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An improved LC-MS/MS method for the determination of mangiferin in rat plasma and its application in nonlinear pharmacokinetics

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A rapid and sensitive LC-MS/MS method was developed and validated for the determination of mangiferin in rat plasma. After simple protein precipitation of the plasma sample (100 μL) with 120 μL acetonitrile containing the internal standard rutin (500 ng/mL), the analytes were separated on a Zorbax SB-C_{18} column (150 × 2.1 mm, 3.5 μm) using an eluent of acetonitrile-0.05% formic acid in water (18:82, v/v), and then detected by electrospray ionization mass spectrometry in the negative multiple reaction monitoring mode with a chromatographic run time of 3.0 min. The method was sensitive, with a lower limit of quantification of 1 ng/mL and good linearity ($r > 0.998$) over the range of 1–250 ng/mL. It was also specific, precise and accurate when it was used to measure mangiferin levels in plasma and to characterize the pharmacokinetic properties following oral administration of mangiferin at a single dose of 5, 15, 45 and 90 mg/kg in rats. In addition, the pharmacokinetics of mangiferin were found to be nonlinear over the above dose range, which provides insight into dose regimen design of this potent compound in new drug development.

1. Introduction

Mangiferin is a natural glucosyl xanthone (2-C-β-D-glucopyranosyl-1,3,6,7- tetrahydroxyxanthone, Fig. 1) (Schieber et al. 2003) existing in *Mangifera indica*, *Anemarrhena asphodeloides*, *Belamcanda chinensis* and other medicinal plants. It has been reported to have various biological activities, including antioxidant, antiinflammatory, antimicrobial, antidiabetic and anticancer activities, with the antioxidant and anticancer activities most predominant (Vyas et al. 2012).

In view of the great clinical potential of mangiferin, it is important to develop a valid analytical method to determine mangiferin in relevant biological samples for the sake of gaining a better understanding about its pharmacokinetic characteristics. Although there have been several studies reporting the determination of mangiferin in biological samples by HPLC-UV (Liu et al. 2012; Hou et al. 2010; Zhang et al. 2010; Wang et al. 2006; Dai et al. 2004), these assays lack the sensitivity (lower limit of quantification, LLOQ > 80 ng/mL) to determine low drug concentrations in an oral pharmacokinetic study. LC-MS/MS has also been reported as a method of determining mangiferin in plasma, but it needs a time-consuming plasma pretreatment (He et al. 2012; Han et al. 2010) and requires a relatively large plasma volume (200 μL, Liu et al. 2010) or long analytical run time (15 min, Sun et al. 2013; 12 min, Suryawanshi et al. 2007).

We developed an improved LC-MS/MS method for rapid and sensitive determination of mangiferin in rat plasma, requiring single-step protein precipitation, a low plasma volume of 100 μL, an LLOQ of 1 ng/mL, and a short analytical time of 3 min. The new method has been successfully applied to a pharmacokinetic study of mangiferin administered orally at doses of 5, 15, 45 and 90 mg/kg in rats. We have also found that the pharmacokinetics of mangiferin are nonlinear over the above dose range.

2. Investigations, results and discussion

2.1. Method development

To determine mangiferin using tandem mass spectrometry, the full scan and product ion spectra of the analyte and internal standard (IS) were investigated. In the negative electrospray ionization mode, mangiferin and rutin mainly formed deprotonated molecular ions $[M–H]^-$ at *m/z* 421.1 and *m/z* 609.2 in full scan mass spectra, respectively. After optimization of the fragmentor voltage and collision energy, the dominant product ions for mangiferin and rutin were *m/z* 301.1 and 299.9, respectively. Thus, the multiple reaction monitoring (MRM) of the precursor-product ion transitions *m/z* 421.1→301.1 for mangiferin and *m/z* 609.2→299.9 for rutin were monitored for quantification. The fragmentation profile of each compound under optimized MS/MS conditions is shown in Fig. 1.

