better understanding the metabolic mechanism of the herb medicine.

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Introduction

Herb medicines have been used to treat ailments for thousands of years. Today, about 80% of medicines and 25% share of the pharmaceutical arsenal around the world are directly or indirectly derived from plants (Bhattaram et al. 2002). *Angelicae Dahuricae Radix* (ADR), the dried roots of *Angelica dahurica* (Fisch. ex Hoffm.) Benth. et Hook. f. or *A. dahurica* (Fisch. ex Hoffm.) Benth. et Hook.

f. var. *formosana* (Boiss.) Shan et Yuan, is used as a food additive and traditional medicine in China, Korea, and Japan. The cultivar of *Angelica dahurica* (Fisch. ex Hoffm.) Benth. et Hook. f. in Hebei province of China was botanically named *Angelica dahurica* (Fisch. ex Hoffm.) Benth. et Hook. f. ex Franch. et Sav. cv. Qibaizhi Yuan et Shan (*A. dahurica* cv. Qibaizhi) and used as one of its substitutes in clinical practice (Editorial Board of Flora of China of Chinese Academy of Sciences 1992). As a well-known traditional Chinese medicine (TCM), ADR is frequently used for the treatment of the common cold, and headache, toothaches, asthma, coryza, hypertension, vitiligo, psoriasis, acne, herpes zoster virus, and freckles (Chinese Pharmacopoeia Commission 2015; He et al. 2008; Wu et al. 2009). As previously described, the main constituents of ADR are coumarins (Zhao et al. 2012). To date, more than 70 coumarins have been isolated and identified from ADR. Among them, imperatorin, phellopterin, isoimperatorin, and oxypeucedanin hydrate, etc. are the major active ingredients. As a class of natural products, coumarins have attracted considerable interest for their various pharmacological activities in recent years. Imperatorin has

Abbreviations: LC–MS/MS, liquid chromatographic–tandem mass; ADR, *Angelicae dahuricae Radix*; MRM, multiple-reaction-mode; TCM, traditional Chinese medicine; GABA, gamma-aminobutyric acid; ERADQ, extract of roots of *Angelica dahurica* (Fisch. ex Hoffm.) Benth. et Hook. f. ex Franch. et Sav. cv. Qibaizhi Yuan et Shan; ACN, acetonitrile; MeOH, methanol; IS, internal standard; CAD, collision activated dissociation; DP, Declustering potential; CE, collision energy; EtOAc, ethyl acetate; QC, quality control; LLOD, lower limit of detection; LLOQ, lower limit of quantification; S/N, signal-noise ratio; RSD, relative standard deviations; FDA, food and drugs administration.

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anti-cancer activity as well as anticonvulsant potential due to increasing level of gamma-aminobutyric acid (GABA) *in vitro* (Choi et al. 2005; Łuszczki et al. 2010; Kleiner et al. 2001; Yang et al. 2006). Isoimperatorin demonstrates various pharmacological effects of anti-inflammatory, analgesic, antispasmodic and anti-cancer activities (Chen et al. 1995; Moon et al. 2011; Moon et al. 2008; Wang et al. 2010). Isoimperatorin affects both the peripheral and central nervous systems and significantly suppresses the smooth muscle spasm of rabbit's isolate intestine induced by BaCl₂. Isoimperatorin also inhibits the reproduction of several tumor cell lines, such as HELA, P388, HL-60, and A549, *in vitro*. Byakangelicin shows potent inhibition on human acetylcholinesterase and butylcholinesterase (Seo et al. 2003). Phellopterin, as well as bergapten, byakangelicin, xanthotoxin, and neobyakangelicin, showed anti-proliferative activity of B16F10 melanoma cells and causes G2/M arrest through an increase in the level of Chk1 phosphorylation and decrease in the level of cdc2 (Tyr 161) phosphorylation (Maho et al. 2014). As the brain GABA type A receptor modulators, imperatorin, isoimperatorin, phellopterin, oxypeucedanin possessed modulative effect of GABA-induced chloride currents on recombinant $\alpha 1\beta 2\gamma 2S$ GABA_A receptors expressed in *Xenopus laevis* (African clawed frog) oocytes (Singhuber et al. 2011). These studies suggest that coumarins may be the main bioactive components contributing to the pharmacological efficacy of ADR.

With increasing knowledge of illative active constituents of TCM and rapid development of analytical methods, pharmacokinetic studies of complex mixtures of compounds in TCM have become available in the last decade. So far, several analytical methods for the determination of coumarins in ADR *in vivo* have been reported. Xie et al (Xie et al. 2007) developed an HPLC–UV method to study the pharmacokinetics of oxypeucedanin hydrate and byakangelicin in mongrel dog plasma after oral administration of ADR extracts. Zhao et al (Zhao et al. 2013) established a GC–MS method for the quantitative determination of eight coumarins including coumarin, isopsoralen, psoralen, xanthotoxin, bergapten, osthole, imperatorin, and oxypeucedanin in rat plasma after oral administration of a mixture of compounds at a dose of 10 mg/kg of each analyte. Wan et al (Wan et al. 2013) published an HPLC–MS/MS method to analyze the plasma and brain pharmacokinetics of three ingredients including imperatorin, isoimperatorin, and cnidilin in mice after oral administration of ADR extract at a dose of 800 mg/kg. Though these studies could not fully reflect the complex pharmacokinetic characteristics of ADR in the body, the methods provided a foundation for the metabolic study of ADR *in vivo*. Therefore, it is necessary to establish a more appropriate analysis method to characterize the pharmacokinetics of ADR *in vivo*.

