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RESEARCH ARTICLE

# Simultaneous determination of usnic, diffractaic, evernic and barbatic acids in rat plasma by ultra-high-performance liquid chromatography–quadrupole exactive Orbitrap mass spectrometry and its application to pharmacokinetic studies

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## Abstract

*Usnea longissima* Ach. (*Usnea*) is used in pharmaceuticals, food and cosmetics. Evernic acid (EA), barbatic acid (BA), diffractaic acid (DA) and usnic acid (UA) are the most typical ingredients in *U. longissima* and exert a wide variety of biological functions. The study aimed to develop a sensitive method for simultaneous analysis of EA, BA, DA and UA in rat plasma and was applied to pharmacokinetic studies after consumption of UA and ethanol extract from *U. longissima* (UE). The samples were separated on a BEH $C_{18}$ column by gradient elution with 0.5% formic acid in water and in methanol. The relative molecular masses of analytes were obtained in full-scan range from 50.0 to 750.0 *m/z* under negative ionization mode by UPLC-Q-Exactive Orbitrap MS. All validation parameters, such as lower limit of quantitation, linearity, specificity, precision, accuracy, extraction recovery, matrix effect and stability, were within acceptable ranges and the method was appropriate for biological specimen analysis. The pharmacokinetic results indicated that the absolute bioavailabilities of UA after oral administration of UA and UE reached 69.2 and 146.9%, respectively. Compared with UA in UE, the relative bioavailability of DA, BA and EA reached 103.7, 10.4 and 0.7% after oral administration of UE.

**KEYWORDS**

depsides, dibenzofuran derivatives, pharmacokinetics, UPLC-Q-Exactive Orbitrap MS, *Usnea longissima* Ach, usnic acid

**Abbreviations:** BA, barbaric acid; DA, diffractaic acid; EA, evernic acid; FDA, US Food and Drug Administration; LLE, liquid–liquid extraction; PPT, protein precipitation (PPT); SPE, solid-phase extraction; UA, usnic acid; UE, ethanol extract from *U. longissima*.

  

## 1 | INTRODUCTION

The use of herbal medicine (such as traditional Chinese medicine) is a traditional way to cure ailments and has been applied for more than five millennia. Even today, medicinal plants are well-known natural sources for the treatment of various diseases and continue to play a major role in primary health care as therapeutic agents in many developing countries. Lichens are among the most fascinating organisms on this planet. The genus *Usnea* (Usneaceae) is a large and hanging hair lichen (part fungus, part algae) that grows throughout the northern temperate zones, especially the sub-arctic and coastal rainforests of Europe, Asia and North America (Choudhary, Shaikh, Jalil, & Rahman, 2005; Guo et al., 2008). *Usnea* plants are valuable plant resources and have been used in food, fodder, dyes, perfumery, cosmetics, preservatives, deodorants, ecological applications and pharmaceuticals, and for miscellaneous purposes throughout the world, particularly in Europe and Eastern countries such as China, Japan and India (Guo et al., 2008; Laxinamujila, Bao, & Bau, 2013; Prateeksha et al., 2016; Shukla, Joshi, Rawat, Bucar, & Gibbons, 2010; Srivastava, Upreti, Dhole, Srivastava, & Nayak, 2013). *Usnea longissima* Ach. and *Usnea diffracta* Vain. (Chinese name: Song Lo) are endemic fruticose lichens found growing luxuriantly in China, mainly distributed in Heilongjiang, Jilin, Shanxi, Gansu, Sichuan, Yunnan, Guangxi, Guizhou, Zhejiang, Fujian, Taiwan Provinces and Inner Mongolia, Xinjiang and Tibet Autonomous Regions (Laxinamujila et al., 2013; Prateeksha et al., 2016; Shukla et al., 2010; Srivastava et al., 2013; Sun, Liu, Sun, Li, & Wang, 2016). As a well-known and customarily used folk medicine of Chinese, Mongolian, Tibetan and Uygur, *Usnea* has been recorded in the *Encyclopedia of Traditional Chinese Medicine* (Nanjing University of Chinese Medicine, 2003), the *Ministerial Drug Standards of the People's Republic of China* (Uygur Medicine Volume) (Chinese Pharmacopoeia Committee, 1998) and the I*llustration of Common Medicinal Plants in Taiwan* (The Committee on Chinese Medicine and Pharmacy Department of Health, Executive Tuan, 2011), as a treatment for various ailments such as dissolving phlegm, relieving cough, purging toxins in liver, detoxifying, clearing heat, stopping bleeding, treating insect infestations, and so on. It also can be used for treatment of human leg and loin injuries, bone fractures and female disease (Aalto-Korte, Lauerma, & Alanko, 2005; Guo et al., 2008; Hager, Brunauer, Turk, & Stocker-Worgotter, 2008), and ulcers (Cabanillas, Fernandez-Redondo, & Toribio, 2006; Halici et al., 2005). Modern pharmacological studies have confirmed that *Usnea* plants and bioactive secondary metabolites exert a wide variety of biological functions, including antibacterial and antimycobacterial, especially to the tubercle bacillus and Gram-positive bacteria (Honda et al., 2010; Ramos & Almeida, 2010), antipyretic, analgesic (Okuyama, Umeyama, Yamazaki, Kinoshita, & Yamamoto, 1995), anti-inflammatory (Vijayakumar et al., 2000), antiprotozoal, apoptosis-inducing and antitumour (Backorova et al., 2012; Singh, Nambiar, Kale, & Singh, 2013), antiviral (Shtro et al., 2014; Sokolov et al., 2012), wound healing promotion (Bruno et al., 2013; Burlando et al., 2009), photoprotective activity (Lohezic-Le, Legouin, Couteau, Boustie, & Coiffard, 2013), antioxidant enzyme activity and mucosal damage (Bayir et al., 2006; Halici et al., 2005; Rabelo et al., 2012; Vijayakumar et al., 2000).

Hundreds bioactive secondary compounds have been discovered from *Usnea* (Laxinamujila et al., 2013). Among them, the most typical secondary compounds unique to lichens are depsides and depsidones evernic acid (EA), barbatic acid (BA) and diffractaic acid (DA), and dibenzofuran derivative usnic acid (UA). The chemical structures of the standard substances are shown in Figure 1. Most of these depsides and dibenzofuran derivatives (especially UA) are responsible for most of the pharmacological effects aforementioned. During recent decades, as an abundant constituent of several lichen species, UA has been formulated into creams, toothpaste, mouthwash, deodorants, antibiotic ointments and sunscreen products. It has also been marketed as a dietary supplement for weight loss because of its ability to increase fat metabolism and to raise basic metabolic rate (Guo et al., 2008). However, recent reports associated with liver-related adverse events including mild hepatic toxicity, chemical hepatitis and liver failure requiring transplant (Foti et al., 2008) have stimulated researchers to discover the mechanism of action of UA and its analogs.