The mobile phase composition and types of column were optimized through several trials to achieve good resolution and symmetric peak shapes for the analytes. As glucoside compounds are to some extent hydrophilic, they would be little retained by lipophilic stationary phase. A number of C_{18} and C_8 columns were tested. After careful comparison of these columns, a Zorbax SB-C_{18} column (150 × 2.1 mm, 3.5 μm) was chosen, on which symmetric peak shapes were achieved. Eventually, a mixture of acetonitrile-0.05% formic acid in water (18:82, v/v) was adopted to achieve efficient chromatographic separation of the analytes and the endogenous plasma components for reducing the matrix effect and shortening the running time. Rutin was

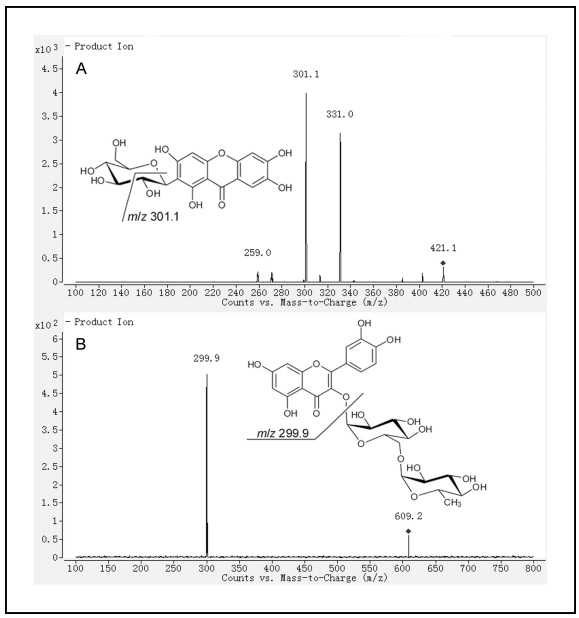

Fig. 1: Structures and product ion spectra of (A) mangiferin and (B) rutin.

chosen as the IS because it was found to have similar retention time (Fig. 2), recovery and ionization properties comparable to mangiferin.

Sample preparation is a critical step for an accurate and reliable LC-MS/MS method. Although protein precipitation (PPT) generally yields a stronger matrix effect than other sample preparation methods like liquid-liquid extraction and solid phase extraction, it is simpler and faster. Hence, PPT was chosen as the isolation procedure. To maintain the symmetric peak shape and avoid a strong matrix effect, the supernatant was diluted with water before being injected into the LC-MS/MS system.

2.2. Method validation

2.2.1. Selectivity

The selectivity of the method was tested by comparing the chromatograms of six different lots of blank plasma and the spiked plasma. All blank plasma lots were found to be free of interferences with the compounds of interest. Figure 2 shows the typical chromatograms of a blank plasma, blank plasma spiked with mangiferin at LLOQ (1 ng/mL) and the IS, and plasma sample obtained at 2 h after single-dose administration of 30 mg/kg mangiferin in a rat. The retention time of mangiferin and the IS under the above conditions was 1.4 and 2.4 min, respectively.

2.2.2. Linearity and LLOQ

A typical calibration equation was $y = 3.4915x - 0.0004$ ($r = 0.999$), where y represents the peak area ratio (analyte/IS) and x represents the relative concentration (analyte/IS). Calibration curves were linear in the range of 1–250 ng/mL with the regression coefficients consistently greater than 0.998. The present method offered an LLOQ of 1 ng/mL with relative standard deviation (RSD) of 8.4% and deviation from the nominal values (bias) of 14.2% in five different rat plasma samples, independent of the calibration curve. The LLOQ was sufficient for a pharmacokinetic study in rats after oral administration of mangiferin.

2.2.3. Precision and accuracy

Five replicate samples at each quality control (QC) concentration were analyzed in three separate days. Table 1 shows the summary of the individual QC data obtained in three days used for the validation. The precision ranged from 7.4 to 8.8% and 8.0 to 9.3% for intra- and inter-day determination, respectively. The accuracy ranged from – 2.1 to 13.9% and – 3.8 to 6.5% for intra- and inter-day, respectively. The QC data indicate the reliability and accuracy of the LC-MS/MS method following PPT in determination of mangiferin in rat plasma.

