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Short communication

Quantification of limonin in human urine using solid-phase extraction by LC–MS/MS

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ABSTRACT

A highly sensitive liquid chromatography-tandem mass spectrometry method was developed and validated for the determination of limonin in human urine using podophyllotoxin as internal standard. The analyte and IS were extracted with solid-phase extraction and separated by a rapid isocratic elution with 1% formic acid/methanol (v:v, 40:60) on an C_{18} column (150 mm × 2.1 mm I.D.). The detection was performed by mass spectrometry in the multi-reaction-monitoring mode. The precursor to product ion transitions of m/z 471.3 → 161.2 and m/z 397.2 → 313.1 were used to measure the analyte and the IS. The assay was linear over the concentration range of 0.0783–10 ng/mL for limonin in human urine. The lower limit of quantification was 0.0783 ng/mL and the extraction recovery was larger than 76.7% for limonin. The inter- and intra-day precision of the method at three concentrations was less than 7.4%. The method was successfully applied to pharmacokinetic study of limonin in humans.

1. Introduction

Limonoids represent a group of triterpene derivatives found in the *Rutaceae* and *Meliaceae* families [1]. Approximately 38 limonoid aglycones and 20 limonoid glucosides have been identified from citrus and its hybrids [2]. Limonin is the most prevalent of the citrus limonoids and the major cause of the bitterness occurring in Citrus fruits and juices [3–5]. It is reported that limonin possesses various biological activities including anti-bacterial, anti-viral, anti-feedant, anti-nociceptive and anti-inflammatory [6–9]. Furthermore, the recent reports provided evidence that limonin has the chemopreventive potential to inhibit colon carcinogenesis [1], induct of phase II detoxifying enzymes [10] and suppress $CD4^+$ T-Cell proliferation and Interleukin-2 production [11].

Early publications have described high performance liquid chromatography equipped with ultraviolet detection (HPLC–UV) for the quantification of limonin in biological samples [2]. The previous HPLC methods suffer from disadvantages such as narrow linearity range, low sensitivity and time consuming. Nowadays, LC–MS methods have been just developed for differentiating some structurally closely related citrus limonoids aglycones due to its high sensitivity, selectivity and reproducibility [12–15]. Liang et al. [16] and Zhao et al. [17] developed LC–MS methods for determination of limonin in rat plasma, with LLOQ of 2 ng/mL for limonin. In the present study, a more sensitive and specific liquid chromatography-tandem mass spectrometry method (LC–MS/MS) was developed for the determination of limonin in human urine after oral administration.

2. Experimental

2.1. Reagents and chemicals

Limonin (purity> 96.8%) was kindly provided by Zhejiang Nanyang Pharmaceutical Group (Zhejiang, China), and podophyllotoxin (internal standard, IS, purity> 95.0%) (Fig. 1) was purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade methanol and acetonitrile was purchased from Merck (Darmstadt, Germany). Ultrapure water was purified by a Milli-Q system (Millipore, Bedford, MA, USA). Formic acid of HPLC-grade was purchased from Tedia (Fairfield, USA).

2.2. LC–MS/MS conditions

Analyses were performed by a series 1200 HPLC system (Agilent technologies, Palo Alto, CA, USA) coupled with a triple-quadrupole tandem API 4000 mass spectrometer (AB/MDS-Sciex, Concord, Ontario, Canada). The Analyst version 1.4.1 software was used for

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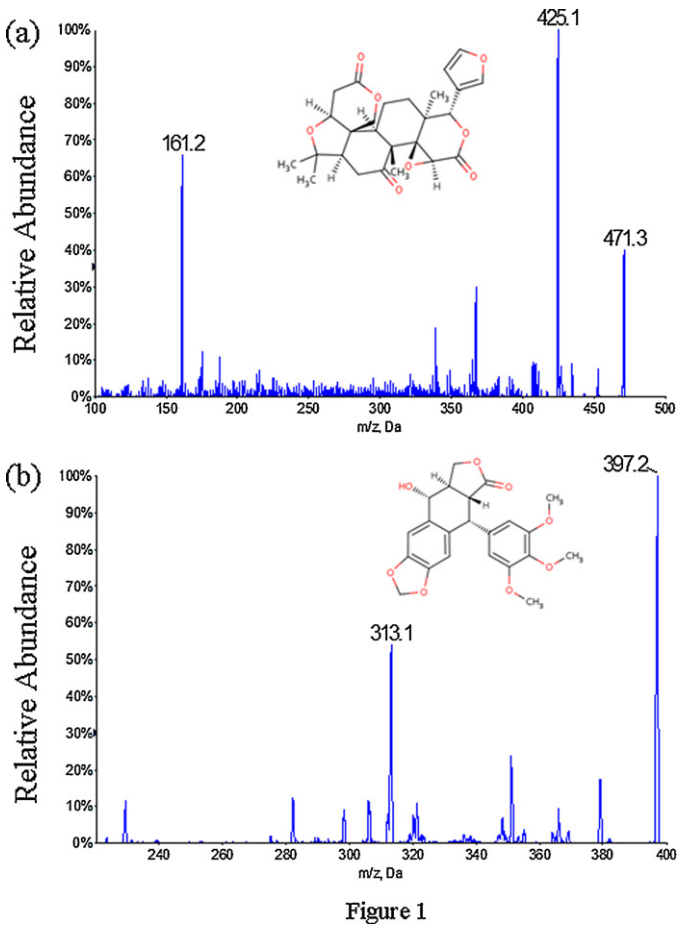