Data concerning UA toxicity mainly deal with the systemic use of UA containing preparations to promote weight loss (Durazo et al., 2004; Guo et al., 2008). Research on toxicity has proved that preparations containing equivalent concentrations of UA produced greater toxicity than pure UA. UA induced contact dermatitis and allergenicity have been reported as weak, mainly owing to the (−)-enantiomer, probably correlated to its general toxicity (Aalto-Korte et al., 2005; Abo-Khatwa, Al-Robai, & Al-Jawhari, 1996). It is necessary to employ new causality assessment strategies and accumulate more high-quality data to clarify the relationship between the toxicity, pharmacological activities and body exposure levels of UA and its analogs. However, there are few *in vivo* data on conventional characteristics of the absorption, distribution, metabolism, and excretion concerning UA and the other organic acids exist in *Usnea* (Foti et al., 2008; Krishna & Venkataramana, 1992; Venkataramana & Krishna, 1993).

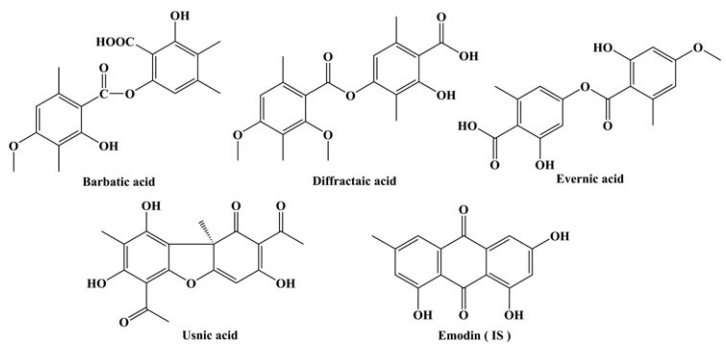


**FIGURE 1** Chemical structures of barbaric acid (BA), diffractaic acid (DA), evernic acid (EA), usnic acid (UA) and emodin (internal standard)

For the reason mentioned above, establishing conditions for the separation and detection of UA and other organic acids from crude materials, preparations and biological samples after consumption of UA-containing products or plant materials such as *U. longissima* was deemed urgent. So far, few studies on the pharmacokinetics or toxicokinetics have been reported for UA and other depsides in animals or humans (Zhao, Song, Tao, Zhang, & Wang, 2011). No research has been conducted to examine the pharmacokinetic differences between UA and depsides. Thus, it is extremely important to perform comparative research on pharmacokinetic and toxicokinetic differences of UA and depsides.

The UA content present in the plant materials and market products was analyzed by high-performance thin-layer chromatography with densitometric detection (Singh et al., 2016), reversed-phase high-pressure liquid chromatography (Cansaran, Kahya, Yurdakulola, & Atakol, 2006; Ji & Khan, 2005; Li et al., 2010; Smeds & Kytöviita, 2010; Zhao et al., 2011), and high-performance capillary electrophoresis (Zhou & Li, 2013). The lichen extracts were analyzed by high-performance liquid chromatography with mass spectrometric detection (HPLC–MS; Smeds & Kytöviita, 2010; Roach, Musser, Morehouse, & Woo, 2006) and by gas chromatography with flame ionization and MS detection (Smeds & Kytöviita, 2010). Recently, an HPLC-electrospray ionization-MS/MS method was developed for simultaneous determination of three phenolic acids, UA, DA, and ramalic acid, in *U. longissima* (Ma et al., 2015). In order to study the pharmacokinetics profiles of UA, DA, BA and EA in *U. longissima*, it is necessary to develop a sensitive and accurate method for simultaneous analysis of UA, DA, BA, and EA.

To the best of our knowledge, there are no publications on the use of ultra-high-performance liquid chromatography quadrupole–exactive orbitrap mass spectrometry (UPLC-Q-Exactive Orbitrap-MS) for the analysis of depsides and dibenzofuran derivatives in biological samples. In this study, we developed and validated a simple and sensitive method for the first time for the quantitatively analysis of UA, DA, BA, and EA with emodin as internal standard (IS) in rat plasma, using UPLC-Q-Exactive Orbitrap-MS in negative ionization full-scan mode. Furthermore, the method was successfully applied to study the pharmacokinetic profiles of the four targeted compounds in rats after intravenous and oral administration of UA and ethanol extract from *U. longissima*.

## 2 | MATERIALS AND METHODS

### 2.1 | Materials

*U. longissima* was collected at NyingChi, Tibet, China, in Aug 2015. The voucher specimen (UL-20150809) was authenticated by Professor Lihong Wu, the Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine and deposited at the Herbarium of Shanghai R&D Center for Standardization of Traditional Chinese, Shanghai, China. (+)-Usnic acid was obtained from Sigma (USA, lot number 3707312). Diffractaic acid was purchased from Shanghai Naiqi Biotechnology Co. Ltd (Shanghai, China, lot number 040070–201607). Emodin (IS, lot number 110756–200110, 98.0%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Evernic acid and BA were isolated from *U. longissima* in our laboratory and characterized by NMR and mass spectral data and comparison with literature values (Narui et al., 1998; Nishitoba, Nishimura, Nishiyama, & Mizutani, 1987). The purities of these compounds were determined to be >98% by HPLC analysis. HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific Co. (Santa Clara, CA, USA). Formic acid of HPLC grade was purchased from ANPEL Laboratory Technologies (Shanghai) Inc. (Shanghai, China). HPLC-grade water was obtained by a Milli-Q Academic System (Millipore, Billerica, MA).

### 2.2 | Preparation and chemical analysis of ethanol extracts from *U. longissima*

For preparation of the ethanol extracts from *U. longissima* (UE), the dried powder of *U. longissima* (50 g) was extracted under reflux with 750 mL ethanol–water (95:5, *v*/v) twice, 1 h each time, and then filtered. The combined filtrate was evaporated to dryness under vacuum and the residue was dried at 70°C to get UE. In order to calculate the administered dose, the contents of the four ingredients in UE were quantitatively determined by a validated HPLC method on an Agilent 1100 Infinity HPLC (Agilent technologies, Waldbronn, Germany) LC system. The separation was conducted on a Diamonsil $C_{18}$ column (250 × 4.6 mm, 5 μm) maintained at 30°C. The mobile phase consisted of acetonitrile (A) and aqueous 0.1% formic acid (B) at a flow rate of 1.0 mL $min^{-1}$ and eluted with gradient elution: 0–3 min (26% A), 3–10 min (26–74% A), 10–25 min (74–90% A) and 25–30 min (90% A). The injection volume was 20 μL. Finally, the contents of UA, EA, BA and DA in UE were determined as 13.7, 2.6, 1.0 and 0.6%, respectively (Wang, 2017).