2.2.4. Matrix effect and extraction efficiency

Knowing that the presence of salts and endogenous materials in analyzing the supernatant from a plasma sample using PPT could cause ion suppression or enhancement leading to higher variations (Van Eeckhaut et al. 2009), assessment of the matrix effect is critical for reliable evaluation of the newly developed LC-MS/MS method. The mean value of absolute

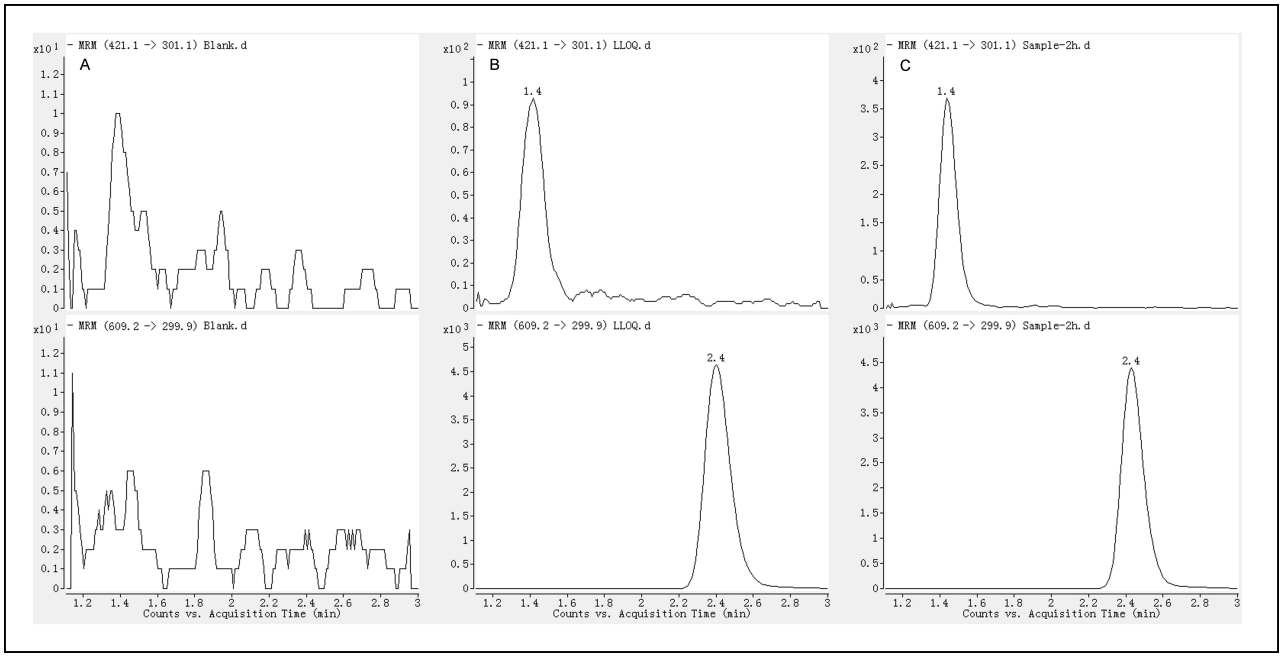

Fig. 2: Representative MRM chromatograms: (A) blank plasma, (B) blank plasma spiked with mangiferin at 1 ng/mL (LLOQ) and IS, and (C) plasma sample collected from a rat at 2 h after a single oral dose of 30 mg/kg mangiferin (concentration: 30.4 ng/mL).

