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# Bioavailability of wilforlide A in mice and its concentration determination using an HPLC-APCI-MS/MS method

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ABSTRACT

Wilforlide A (WA), an active compound in *Tripterygium wilfordii* Hook F (TW) which is a traditional Chinese medicine for treatment of autoimmune diseases, is a quality control marker for TW product. At present, the bioavailability/pharmacokinetics of WA is not known. Such information is not only essential to evaluate the relevance of WA as a quality control maker, but also important for future clinical efficacy studies. Therefore, a high-performance liquid chromatography-atmospheric pressure chemical ionization tandem mass spectrometric method (HPLC-APCI-MS/MS) was developed and applied to a bioavailability/pharmacokinetic study of WA.

WA and celastrol (the internal standard, IS) were extracted by a liquid-liquid extraction method using methyl tert-butyl ether. Multiple reaction monitoring (MRM) scanning in positive ionization mode was used to monitor the transition of *m/z* 455.1 to 191.3 for WA and 451.3 to 201.2 for IS. This method was validated and applied to a pharmacokinetic study of WA in mice following intravenous administration (IV, 1.2 mg/kg), intraperitoneal injection (IP, 6 mg/kg) and oral administration (PO, 30 mg/kg).

The lower limit of quantification (LLOQ) for WA was 10 ng/ml. The intra- and inter-day precision was found to be within 15.4% while the accuracy within 94.1–115.7% for all the quality control and LLOQ samples. The samples were stable under all the usual storage and experimental conditions. The terminal elimination half-lives were 14.7, 9.1 and 22.7 min following IV, IP and PO dosing, while the absolute bioavailability for IP and PO WA were 9.39% and 0.58% respectively.

These results indicated that the HPLC-APCI-MS/MS assay was suitable for the pharmacokinetic study of WA. WA was found poorly absorbed when given orally and therefore it may not be a relevant marker for the oral TW products in the market.

## 1. Introduction

Wilforlide A (WA) and triptolide are two active components in *Tripterygium wilfordii* Hook F (TW, also called Thunder God Vine), a traditional Chinese medicine (named Lei Gong Teng in Chinese) known for treatment of autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus and psoriasis for years (Fig. 1) [1–3]. Currently, a product called *Tripterygium wilfordii* glycosides tablet or Lei Gong Teng Duo Gan Tablet is readily commercially available in China. In our previous study, the TW extract has also been found to sensitize prostate cancer resistant to docetaxel [4]. However, the use of TW extract has been associated with serious side effects such as immunosuppression and hepatotoxicity as well as quality control issues of herbal extracts [5–7]. Thus its active chemical markers, such as WA (content of 0.056 mg/g in root of TW) and triptolide (content of 0.067 mg/g to 0.8 mg/g in root of TW) would be attractive for further potential development [8,9]. Of the two chemical markers, triptolide has been reported to cause more side effects, such as immune suppression and anti-fertility than WA [10,11]. Its content is restricted to be no more than 10 μg per 10 mg (one tablet) in the commercial tablets [12]. In comparison, the recommended content of WA in the TW tablet is greater than 10 μg per 10 mg, consistent with a safer projection of this maker. In previous studies, WA has shown efficacious anti-inflammatory activities in carrageenan-induced rat pedal swelling and in tampon-induced rat granulation models with no significant immune suppressive effect [13]. Furthermore, we have observed an enhanced anticancer effect when combined with a conventional chemotherapeutic drug [14]. Thus, WA seems to be a good drug candidate to be further developed due to its various favorable activities, but less toxicity. However, there is no absorption and pharmacokinetic information available for WA. Such information will be useful for planning preclinical efficacy studies as well as future clinical trials in human

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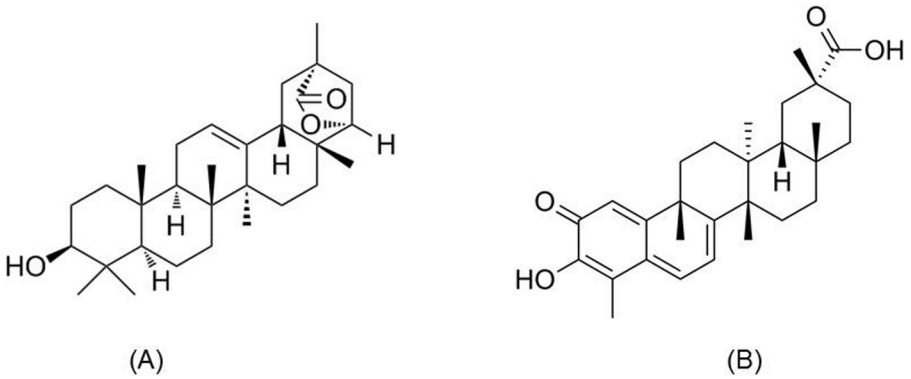


**Fig. 1.** Chemical structure of WA and celastrol.

subjects. In addition, the oral pharmacokinetic profile of WA will be relevant to its designation as a quality control marker for the TW product when given orally [15]. Therefore, in this study the bioavailability of WA following its oral and intraperitoneal administration is investigated using a newly developed high-performance liquid chromatography-atmospheric pressure chemical ionization tandem mass spectrometric assay (HPLC-APCI-MS/MS).