### 2.3 | Animals and ethics statement

Sprague–Dawley rats (male and female, 180–230 g) were provided by the Experimental Animal Center of Shanghai University of Traditional Chinese Medicine [permit number SCXK (Hu) 2013–0016]. The animals were housed with free access to food and water and maintained on a 12 h light and dark cycle (lights on from 7:00 to 19:00) at environmental temperature (22–24°C) and 60–65% relative humidity for 7 days. Before the experiments, all rats were fasted for 12 h with free access to water and housed in wire-bottomed cages. Animal maintenance and experiments were approved by the Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (approval number ACSHU-2015-002) and guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

### 2.4 | Instrument and chromatographic conditions

For separation, chromatographic analysis was performed with an Acquity UPLC BEH $C_{18}$ column (50 × 2.1 mm, 1.8 μm, Waters Corporation, Milford, MA, USA) tandem with a guard column using a Thermo Scientific™ Q Exactive™ Quadrupole-Orbitrap Mass Spectrometer system (Thermo Fisher Scientific Inc., Grand Island, NY, USA). The UPLC system consisted of a Thermo Scientific Dionex Ultimate 3000 Series RS pump coupled with a Thermo Scientific Dionex Ultimate

3000 Series TCC-3000RS column compartments and a Thermo Fisher Scientific Ultimate 3000 Series WPS-3000 autosampler controlled by Chromeleon 7.2 Software. The cooling autosampler was set at 10°C and protected from light, and the column heater was set at 30°C. A 0.5% formic acid in methanol solution and 0.5% formic acid in deionized water were treated as mobile phases A and B, respectively. The mobile phase gradient elution was programmed as follows: 0–4min, linear from 70 to 90% A; 4–5 min, 90% A; 5–5.1 min, linear from 90 to 70% A; 5.1–6.5 min, 70% A. The flow rate of the mobile phase was set at 0.3 mL/min and the injection volume was 3 μL.

A Thermo Scientific™ Q Exactive™ Quadrupole-Orbitrap Mass Spectrometer system (Thermo Fisher Scientific Inc., Grand Island, NY USA) was connected to the UPLC system via a heat electrospray ionization interface and controlled by Xcalibur 4.1 software was used for data capture and analysis. The electrospray ionization source was operated optimized in negative ionization mode using a full-scan mass spectrum: full-scan range, 50.0–750.0 *m/z*; scan resolution, 70,000 FWHM (*m/z*/s); high quality (internal standard calibration accuracy <10 ppm); sheath gas ($N_2$) flow rate, 35 arbitrary units; auxiliary gas ($N_2$) flow rate, 10 arbitrary units; spray voltage, 2.00 kV; capillary temperature, 320°C; s-lens RF level, 50.0; auxilliary gas heater temperature, 300°C.

## 2.5 | Stock solutions, standards samples, and quality control samples

Stock solutions of BA, DA, EA and UA with concentrations of 204.0, 197.6, 206.8 and 203.6 μg $mL^{-1}$ were prepared separately in a 25 mL brown volumetric flask by dissolving the proper amount of each standard substance in methanol. A series of working solutions were obtained and serially diluted with the initial mobile phase (70% methanol) to provide the required concentrations for calibration standards (CS) and quality controls (QC). Stock solution of emodin (internal standard, IS) with a concentration of 82.4μg $mL^{-1}$ was prepared by dissolving the proper amount of the standard substance in a 25 mL volumetric flask with methanol. IS working solution (103.0 ng $mL^{-1}$) was prepared by diluting the stock solutions in methanol. All of the solutions were stored at 4°C and brought to room temperature before use.

## 2.6 | Sample preparation

For CS and QC samples, 50 μL aliquots of CS or QC working solutions were added to 20μL of blank plasma, followed by the addition of IS working solution (100 μL), formic acid (10 μL) and methanol (150 μL). For unknown samples, 20 μL of plasma was spiked with 100 μL IS solution, 10 μL of formic acid and 200 μL of methanol. The mixtures were vortexed for 30 s and centrifuged at 15,000 ***g*** for 10 min at 4 °C. The supernatant (250 μL) was evaporated to dryness with a gentle stream of nitrogen (37°C). The residues were dissolved in 200 μL of initial mobile phase and centrifuged at 15,000 ***g*** for 10 min. The supernatants (3 μL) were applied to the UPLC-Q-Exactive Orbitrap-MS analysis.

## 2.7 | Full bioanalytical method validation

The method was validated in accordance with current acceptance criteria for bioanalytical method validation by the US Food and Drug Administration (FDA). For all validation tests, imprecision is expressed by the RSD (%) on results tables, and inaccuracy as the mean percentage of error RE (bias) with regard to the theoretical (or nominal) values. Following the FDA guidance for industry, the imprecision (RSD) must not exceed 15% for all levels [20% for the lower limit of quantitation (LLOQ) is the exception], and the inaccuracy (RE) must be within ±15% of the nominal value for all levels (±20% of the nominal value for the LLOQ as exception).

### 2.7.1 | Selectivity and carry-over

The selectivity was evaluated by comparing the chromatographic behavior of the blank plasma, the drug-containing plasma and blank plasma spiked with 5.0 ng $mL^{-1}$ (LLOQ) of standards and 103.0 ng $mL^{-1}$ of IS, to investigate whether there were endogenous substances present. The imprecision (RSD) had to be <20%, and the inaccuracy (RE) must be within ±20% of the nominal value.

The carry-over test was performed in triplicate with one blank preparation sample injected immediately after the ULOQ sample (3200 ng $mL^{-1}$ for BA, DA, EA and UA) along with the IS. The peak area in blank sample injected after the ULOQ calibrator had to be <20% of the peak area of the LLOQ calibrator for all standard compounds, and <5% for IS.

### 2.7.2 | Linearity, lower limit of detection and lower limit of quantification

Eight CS working solutions were prepared in five replicates of each concentration and the calibration curves were established by plotting peak area ratios (*y*) of the product to the internal standard vs the respective true standard concentration (*x*) using weighed (1/*x*) least squares regression analysis or quadratic equation of nonlinear regression analysis. The lower limit of detection (LLOD) was defined as the lowest concentration of the analytes with a signal to-noise ratio at 3:1, and the LLOQ was defined as the lowest concentration on the calibration curves for which an acceptable accuracy (within 80–120%) and precision (< 20%) were obtained.

### 2.7.3 | Within-run and between-run precision

Precision was assessed by analyzing the replicates of QC samples ($n = 5$) at five concentration levels (5.0, 10.0, 250.0, 1600.0 and 3200.0 ng $mL^{-1}$ for BA, DA, EA and UA). The within-run precision was evaluated by repeating analysis of the standards five times during a single analytical run, and the between-run precision was determined by repeating analysis of the standards during three consecutive days. The imprecision was required to not exceed 15% (20% at the LLOQ) and the inaccuracy had to be within ±15% (±20% at the LLOQ) of the nominal value. The reproducibility was assessed on the same QC determinations on at least three series during 3 days.

### 2.7.4 | Extraction recovery and matrix effects

The extraction recoveries of BA, DA EA and UA were determined at three QC levels (10.0, 250.0 and 1600.0 ng $mL^{-1}$ for BA, DA, EA and UA) with five replicates by comparing the mean peak areas from blank plasma samples spiked before extraction with those from blank plasma

samples spiked after extraction. The extraction recovery of the IS was determined using the same method.

The effect of rat plasma constituents on BA, DA, EA, UA and IS was determined by comparing the full-scan mass peak responses of the pretreatment plasma standard QC samples mixed with rat plasma ($n = 5$) with those of the corresponding analytes in the initial mobile phase ($n = 5$). The matrix effects were implied if the ratio was <85% or >115%.