Table 1: Precision and accuracy for mangiferin in rat plasma

Spiked concentration (ng/mL)	Intra-day ($n = 5$)			Inter-day ($n = 15$)		
	Mean ± SD (ng/mL)	RSD (%)	Bias (%)	Mean ± SD (ng/mL)	RSD (%)	Bias (%)
2	2.28 ± 0.17	7.4	13.9	2.13 ± 0.17	8.0	6.5
20	19.6 ± 1.7	8.8	− 2.1	19.2 ± 1.8	9.3	− 3.8
200	200 ± 17	8.5	0.4	202 ± 42	8.3	1.2

matrix effect for mangiferin at 2, 20 and 200 ng/mL was 157.6 ± 6.7%, 158.3 ± 9.7% and 162.8 ± 8.4%, respectively, suggesting that there was a significant effect of ion enhancement under the experimental conditions, but the matrix effect was consistent and concentration-independent. Thus, despite the matrix effect observed, the present analytical method is stable and reliable. The extraction efficiency for mangiferin ranged from 90.8 ± 4.2% to 92.6 ± 6.5% over the entire QC concentration range. The matrix effect for the IS (500 ng/mL) was 99.8–103.4% and the extraction efficiency exceeded 75.0%.

2.2.5. Stability

The stability of mangiferin at three QC levels (2, 20 and 200 ng/mL) was studied under various conditions. The percentage deviation over spiked concentrations was − 2.1% to 0.3% at room temperature for 4 h, − 4.3% to 1.5% at − 20 °C for two weeks, 1.1% to 2.4% in the autosampler at room temperature for 12 h after PPT, and − 2.3% to 1.0% after three freeze-thaw cycles. The precision (RSD) did not exceed 6.4% in all stability tests of QC samples. The results indicate that mangiferin remained considerably stable under the conditions applied.

2.3. Application

The improved method has been successfully applied to analyze samples obtained from rats treated orally with mangiferin at doses of 5, 15, 45 and 90 mg/kg. The mean plasma concentration-time profiles at different doses are shown in Fig. 3. The pharmacokinetic parameters determined by noncompartmental analysis are listed in Table 2. The elimination half-life time ($t_{1/2}$) and mean residence time (MRT) remained roughly unaltered and were independent of the administered dose, implying that the elimination process was linear. Although the maximum plasma concentration (C_{max}) and area under the curve (AUC) were increased over the dose range, the increase in AUC with dose was smaller than the proportional change. In addition, the dose-normalized $AUC_{0\text{-}\infty}$ value at the 5 and 15 mg/kg doses was 3.1-fold and 3.5-fold higher than that for the 90 mg/kg dose, respectively. The result of linear regression also showed that there was not a good linear correlation between the dose and the value of $AUC_{0\text{-}\infty}$ ($r^2 = 0.836$) or C_{max} ($r^2 = 0.883$). These findings provide the explicit evidence for the nonlinear pharmacokinetics of mangiferin over the oral dose range of 5–90 mg/kg. Therefore, this characteristic should be considered in dose regimen design for new drug research. A saturable transport in the intestinal absorption could be the underlying cause for the observed nonlinear pharmacokinetic profile (Stewart et al. 1993; Ruiz-Carretero et al. 2004), but further detailed absorption studies are needed to confirm this.

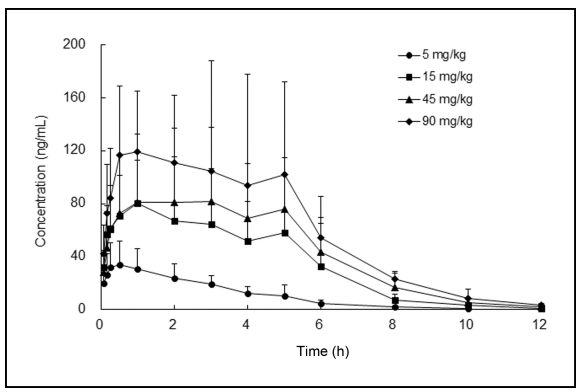

Fig. 3: Mean plasma concentration-time profiles of mangiferin after oral administration at different doses in rats ($n = 6$).

2.4. Conclusion

A rapid and sensitive LC-MS/MS method has been developed and validated for the determination of mangiferin in rat plasma. The method has proved to be highly sensitive with an LLOQ of 1 ng/mL for mangiferin. Plasma samples were pretreated with single-step PPT and analyzed under isocratic LC conditions. The total analysis time was 3.0 min per sample, thus offering an attractive procedure for high-throughput bioanalysis of mangiferin. The assay validation results were within the acceptable ranges for bioanalytical purposes. Mangiferin exhibited nonlinear pharmacokinetics in rats after oral administration at 5–90 mg/kg doses, which would provide insight into dosing regimen design for this potent compound in new drug development.