In the present study, we developed a sensitive, rapid, and selective LC–MS/MS method for simultaneous determination of sixteen active ADR ingredients (Fig. 1) in rat plasma following oral administration of the extract of the roots of *Angelica dahurica* (Fisch. ex Hoffm.) Benth. et Hook. f. ex Franch. et Sav. cv. Qibaizhi Yuan et Shan (ERADQ). It was anticipated that this study would provide data for mechanism of action and pharmacological effects of ADR *in vivo*. To our knowledge, it is the first study with detailed pharmacokinetic characterizations of the sixteen coumarins in rat plasma.

Materials and methods

Chemicals and reagents

LC–MS grade acetonitrile (ACN) and methanol (MeOH) were obtained from J. T. Baker (Phillipsburg, USA). HPLC-grade formic acid was purchased from Dikma Tech. Inc. (Beijing, China). Water (H₂O) was purified by a Milli-Q system (Millipore, Billerica, MA, USA) in our laboratory. Other reagents were of analytical grade.

The standards of xanthotoxin (1), oxypeucedanin hydrate (2), 5-hydroxy-8-methoxypsoralen (3), (-)-marmesin (4), byakangelicin (5), columbianetin (6), psoralen (7), xanthotoxin (8), neobyakangelicin (9), isoimipinellin (10), bergapten (11), heraclenol (12), oxypeucedanin ethanolate (13), imperatorin (14), phellopterin (15), isoimperatorin (16) were isolated and identified from the roots of *A. dahurica* cv. Qibaizhi and *A. dahurica* Benth. et Hook. f. ex Franch. et Sav. (Zhao et al. 2012; Zhao et al. 2014). The purities of all standards were more than 98.5%, making them suitable for LC–MS/MS analysis. Daidzein (internal standard, IS) was purchased from National Institutes for Food and Drug (Beijing, China) with purity > 99.0%.

The roots of *A. dahurica* cv. Qibaizhi were collected from Anguo city of Hebei province, China, in 2008 and identified by Prof. Wang Wen-quan in Beijing University of Chinese Medicines. Voucher specimen (No. 20081025Q) was deposited at the State Key Laboratory of Natural & Biomimetic Drugs, Peking University (Beijing, China).

Preparation of *Angelica dahurica* extract and the solutions for pharmacokinetic study

The dried roots of *A. dahurica* cv. Qibaizhi (1 kg) were refluxed with 3 l 70% ethanol for four times. The solution was filtrated and evaporated under reduced pressure in a Buchi R-210 rotary evaporator (Buchi Ltd., Labortechnik AG, Switzerland). Subsequently, the concentrated extract was lyophilized using a lyophilizer. (Four-ring Science Instrument Plant Beijing Co., LTD., Beijing, China). The lyophilized powder was then dissolved in H₂O to form a 1.2 g/ml (crude drug 2.4 g/ml) suspension.

Apparatus and chromatographic conditions

Detection and quantification of the analytes were performed on a LC–MS system. The analytical DIONEX Ultimate 3000 HPLC system equipped with an Ultimate 3000 Pump, a DIONEX Ultimate 3000 Autosampler and a DIONEX Ultimate 3000 Compartment. The chromatograph was connected online to a 4000QTRAP triple quadrupole tandem mass spectrometer (Applied Biosystems/MDS Sciex, Canada) equipped with an electrospray ionization (ESI) source for the mass analysis and detection. Analyst 1.5.1 software (Applied Biosystems/MDS Sciex, Canada) was used for data collection and analysis. The separation was performed on a Diamonsil ODS C₁₈ column (250 × 4.6 mm i.d., 5 μm; Dikma, China). The mobile phase was a mixture of H₂O containing 0.1% formic acid (v/v) (A) and ACN (B). The gradient program of mobile phase was carried out as follows: 0–5 min, 10–40% B; 5–10 min, 40–50% B; 10–15 min, 50–70% B; 15–20 min, 70–80% B; 20–20.1 min, 80–10% B; 20.1–27 min, 10% B. The flow rate was set at 1.0 ml/min and the injection volume was 5 μl. Ionization was performed in the positive electrospray mode and the turbo ionspray source was set as follows: capillary voltage 4.5 kV, source temperature 600 °C, collision activated dissociation (CAD) 2.0 with nitrogen as collision gas. Nitrogen was also used as nebulizing gas, curtain gas and heater gas with pressures of 60, 15, and 50 psi, respectively. Declustering potential (DP) and collision energy (CE) were optimized by infusing the standard solution of each individual compound into the mass spectrometer separately with a syringe pump at a flow rate of 10 μl/min. The quantification was carried out with multiple reactions monitoring (MRM) mode.

Preparation of samples

20 μl IS solution (20 ng/ml) and 900 μl ethyl acetate (EtOAc) were added into 300 μl rat plasma sample. The mixture was vortexed for 1.0 min and then centrifuged at 16,000 g for 10 min. The

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