#### 2.7.5 | Dilution test

In order to assess the reliability of the method at concentration levels outside the calibration range, 10 replicates of QC samples at 5000 ng $mL^{-1}$ were prepared. Five were diluted at 1:10 and five at 1:100 with post-extracted blank plasma solution containing IS to bring the concentration into the required calibration range. Following the FDA guidance for industry, the mean concentration, the imprecision (RSD) and the inaccuracy (RE) were calculated for each dilution factor. The imprecision (RSD) had to not exceed 15%, and the inaccuracy (RE) had to be within 15% of the nominal value.

#### 2.7.6 | Biological sample stability

The stability of BA, DA, EA and UA in rat plasma was assessed by analyzing replicates ($n = 5$), which were spiked with standards at low, medium and high QC samples (10, 250 and 1600 ng $mL^{-1}$ for BA, DA, EA and UA) during the sample storage and processing procedures. The stability validation of BA, DA, EA and UA was investigated under different conditions. Five aliquots of QC samples were stored at −20°C for 1 month to test long-term stability and stored for 24 h at room temperature (AT, 25 ± 2°C) for short-term stability. The freeze–thaw stability was determined after thawing at room temperature and freezing at −20°C for three cycles on consecutive days. Post-preparation stability was estimated by analyzing QC samples within 3 days in the autosampler at 4°C. The calibration curves of freshly prepared standards were used for all stability tests of QC samples. The acceptable precision and accuracy should be within ±15%, and the low QC samples should not exceed 20%.

### 2.8 | Pharmacokinetic study

Pharmacokinetic experiments were performed in 30 rats that were randomly divided into three groups: one intravenous dosage group was intravenously injected UA at dosage of 0.5 mg $kg^{-1}$ (dissolved in normal saline and adjusted pH to 6.5 with 0.1 M NaOH) to rats by vena caudalis and two oral dosage groups were orally administered UA at a dosage of 5.0 mg $kg^{-1}$ and UE at dosage of 35 mg $kg^{-1}$ (dissolved in normal saline and adjusted pH to 6.5 with 0.1 M NaOH, at equivalent dose of UA 5.0 mg $kg^{-1}$) by gavage with gauge syringe, separately. Blood samples (~0.15 mL in each sampling time point) from the retinal vein plexus were collected into a 1.5 mL EP tube pretreated with heparin sodium, according to the specific scheduled time intervals (at different time intervals of 2, 5, 10, 15, 20, 30, 60, 120, 240, 480, 720, 1440 and 2160 min after intravenous administration of UA and 2, 5, 15, 30, 45, 60, 120, 240, 480, 720, 1440, 2160 after oral administration of UA and UE). After each blood sampling, 0.15 mL sterile isotonic saline was injected intraperitoneally to compensate for blood loss. The blood samples were centrifuged at 5000 ***g*** at 4°C for 10 min to obtain plasma. The plasma samples were stored at −20°C until UPLC-Q-Exactive Orbitrap-MS analysis.

The intravenous and oral administration doses applied in present animal study were extrapolated as 1/500 and 1/50 from the median lethal dose ($LD_{50}$) of UA (Cheng et al., 2009), respectively. A preliminary experiment was performed and the results indicated that these doses of animal experiment did not show any toxicity properties after oral administration of UA and UE (data not shown).

### 2.9 | Data analysis

Experimental data and the pharmacokinetic parameters were expressed as the mean ± standard deviation. The pharmacokinetic (PK) parameters, including the maximum peak concentration ($C_{max}$), the time of maximum plasma concentration ($T_{max}$), the terminal elimination half-life ($t_{1/2}$), the elimination rate constant ($K_e$), the mean residence time (MRT), the volume of distribution at terminal phase ($Vz$), the clearance rate ($CL$), area under the plasma concentration vs time curve from zero to time $t$ ($AUC_{0-t}$) and area under the plasma concentration vs time curve extrapolated to infinity ($AUC_{0-\infty}$) were determined by noncompartmental analysis using WinNonlin software (version 5.2.1, Pharsight Corporation, St Louis, MO, USA). The $AUC_{0-t}$ was obtained directly from the observed concentration vs time data. The $AUC_{0-\infty}$ was calculated by means of the trapezoidal rule with extrapolation to infinity with the elimination rate constant ($K_e$).

## 3 | RESULTS AND DISCUSSION

### 3.1 | Chromatography performance, selectivity and carry-over

The representative chromatograms of the blank plasma, blank plasma spiked with the four targeted analytes at 5.0 ng $mL^{-1}$ (LLOQ) and internal standard at 103.0 ng $mL^{-1}$, and the drug-containing plasma sample (concentrations of each analyte were about 43.86, 313.58, 24.47 and 650.04ng $mL^{-1}$ for BA, DA, EA, and UA, respectively) are shown in Figure 2. Satisfactory performances in terms of peak intensity, shape and elution time were found, with the use of 0.5% formic acid in water as solvent A and 0.5% formic acid in methanol as solvent B. A gradient elution program within 8.0 min, including a reconditioning time for the next analysis, was further established and optimized to ensure better separation and fast elution of all of the target analytes and IS. The analytes and IS peaks were defined with retention times of 2.89 min (EA), 3.26 min (IS), 3.54 min (DA), 4.40 min (UA) and 4.70 min (BA). In drug-free plasma sample there were no peaks or interference from endogenous substances observed in the positions of all analyte channels. The carry-over test for BA, DA, EA, UA and IS at the upper limit of quantification did not show any carry-over effect to the blank sample.

A difficult problem was encountered with peak trailing for UA but not for BA, DA, EA and IS. Various mobile phases were evaluated for chromatographic behavior and the ionization responses of BA, DA, EA, UA and IS: methanol–water (1), acetonitrile–water (2), acetonitrile (0.1% formic acid)–water (0.1% formic acid) (3), methanol (0.1% formic

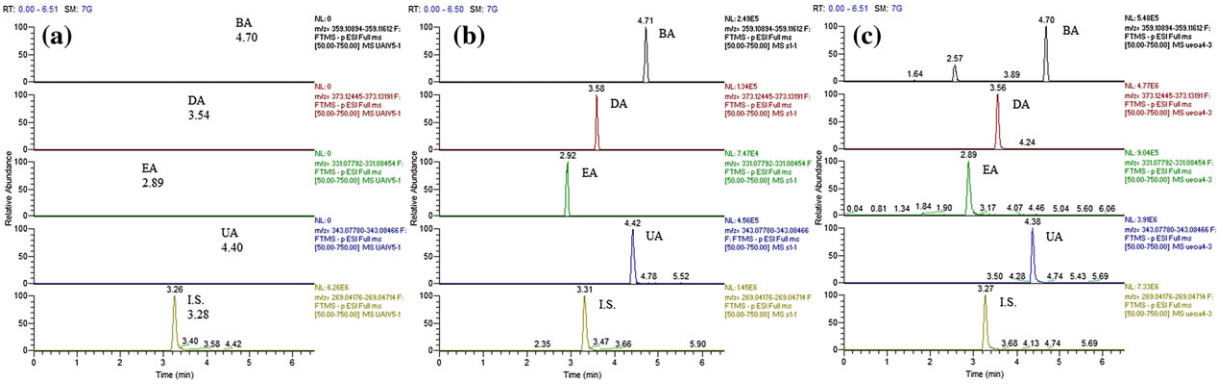


**FIGURE 2** Representative full-scan chromatograms of blank rat plasma sample (a), blank plasma spiked with BA, DA, EA and UA at 250 and 103.0 ng $mL^{-1}$ of IS (b) and plasma sample spiked with IS after oral administration of UE at a dose of 35 mg $kg^{-1}$ (c)