3. Experimental

3.1. Materials

Mangiferin (99.0% purity) and the IS rutin (99.0% purity) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile (HPLC grade) was obtained from Merck (Darmstadt, Germany). Formic acid was purchased from Tedia (Fairfield, OH, USA). Ultrapure water was produced by a Milli-Q Reagent Water System (Milford, MA, USA). All other chemicals were of analytical grade.

3.2. Analytical system

An Agilent LC-MS/MS system (Agilent Technologies, Palo Alto, CA, USA) consists of 1200 series liquid chromatography and a 6410 triple quadrupole mass spectrometer equipped with an electrospray ionization source. Data were acquired and analyzed with MassHunter Workstation Software (Agilent Technologies). The chromatographic separation was performed on an Agilent Zorbax SB-C_{18} column (150 × 2.1 mm, 3.5 μm) with an Agilent C_{18} guard column (12.5 × 2.1 mm, 5 μm) eluted at 0.3 mL/min with a mobile phase of acetonitrile-0.05% formic acid in water (18:82, v/v). The column temperature was maintained at 25 °C and the injection volume was 10 μL. The total analysis time was 3.0 min per sample.

The mass spectrometer was operated in the negative ion mode. Experiments were carried out carefully to optimize the instrument parameters for maximal generation of deprotonated molecules. The capillary voltage was set at 4000 V. Nitrogen served as nebulizing gas set at 40 psi and drying gas kept at 350 °C with 10 L/min gas flow for solvent evaporation. Deprotonated analyte molecules were subjected to collision induced dissociation using high purity nitrogen as the collision gas at 0.1 MPa to yield product ions. The MRM of the precursor-product ion transitions *m/z* 421.1→301.1 for mangiferin and *m/z* 609.2→299.9 for the IS was monitored for quantification. The fragmentor voltage was 125 V for mangiferin and 100 V for the IS. The optimized collision energy was 23 eV for mangiferin and 35 eV for

Table 2: Pharmacokinetic parameters of mangiferin after oral administration at different doses in rats (mean ± SD, $n = 6$)

Parameter	Oral dose (mg/kg)			
	5	15	45	90
T_{max} (h)	0.6 ± 0.3	1.0 ± 0.6	3.3 ± 2.0	1.5 ± 1.5
C_{max} (ng/mL)	37.3 ± 17.8	86.7 ± 41.5	93.7 ± 53.2	151.5 ± 76.8
$AUC_{0\text{-}t}$ (ng h/mL)	120.7 ± 46.4	414.8 ± 252.5	521.1 ± 296.0	715.2 ± 355.6
$AUC_{0\text{-}\infty}$ (ng h/mL)	122.6 ± 47.1	417.7 ± 251.9	523.9 ± 296.2	720.9 ± 355.5
$AUC_{0\text{-}\infty}$/dose (mg h/mL)	24.5 ± 9.4	27.8 ± 16.8	11.6 ± 6.6	8.0 ± 3.9
$t_{1/2}$ (h)	1.5 ± 0.4	1.3 ± 0.3	1.4 ± 0.3	1.5 ± 0.4
$MRT_{0\text{-}\infty}$ (h)	2.8 ± 0.3	3.3 ± 0.4	3.8 ± 0.4	3.7 ± 0.4

the IS. The chemical structure and product ion mass spectra for mangiferin and the IS are shown in Fig. 1.

3.3. Preparation of standards and quality controls

The stock solution of mangiferin was prepared in methanol-dioxane (80:20, v/v) to obtain a final concentration of 200 μg/mL. Working standard solutions at 20, 50, 100, 200, 500, 1000, 2000 and 5000 ng/mL were prepared from the stock solution with acetonitrile-water (50:50). A stock solution of 200 μg/mL for rutin (IS) was prepared in methanol and then was further diluted with acetonitrile to yield a working solution of 500 ng/mL.