Fig. 1. Full-scan product ion spectra of $[M+H]^+$ ions and fragmentation pathways for limonin (A) and podophyllotoxin (B).

instrumental control, acquisition and processing of the data. The LC separation was performed on an Agilent Zorbax SB-C_{18} column (150 mm × 2.1 mm I.D., 5 μm, Agilent Technologies, Wilmington, DE, USA) with a security guard column (12.5 mm × 2.1 mm I.D., 5 μm, Agilent Zorbax SB-C_{18}, DE, USA). The mobile phase consisted of methanol and deionized water (v:v, 60:40) containing 1% formic acid at a flow rate of 0.35 mL/min. The autosampler temperature was maintained at 15 °C. The total LC run time was 5 min with the column temperature kept at 30 °C.

A MS detector with an electrospray ionization (ESI) interface in positive ion mode was used for quantitative analysis. Quantitation was performed using the multi-reaction-monitoring (MRM) mode of transitions of m/z 471.3 → 161.2 for limonin, m/z 397.2 → 313.1 for podophyllotoxin. The optimized conditions used for the ESI^+ source were as follows: capillary voltage: 4.5 kV; turbo heater temperature: 400 °C; curtain gas (CUR): 20 psi; collision activation dissociation (CAD): 10 psi; declustering potential (DP): 100 V; collision energy (CE): 30 eV for limonin and 27 eV for IS, respectively.

2.3. Preparation of standard and quality control (QC) samples

The standard stock solutions were prepared by dissolving limonin (197.6 μg/mL) and podophyllotoxin as an IS (988 μg/mL) in methanol. The stock solution of limonin was successively diluted with methanol to a series of proper concentrations of working solutions. All working solutions were stored in a refrigerator (4 °C). These solutions were spiked into drug-free human urine samples to obtain final concentrations levels of 0.0783, 0.157, 0.313, 0.625, 1.25, 2.5, 5, and 10 ng/mL. Quality control (QC) samples were prepared at concentrations of approximately 0.1, 1 and 8 ng/mL in the same way as the urine samples for calibration. All the standard calibration samples and QC samples were stored at −20 °C.

2.4. Sample preparation

Urine samples were subjected to solid-phase extraction (SPE) prior to derivatization in order to remove salts and other interferences. SPE was performed using Oasis HLB 30 mg cartridges (Waters, Etten-Leur, The Netherlands). The cartridges were conditioned and equilibrated with 1.0 mL of methanol and 1.0 mL of water, respectively. Subsequently, an aliquot of 1.0 mL urine and 10 μL of IS (0.988 μg/mL) were added into a 10 mL centrifuge tube. After vortex-mixing for 30 s, the mixed samples were loaded, and the cartridges were washed with 1.0 mL of 5% methanol and vacuum dried for 1 min. The analytes were eluted with 1.0 mL of methanol and the eluates were evaporated to dryness in the centrifugal thickener (Centrivap console, Labconco Co., USA) at 50 °C for 40 min, the residue was reconstituted in 100 μL mobile phase and vortexed for 2 min, and then centrifuged for 10 min at 12,000 × g. A 5 μL supernatant was injected onto the LC–MS/MS system for analysis.

2.5. Method validation

The method was fully validated for selectivity, matrix effect (M.E.), linearity, lower limits of quantification (LLOQ), accuracy and precision, recovery and stability, which was carried out according to FDA guidance for bioanalytical method validation.

To evaluate assay specificity, eight independent lots of human blank urine were analyzed for excluding any enhumanenous co-eluting interference by comparing them with the assay of a urine sample spiked with analytes and a urine sample obtained after oral administration.

The matrix effect was defined as the ion suppression/enhancement on the ionization of analytes, which was evaluated by comparing the area response of post-extraction blank urine samples spiked with limonin at three QC levels (A) to those of the equivalent concentration standard solutions dried directly and reconstituted with the same mobile phase (B). The ratio ($A/B \times 100$)% was used to evaluate the matrix effect. The same procedure was performed for the IS.

Five calibration curve of limonin was performed with eight non-zero concentrations. Calibration curve was constructed by plotting the area ratios of the analyte/internal standard (Y) versus the concentrations of the analyte (X) in the form of $Y = A + BX$, using weighted ($1/x^2$) least squares linear regression. The LLOQ was defined as the minimum concentration at which the analyte could be quantified with acceptable accuracy and precision (R.S.D. < 20%).

The precision was described as the relative standard deviation (R.S.D.) of replicate measurements and the accuracy was evaluated as the ratio of calculated versus theoretical concentrations. Three QC samples ($n = 5$) were analyzed in each batch assay to determine within-run precision (R.S.D.) and accuracy, and analyzed in each of three different batch assays to determine between-run precision and accuracy of the method.