acid)–water (0.1% formic acid) (4), acetonitrile (0.5% formic acid)–water (0.5% formic acid) (5) and methanol (0.5% formic acid)–water (0.5% formic acid) (6). Better peaks shape and ionization responses were obtained when using methanol system and reducing the mobile phase pH, which reduces interactions with silanols that cause peak tailing. The best response and peak shape were obtained from a gradient delivery of a mixture of methanol (0.5% formic acid)–water (0.5% formic acid). The overall chromatographic run time was <6.5 min.

## 3.2 | Optimization of plasma sample preparation method

Biological sample preparation is a critical procedure for development of reliable and accurate LC–MS assays to eliminate interference from the sample matrix. A rapid, simple and cost-effective sample preparation method is necessary for pharmacokinetic studies, especially when a large number of samples must be analyzed. Liquid–liquid extraction (LLE), protein precipitation (PPT), and solid-phase extraction (SPE) are commonly used techniques in the preparation of biological samples for improving the sensitivity and robustness of evaluation. Firstly, LLE was employed to extract the targeted analytes from plasma with ethyl acetate as organic solvent, in order to obtain satisfactory recoveries and matrix effects. Actually, satisfactory matrix effects ranging from 97.5 to 118.4% for BA, DA, EA, UA and IS were obtained. However, very unsatisfactory extraction yields (recoveries) scattered from 8.4 to 69.6% for BA, DA, EA, UA and IS. Lastly, a one-step PPT method was adopted which provided high recoveries and satisfactory matrix effects for the four target analytes and IS in the present study. Compared with LLE and SPE, the sample preparation procedure of a PPT method is simple and low-cost. Methanol rather than acetonitrile was chosen as the protein precipitant for its satisfactory efficiency in precipitating.

## 3.3 | Linearity, LLOD and LLOQ

The seven-point calibration curves showed good linearity within the tested ranges of 5–3200 ng $mL^{-1}$ with $R^2$ from 0.9970 to 0.9986 for BA, DA and EA in rat plasma. The slopes and intercepts obtained from typical calibration curves of all analytes are shown in Table 1. It can be seen that UA shows a curve response with quadratic regression. The data were fitted with quadratic equation and showed a good relationship between mass response and UA concentration. The LLOQs were 5.10, 4.94, 5.17 and 5.09 ng $mL^{-1}$ and the LLODs were 1.02, 0.95, 1.02 and 1.03 ng $mL^{-1}$ for BA, DA, EA and UA separately, with acceptable limits of accuracy and precision. The result confirmed that the UA blood concentration and the mass spectrum signal response could be more accurately fitted with a quadratic equation. Different calibrator concentrations were observed to impact the accuracy of quadratic regression for liquid chromatography–mass spectrometry bioanalysis (Cunliffe, Noren, Hayes, Clement, & Shen, 2009; Tan, Awaiye, & Trabelsi, 2014; Yuan, Zhang, Jemal, & Aubry, 2012). However, the root cause is not clear, and it requires further investigation.

## 3.4 | Within-run and between-run precision

At all concentration levels, the within- and between-run imprecision and inaccuracy of BA, DA, EA and UA were evaluated at five concentration levels (5.0, 10.0, 250.0, 1600.0 and 3200.0 ng $mL^{-1}$) by repeated determination ($n$ = 5) of QC samples. The within-run imprecision expressed as intra-run RSD did not exceed 7.1% at the LLOQ, and

**TABLE 1** Representative calibration curves, lower limit of detection (LLOD) and lower limit of quantitation (LLOQ) of barbaric acid (BA), diffractaic acid (DA), evernic acid (EA), usnic acid (UA) ($n$ = 5)

| Analytes | Equation | $R^2$ | Linear range (ng $mL^{-1}$) | LLOD (ng $mL^{-1}$) | LLOQ (ng $mL^{-1}$) |
|---|---|---|---|---|---|
| BA | $y = 0.0013 + 0.0313\times$ | 0.9980 | 5.10–3264.00 | 1.02 | 5.10 |
| DA | $y = 0.0523 + 0.0408\times$ | 0.9970 | 4.94–3161.60 | 0.95 | 4.94 |
| EA | $y = 0.0459 + 0.0386\times$ | 0.9986 | 5.17–3308.80 | 1.02 | 5.17 |
| UA | $y = 0.1900 + 0.0540\times - 6.1751^{e-006}\times^2$ | 0.9925 | 5.09–3257.60 | 1.03 | 5.09 |

10.1% at other concentration levels. Inaccuracy expressed as intra-run RE was between −13.4 and 5.1% (Table 2). The between-run imprecision expressed as inter-run RSD was <12.0%, and the inaccuracy expressed as inter-run RE was between −7.1 and 6.3% (Table 2). Both within- and between-run inaccuracy and imprecision of the assay were within FDA bioanalytical method validation guidance acceptance criteria, which demonstrated that the method is consistent and precise at different sample concentrations.

## 3.5 | Extraction yield and matrix effect

The extraction yield of the assay was expressed as the recovery rate of QC samples at low, medium and high concentration levels (10.0, 250.0 and 1600.0 ng $mL^{-1}$ for BA, DA, EA and UA). The recoveries of BA, DA, EA and UA were within the ranges of 98.4–100.4%, 99.2–102.5%, 101.0–102.1% and 98.0–102.4%, respectively (Table 2). The extraction yield for IS was 94.22%. Thus, the recoveries of all analytes were consistent and reproducible across the entire range.

Matrix effect was tested as described in Section 2.7.4. The values of mean (B)/mean (A) 100% of all analytes at three concentrations of QC samples and at a single concentration of IS were within the acceptable limits (95.3–107.5%, Table 2). Thus, no significant matrix effect for BA, DA EA, UA and IS was observed, indicating that ion suppression and enhancement from plasma were negligible for this method.

## 3.6 | Dilution test

Ten pooled blank rat plasma QC samples higher than the upper limit of the calibration curve at concentration of 5000 ng $mL^{-1}$ for BA, DA EA and UA were diluted by factors of 10 and 100 with post-extracted blank plasma solution containing the IS. The resulting concentrations were multiplied by the dilution factor. For both dilution factors, the imprecision (RSD) was <5.19% and the inaccuracy (RE) was between −0.9 and 1.8% (Table 3). Thus, the dilution had no effect on the precision and accuracy of the results.