Calibration standards were prepared by spiking 95 μL rat blank plasma with 5 μL working standard solutions of mangiferin to give nominal concentrations of 1, 2.5, 5, 10, 25, 50, 100 and 250 ng/mL. For each validation and assay run, the calibration curve standards were prepared freshly from the working standard solutions. QC samples used in validation and pharmacokinetic study were independently prepared at three level concentrations of 2 (low QC), 20 (medium QC) and 200 ng/mL (high QC). The QC samples were stored at − 20 °C and brought to room temperature before being processed together with the biological samples.

3.4. Sample preparation

A simple and rapid protein precipitation method was used for the preparation of plasma samples. A 100 μL aliquot of plasma was treated with 120 μL acetonitrile containing the IS rutin (500 ng/mL). The mixture was vortex mixed for 2 min and centrifuged at 13,000 × *g* for 10 min. Then 150 μL supernatant was added to 300 μL water. After mixing, the solution was transferred to a 1.5 mL autosampler vial and 10 μL of the solution was injected into the LC-MS/MS system for analysis.

3.5. Validation procedures

A full validation was performed to evaluate the performance of the method in accordance to the recommendations published by FDA (2001). A calibration curve ranging from 1 ng/mL to 250 ng/mL of mangiferin was used in each run by plotting the peak area ratios of the analyte to IS against the nominal standard curve concentrations. Least-squares linear regression was used for curve fitting with $1/x$ as the weighting factor.

Intra- and inter-day validation study for precision and accuracy was performed at three QC levels (2, 20 and 200 ng/mL) with five replicate samples analyzed in each day. The criteria for acceptability of data induced a precision within 15% RSD and accuracy within ± 15% bias. The LLOQ of the assay was determined as the lowest concentration on the standard curve that could be quantitated with a precision of ≤20% and accuracy of 80–120%.

The matrix effect and extraction efficiency were examined by comparing the peak areas of the analytes between three different sample sets at three levels (Matuszewski et al. 2003). The first set (set 1) was prepared in the mobile phase; the second set (set 2) was prepared in plasma extracts originating from five different rats and spiked after extraction; and the third set (set 3) was prepared in plasma from the same five different rats as in set 2, but the plasma samples were spiked before extraction. The absolute matrix effect was assessed by comparing the mean peak areas of the analyte in set 2 to that of the neat standards in set 1. The extraction efficiency of mangiferin and the IS was determined by calculating the ratios of the mean peak areas of the regularly prepared plasma samples in set 3 to that of the spiked post-extraction samples in set 2.

Analyte stability evaluation comprised short-term stability, long-term stability, autosampler stability and freeze-thaw stability, which were determined by analyzing three QC levels in quintuple. The mean values and standard deviations of the ratios between the measured concentrations and the spiked concentrations were used for stability evaluation. The QC samples were analyzed after storage at room temperature for 4 h, at − 20 °C for two weeks, in the autosampler at room temperature for 12 h after protein precipitation and after three freeze-thaw cycles, which consisted of storage at − 20 °C for a minimum of 12 h, followed by thawing at room temperature.

3.6. Pharmacokinetic study

The improved LC-MS/MS method was used to investigate the plasma profiles of mangiferin after oral administration at doses of 5, 15, 45 and 90 mg/kg (suspended in 0.5% CMC-Na) in 24 clean grade male SD rats (200–220 g, SCXK2007–0005) obtained from the Animal Center of the Chinese Academy of Sciences in Shanghai, China. The experiments were carried out according to the Guidelines for the Care and Use of Laboratory Animals, and were approved by the Animal Ethics Committee of the Second Military Medical University. The animals were fasted overnight and equally randomized to four groups prior to dosing. Blood samples (about 0.25 mL) were collected from the postorbital venous plexus veins in heparinized tubes before administration and at 0.083, 0.17, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 8, 10 and 12 h after administration. Plasma was separated at 4 °C and stored at − 20 °C until analysis. The pharmacokinetic parameters of mangiferin were calculated from the plasma concentration-time data by non-compartmental analysis using the DAS 2.0 software program (Mathematical Pharmacology Professional Committee of China).

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