## 2. Method

### 2.1. Materials and instruments

WA and celastrol were purchased from the National Institutes for Food and Drug Control (Beijing, China) with purities of greater than 95%. The analytical grade formic acid was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) with a purity of more than 99%. HPLC-grade methanol and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA, USA). Methyl tert-butyl ether (MTBE) was purchased from VWR International, LLC. (Brisbane, CA, USA). The blank mouse plasma (CD-1) was got from Innovative Research (Novi, MI, USA). The deionized water was prepared using a Barnstead Nanopure Diamond™ water purification system (APS Water Services Corporation, Van Nuys, CA, USA) and used throughout the study.

The HPLC-APCI-MS/MS system consisted of an API 3200 LC/MS/MS system (Sciex, Framingham, MA, USA) with an APCI (atmospheric pressure chemical ionization) probe and two Shimadzu LC-20AD Prominence Liquid Chromatograph pumps equipped with an SIL-20A Prominence autosampler (Shimadzu, Carlsbad, CA, USA). The chromatographic separation was conducted using a Zorbax SB $C_{18}$ column (150 × 2.1 mm, 5 μm, Zorbax, Agilent, Santa Clara, CA, USA) protected with a SB-$C_{18}$ Guard Cartridge (12.5 × 2.1 mm, Zorbax, Agilent, Santa Clara, CA, USA).

### 2.2. Sample preparation

The stock solution of WA was prepared by dissolving 5 mg of its standard into 100 ml methanol to yield a concentration of 50 μg/ml (heat up to 60 °C in water bath if needed), while the stock solution of celastrol (1 mg/ml) (internal standard, IS) was prepared by dissolving 1 mg of celastrol standard in 1 ml of methanol. All the stock solutions were stored at −20 °C and subsequently found to be stable for at least 6 months.

The working solutions for construction of standard curves were prepared by diluting its stock solution (50 μg/ml) with methanol to generate 0.1, 0.5, 1, 5, and 10 μg/ml. The working solutions for quality control (QC) samples were prepared separately at concentrations of 0.2, 2, and 50 μg/ml which were used for low (LQC), medium (MQC), and high (HQC) samples respectively. The working solution (1 μg/ml) of celastrol was prepared by diluting the stock solution using 50% methanol.

The standard calibration samples were prepared by spiking 100 μl of the blank mice plasma with 10 μl of the working solutions listed above to yield six different concentrations, i.e. 10, 50, 100, 500, 1000, and 5000 ng/ml. The QC samples were prepared using the same method to generate concentrations of 20, 200, and 5000 ng/ml. Afterwards, 10 μl of celastrol working solution (1 μg/ml) was added and mixed well. For the mice plasma samples, 10 μl of 50% methanol and 10 μl of celastrol working solution were added and mixed thoroughly.

After mixing, 300 μl MTBE were added to each sample mixture. The samples were then mixed using a vortex mixer for 3 min followed by centrifuging at 5000 rpm for 3 min. The upper clear solvent layer was transferred into a clean glass tube and evaporated under the air blow until dryness. Then 0.1 ml of 80% acetonitrile was added to each tube and vortexed for 1 min. The mixture was centrifuged at 10,000 rpm for 5 min and the supernatant (10 μl) injected into HPLC-MS/MS system for analysis.

### 2.3. Liquid chromatography and mass spectrometry

An isocratic elution was carried out using a mobile phase containing acetonitrile (80%) and 0.1% formic acid (20%) with the flow rate of 0.35 ml/min. The temperatures of the analytical column and autosampler were both set at room temperature.

All of the liquid chromatographic eluent was then introduced into the APCI source. The mass spectrometric conditions were: collision gas, nitrogen (5 psi), gas 1, air (30 psi); gas 2, air (30 psi); nebulizer current (5 mA); ion source temperature, 400 °C; curtain gas, nitrogen (25 psi). Multiple reaction monitoring (MRM) scanning in positive ionization mode was used to monitor the transition of *m/z* 455.1 to 191.3 for WA and 451.3 to 201.2 for celastrol (Fig. 2) with the optimized MS/MS conditions listed in Table 1.

### 2.4. Validation of the assay method

The validation of the assay method was assessed in terms of linearity, sensitivity, precision, accuracy, recovery, stability. The QC samples at concentrations of LLOQ, 20, 200 and 5000 ng/ml were utilized and analyzed for these tests.

#### 2.4.1. Standard curve and linearity

For construction of the standard calibration curve, all the samples were prepared in triplicates using calibration standard solutions. The calibration curve was constructed using the analyte/IS peak area ratio versus the nominal concentrations of WA, and fitted by linear least-squares regression analysis with weighing factor of $1/x^2$ (x is the value of the nominal concentration). The linearity was determined by coefficient of determination ($R^2$) which should be greater than 0.99.