## 3.7 | Biological sample stability

Five replicates of rat plasma QC samples at low, medium and high concentration levels (10.0, 250.0 and 1600.0 ng $mL^{-1}$ for BA, DA, EA and UA) were tested in rat plasma under different conditions that the samples might experience between preparation and analysis. As summarized in Table 4, all three levels of analytes in rat plasma were stable at room temperature up to 24 h with RSD <7.0%. All analytes at three levels were stable when kept in the autosampler (4 °C) for 3 days with RSD <8.9%. After three cycles of freeze and thaw for QC samples, all analytes at three levels were stable in plasma with RSD <6.3%. All analytes were stable at −20°C for at 1 month with RSD <8.7%. The results from all stability tests indicated that the analytes were stable under routine laboratory conditions. The method therefore proved to be applicable for routine analysis.

**TABLE 3** Dilution test (mean ± SD, $n = 5$)

| Analytes | Nominal concentration (ng $mL^{-1}$) | Dilution factor | Detected concentration (ng $mL^{-1}$) | RSD (%) | RE (%) |
|---|---|---|---|---|---|
| BA | 5100 | 10× | 5025.58 ± 186.66 | 4.4 | −0.9 |
| | | 100× | 5084.70 ± 263.85 | 5.2 | −0.3 |
| DA | 4940 | 10× | 5008.31 ± 178.68 | 3.6 | 1.4 |
| | | 100× | 5033.09 ± 105.64 | 2.1 | 1.8 |
| EA | 5170 | 10× | 5190.89 ± 155.91 | 3.0 | 0.4 |
| | | 100× | 5147.25 ± 148.37 | 2.9 | −0.4 |
| UA | 5090 | 10× | 5145.21 ± 190.50 | 3.7 | 1.1 |
| | | 100× | 5180.58 ± 241.97 | 4.7 | 1.8 |

**TABLE 2** Results of within-run, between-run precision of quality control (QC) samples, recoveries and matrix effects ($n = 5$)

| Analytes | Nominal level (ng $mL^{-1}$) | Within-run precision Re (%) | Within-run precision RSD (%) | Between-run precision (%) | Between-run precision (%) | Recovery (%) | Matrix effect (%) |
|---|---|---|---|---|---|---|---|
| BA | 5.10 (LLOQ) | −8.3 | 6.0 | −6.1 | 6.5 | | |
| | 10.20 (QCL) | −4.3 | 5.5 | 0.4 | 5.3 | 100.2 ± 5.2 | 100.1 ± 3.1 |
| | 255.00 (QCM) | −5.3 | 5.0 | −1.8 | 5.2 | 100.4 ± 5.2 | 104.1 ± 3.6 |
| | 1632.00 (QCL) | −3.7 | 7.1 | −2.8 | 2.8 | 98.4 ± 4.9 | 95.3 ± 2.2 |
| | 3264.00 (ULOQ) | 3.4 | 8.7 | 6.3 | 2.6 | | |
| DA | 4.94 (LLOQ) | −13.4 | 4.8 | 0.4 | 12.0 | | |
| | 9.88 (QCL) | −9.0 | 6.4 | −0.6 | 7.3 | 102.5 ± 7.4 | 105.1 ± 9.9 |
| | 247.00 (QCM) | −4.9 | 7.9 | −3.3 | 4.1 | 100.4 ± 4.2 | 101.9 ± 6.0 |
| | 1580.00 (QCH) | −3.0 | 7.0 | −2.5 | 2.0 | 99.2 ± 6.6 | 96.1 ± 5.1 |
| | 3161.60 (ULOQ) | 6.2 | 6.3 | 4.3 | 1.8 | | |
| EA | 5.17 (LLOQ) | −11.7 | 7.1 | −6.0 | 5.8 | | |
| | 10.34 (QCL) | −5.9 | 10.1 | −3.8 | 3.0 | 102.1 ± 12.1 | 96.7 ± 6.1 |
| | 258.50 (QCM) | −2.9 | 8.7 | −6.4 | 3.8 | 101.0 ± 7.1 | 100.6 ± 4.0 |
| | 1654.40 (QCH) | −1.1 | 6.1 | −7.1 | 6.4 | 101.6 ± 3.5 | 99.0 ± 5.0 |
| | 3308.80 (ULOQ) | 2.7 | 6.6 | −2.2 | 4.5 | | |
| UA | 5.09 (LLOQ) | 5.1 | 6.7 | 1.5 | 4.0 | | |
| | 10.18 (QCL) | −2.7 | 5.2 | −1.1 | 6.2 | 102.4 ± 7.4 | 101.1 ± 9.9 |
| | 254.50 (QCM) | −11.7 | 5.4 | −4.3 | 7.4 | 98.0 ± 11.2 | 100.0 ± 9.4 |
| | 1628.80 (QCH) | −1.3 | 7.5 | −6.6 | 5.1 | 100.5 ± 4.1 | 95.8 ± 3.9 |
| | 3257.60 (ULOQ) | 5.5 | 2.0 | −3.1 | 7.8 | | |
| IS | 103.0 | / | 5.4 | / | 4.3 | 94.2 ± 7.1 | 107.5 ± 10.1 |

QCH, Quality control high; QCL, quality control low; QCM, quality control medium; IS, internal standard.

**TABLE 4** Stability of targeted analytes in rat plasma under different storage conditions ($n = 5$)

| | | BA | | DA | | EA | | UA | |
|---|---|---|---|---|---|---|---|---|---|
| Conditions | Levels | Mean ± SD (ng mL$^{-1}$) | RSD (%) | Mean ± SD (ng mL$^{-1}$) | RSD (%) | Mean ± SD (ng mL$^{-1}$) | RSD (%) | Mean ± SD (ng mL$^{-1}$) | RSD (%) |
| AT | QCL | 11.77 ± 0.56 | 4.8 | 11.26 ± 0.64 | 5.7 | 12.46 ± 0.87 | 7.0 | 12.98 ± 0.07 | 0.5 |
| | QCM | 274.15 ± 11.20 | 4.1 | 259.37 ± 10.22 | 3.9 | 268.75 ± 9.33 | 3.5 | 290.29 ± 7.51 | 2.6 |
| | QCH | 1677.30 ± 64.32 | 3.8 | 1579.19 ± 60.72 | 3.8 | 1683.61 ± 70.34 | 4.2 | 1883.69 ± 38.81 | 2.1 |
| 4 °C | QCL | 9.72 ± 0.59 | 6.1 | 8.98 ± 0.64% | 7.1 | 9.98 ± 0.87 | 8.7 | 10.84 ± 0.71 | 6.6 |
| | QCM | 245.58 ± 17.49 | 7.1 | 239.46 ± 16.73 | 7.0 | 255.70 ± 15.50 | 6.1 | 251.29 ± 18.81 | 7.5 |
| | QCH | 1534.70 ± 129.06 | 8.4 | 1488.83 ± 133.06 | 8.9 | 1637.21 ± 98.23 | 6.0 | 1432.31 ± 83.87 | 5.9 |
| Freeze–thaw | QCL | 9.71 ± 0.56 | 5.8 | 10.70 ± 0.44 | 4.1 | 10.08 ± 0.41 | 4.1 | 12.84 ± 0.30 | 3.0 |
| | QCM | 233.48 ± 14.61 | 6.3 | 246.65 ± 12.24 | 5.0 | 236.31 ± 14.80 | 6.3 | 273.45 ± 4.09 | 0.3 |
| | QCH | 1446.37 ± 74.46 | 5.2 | 1529.42 ± 95.79 | 6.3 | 1597.13 ± 89.70 | 5.6 | 1550.14 ± 209.97 | 1.5 |
| −20 °C | QCL | 8.90 ± 0.66 | 7.4 | 11.35 ± 0.85 | 7.4 | 9.00 ± 0.79 | 8.7 | 11.52 ± 1.00 | 8.6 |
| | QCM | 237.86 ± 10.51 | 4.4 | 251.34 ± 11.63 | 4.6 | 256.69 ± 16.76 | 6.5 | 232.29 ± 15.45 | 6.6 |
| | QCH | 1495.56 ± 108.01 | 7.2 | 1519.74 ± 66.67 | 4.4 | 1627.67 ± 84.03 | 5.2 | 1640.17 ± 168.77 | 10.3 |

## 3.8 | Pharmacokinetic study

### 3.8.1 | Pharmacokinetics after intravenous and oral administration of UA

The UPLC-MS/MS method was then applied to an *in vivo* pharmacokinetic study of UA in rats after a single intravenous and oral administration. The intravenous and oral doses were determined as 0.5 and 5 mg kg$^{-1}$ through the pre-experiment, which ensured the detection of the components, while not causing adverse reactions owing to the overdosing of UA.

The plasma concentration vs time profiles and the pharmacokinetic parameters derived from the plasma profiles after a single intravenous administration of UA at dose of 0.5 mg kg$^{-1}$ in rats are shown in Figure 3(a) and Table 5, respectively. As illustrated in Figure 3(a), the UA plasma concentration vs time curve produced a sharp decline in concentration followed by a slower phase of decrease with $T_{1/2ke}$ of 2.06 ± 0.54h until the levels fell below the detection limits, within 24 h after intravenous administration. The $AUC_{0-\infty}$ value of UA after intravenous injection of UA at a dose of 0.5 mg kg$^{-1}$ was 1689.02 ± 502.79 ng h mL$^{-1}$.

After oral administration of UA at dose of 5.0 mg kg$^{-1}$, UA could be absorbed into blood and was found in rat plasma at various sampling points, reaching a maximum plasma concentration ($C_{max}$) of 1126.03 ± 358.86 ng mL$^{-1}$ within 6.67 ± 1.51 h. After reaching $C_{max}$, UA plasma concentration declined with a $T_{1/2ke}$ of 3.48 ± 0.75 h. The $AUC_{0-\infty}$ value of UA after intravenous injection of UA at a dose of 5.0 mg kg$^{-1}$ was 11,685.96 ± 3447.93 ng h mL$^{-1}$. The absolute bioavailability ($F_{ab}$) values of UA derived from the AUC (Table 5) of rats that had received UA after oral administration at dose of 5.0 mg kg$^{-1}$ was 69.2%, in comparison with the $AUC_{0-\infty}$ following intravenous injection at a dose of 0.5 mg kg$^{-1}$.

### 3.8.2 | Pharmacokinetics of BA, DA, EA and UA after oral administration of UE

After oral administration of UE at doses of 35 mg kg$^{-1}$, equivalent to doses of 0.35 mg kg$^{-1}$ for BA, 0.22 mg kg$^{-1}$ for DA, 0.90 mg kg$^{-1}$ for EA and 4.81 mg kg$^{-1}$ for UA, the four target analytes were found in rat plasma at various sampling points. The plasma concentration vs time profiles and the pharmacokinetic parameters derived from the plasma profiles of UA, BA, DA and EA after oral administration of UE are shown in Figure 3(b) and Table 5.

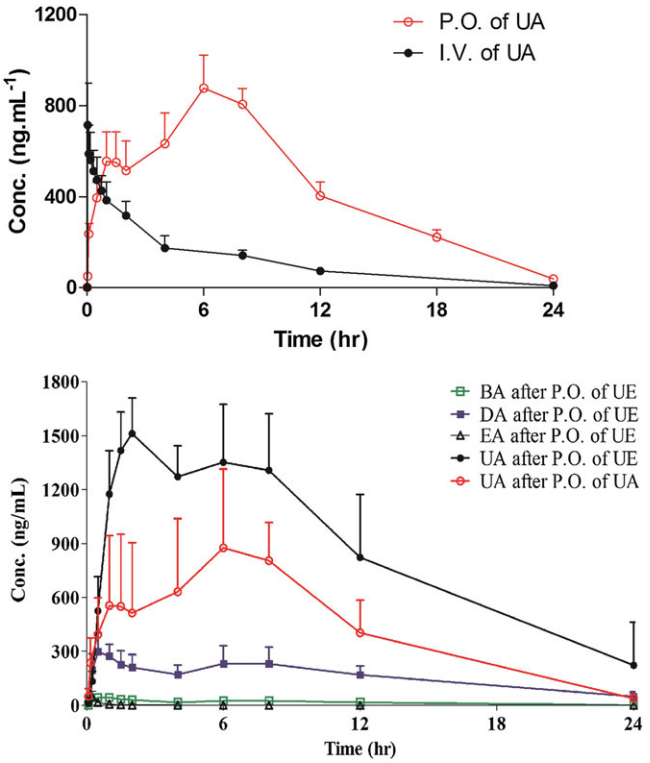


**FIGURE 3** Mean plasma concentration–time profiles of UA after intravenous and oral administration of UA at doses of 0.5 and 5 mg kg$^{-1}$ respectively in rats (a) and mean plasma concentration–time profiles of UA, BA, DA and EA after oral administration of UE at a dose of 35 mg kg$^{-1}$ and UA at a dose of 5 mg kg$^{-1}$ in rats (b)

After oral administration of UE at a dose of 35.0 mg kg$^{-1}$, EA could be absorbed rapidly into blood and reached a $C_{max}$ of 54.09 ± 36.65 ng mL$^{-1}$ within 0.19 ± 0.09 h. After reaching $C_{max}$, EA plasma concentration declined with a $T_{1/2ke}$ of 0.75 ± 0.52 h until the levels fell below the detection limits, within 12 h after administration. BA could be absorbed into blood, but more slowly than EA, and was found in rat plasma, reaching a $C_{max}$ of 50.88 ± 17.85 ng mL$^{-1}$ within 0.58 ± 0.20h. After reaching $C_{max}$, BA plasma concentration declined slowly with a

**TABLE 5** Pharmacokinetics parameters of UA after intravenous and oral administration at dose of 0.5 and 5 mg/kg as well as the pharmacokinetics parameters of BA, DA, EA, and UA after oral administration of UE at dosage of 35 mg/kg in rats (Mean ± SD, $n = 8$)

| Pharmacokinetics parameters | Intravenous administration | Oral administration | | | | |
|---|---|---|---|---|---|---|
| | UA | UA | BA from UE | DA from UE | EA from UE | UA from UE |
| Dose (mg $kg^{-1}$) | 0.5 | 5.0 | 0.35 | 0.22 | 0.90 | 4.81 |
| $T_{max}$ (h) | / | 6.67 ± 1.51 | 0.58 ± 0.20 | 1.94 ± 2.51 | 0.19 ± 0.09 | 3.19 ± 1.89 |
| $C_{max}$ (ng $mL^{-1}$) | / | 1126.03 ± 358.86 | 50.88 ± 17.85 | 335.21 ± 85.47 | 54.09 ± 36.65 | 1573.76 ± 200.44 |
| $K_e$ ($h^{-1}$) | 0.36 ± 0.10 | 0.20 ± 0.04 | 0.10 ± 0.06 | 0.10 ± 0.04 | 1.46 ± 1.12 | 0.15 ± 0.08 |
| $t_{1/2}$ (h) | 2.06 ± 0.54 | 3.48 ± 0.75 | 10.24 ± 8.67 | 9.01 ± 6.28 | 0.75 ± 0.52 | 7.48 ± 7.26 |
| $AUC_{0-t}$ (ng h $mL^{-1}$) | 1651.31 ± 471.97 | 11,451.13 ± 3323.41 | 308.01 ± 85.71 | 3797.34 ± 846.96 | 29.91 ± 11.12 | 19,955.82 ± 3945.01 |
| $AUC_{0-\infty}$ (ng h $mL^{-1}$) | 1689.02 ± 502.79 | 11,685.96 ± 3447.93 | 466.13 ± 137.84 | 4604.28 ± 836.27 | 31.50 ± 11.39 | 24,169.76 ± 8108.70 |
| MRT (h) | 2.41 ± 0.41 | 8.56 ± 1.46 | 6.10 ± 1.51 | 9.00 ± 0.76 | 0.63 ± 0.29 | 8.31 ± 1.30 |
| *Vz* (mL $kg^{-1}$) | 908.23 ± 202.06 | 2238.20 ± 405.02 | 27,135.45 ± 17,325.86 | 618.89 ± 405.18 | 13,837.29 ± 12,694.86 | 1371.45 ± 822.32 |
| *CL* (mL·h $kg^{-1}$) | 323.42 ± 107.00 | 460.65 ± 122.77 | 2068.96 ± 567.20 | 49.52 ± 11.25 | 12,217.68 ± 3840.16 | 159.07 ± 49.66 |
| $F_{ab}$ (%) | / | 69.2 | / | / | / | 146.9 |
| $F_{re}$ (%)[a] | / | / | / | / | / | 212.4 |
| $F_{re}$ (%)[b] | / | / | 10.4 | 103.7 | 0.7 | 100 |

[a]Relative to $AUC_{0-\infty}$ of UA after oral administration of pure UA;

[b]relative to $AUC_{0-\infty}$ of UA after oral administration of UE.

$T_{max}$, Time of maximum plasma concentration; $C_{max}$, maximum peak concentration; $K_e$, elimination rate constant; $t_{1/2}$, terminal elimination half-life; $AUC_{0-t}$, area under the plasma concentration vs time curve from zero to time $t$; $AUC_{0-\infty}$, area under the plasma concentration vs time curve extrapolated to infinity; MRT, mean residence time; *Vz*, volume of distribution at terminal phase; *CL*, clearance rate; $F_{ab}$, absolute bioavailability; $F_{re}$, relative bioavailability.

$T_{1/2ke}$ of 10.24 ± 8.67 h until the levels fell below the detection limits, within 24 h after administration. Compared with EA and BA, with the lowest administration dose among the four target markers in UE, DA presented higher exposure levels in the body after oral administration of UE, which could be absorbed rapidly into blood and was found in rat plasma, reaching a $C_{max}$ of 335.21 ± 85.47 ng $mL^{-1}$ within 1.94 ± 2.51 h. After reaching $C_{max}$, the DA plasma concentration declined slowly with a $T_{1/2ke}$ of 9.01 ± 6.28 h, until the levels at 24 h after administration could also be detected. From the view of the concentration in UE, the UA content and dose administered in UE was the highest. After oral administration of UE, UA could be absorbed rapidly into blood and reached a $C_{max}$ of 1573.76 ± 200.44 ng $mL^{-1}$ within 3.19 ± 1.89 h. After reaching $C_{max}$, UA plasma concentration declined with a $T_{1/2ke}$ of 7.48 ± 7.26 h. Compared with the $AUC_{0-\infty}$ value (11,685.96 ± 3447.93 ng h $mL^{-1}$) after oral administration of pure UA alone, a larger $AUC_{0-\infty}$ value (24,169.76 ± 8108.70 ng h $mL^{-1}$) of UA after oral administration of UE was obtained. By the dose conversion method, the absolute bioavailability ($F_{ab}$) and relative bioavailability ($F_{re}$) after oral administration of UA from UE reached 146.9 and 212.4%, respectively. The results indicate that some unknown ingredients present in UE could prompt UA absorption after oral administration of UE or delay UA elimination from body. In addition, compared with the $F_{re}$ value of UA in UE, the relative bioavailability of DA reached 103.7%; however, the relative bioavailability of BA and EA only reached 10.4 and 0.70%. In other words, the bioavailability of DA was equivalent to that of UA, but the bioavailabilities of BA and EA were lower than that of UA.

As common phenolic acids present in *U. longissima*, it can be seen from Figure 3 and Table 5 that UA, BA, DA, and EA showed distinct pharmacokinetic characteristics. These may be not due only to differences in structure, but also to the pharmacokinetic interaction within the concurrent ingredients in *U. longissima*. The accurate mechanism is poorly understood further work is necessary to discovered it.

## 4 | CONCLUSION

A UPLC-Q-Exactive Orbitrap-MS method was developed and validated for the simultaneous determination of BA, DA, EA and UA in plasma. The method developed here is selective, precise, accurate, specific and capable of producing reliable results. The results indicated that BA, DA, EA and UA could be absorbed into blood after oral administration of UE. The absolute bioavailability of UA after oral administration was 69.2%. The absolute bioavailability ($F_{ab}$) and relative bioavailability ($F_{re}$) after oral administration of UA from UE reached 146.9 and 212.4%, respectively. UA, BA, DA and EA showed distinct pharmacokinetic characteristics, possibly not only owing to its difference in structure, but also owing to pharmacokinetic interaction within the concurrent ingredients presented in *U. longissima*. However, the unknown mechanism requires further investigation.

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## DECLARATION OF INTEREST

The authors report that they have no conflict of interest.

## AUTHORS' CONTRIBUTIONS

Changhong Wang, Xinhong Wang, Xuemei Cheng, and Hanxue Wang conceptualized, planned and designed the study; Sukfan Kwong prepared the extract; Hanxue Wang and Tao Yang carried out the experiments and drafted the manuscript; Chenghai Liu and Rui An assisted in the analysis of data; Hanxue Wang, Guowen Li, Xinhong Wang, and Changhong Wang finalized the manuscript. All the authors read and approved the final manuscript prior to submission.

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