The extraction recovery experiments were estimated by comparing the analytical results of extracted samples at three concentrations with pure standards without extraction.

The freeze and thaw stability study QC samples ($n = 5$) were stored at −20 °C and subjected to two freeze-haw cycles. The short-term stability of limonin, was performed by repeated injection every 4 h for a period of 24 h during storage in the autosampler at 4 °C. The long-term stability of limonin in urine was assessed in three concentration levels after storage at −20 °C for 1 month.

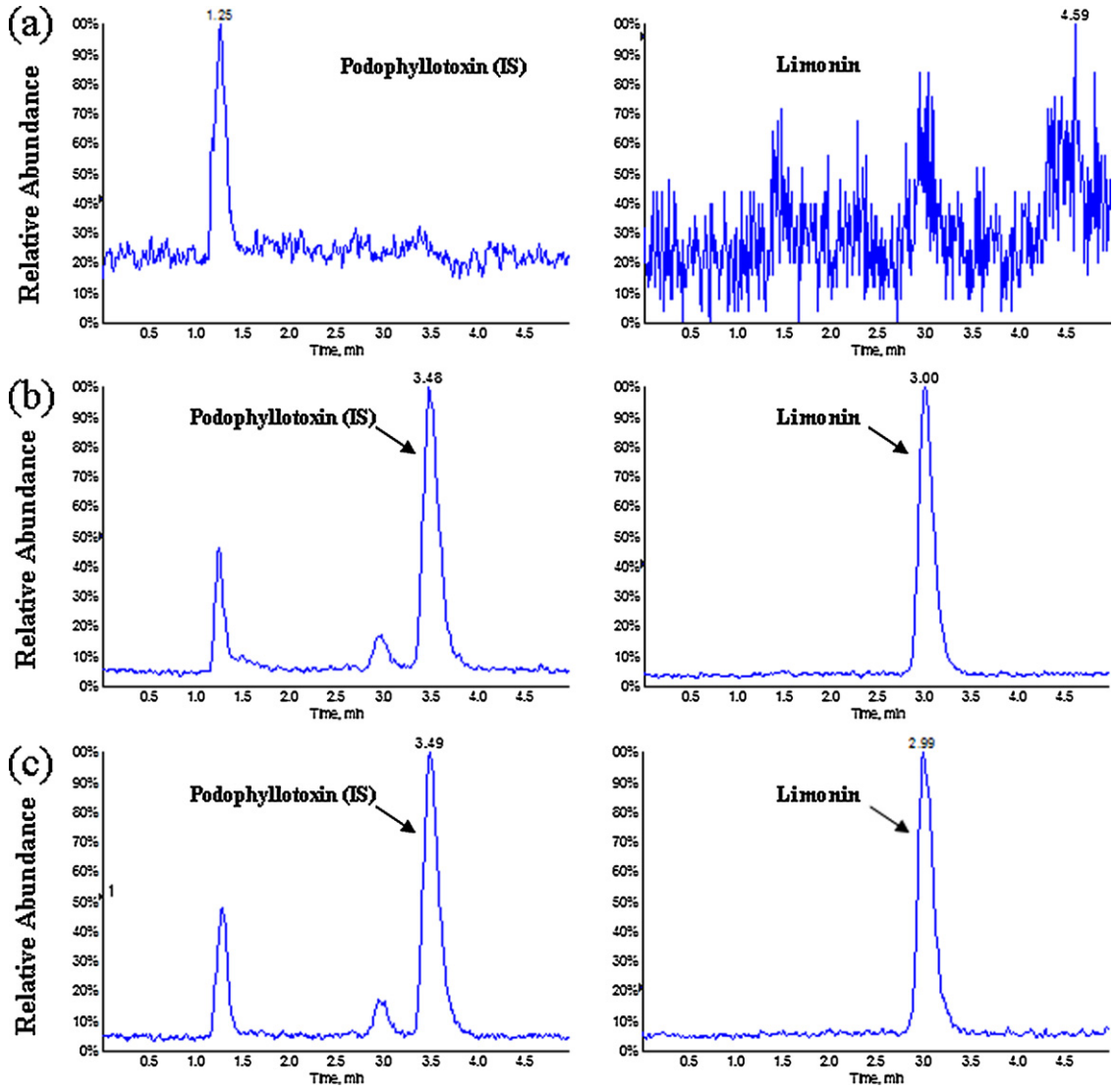

Fig. 2. Typical MRM chromatograms of limonin and podophyllotoxin. (A) A blank human urine sample; (B) a blank urine spiked with limonin (0.1 μg/mL^{-1}) and IS (0.988 μg/mL^{-1}); (C) a human urine sample from the intervals of 4–12 h after oral administered of 120 mg limonin capsules spiked with IS.

2.6. Application to clinical studies

2.6.1. Test persons

The developed HPLC-ESI/MS method was applied to determine the urine concentrations of limonin from the Phase I clinical trial study in which 8 volunteers were enrolled (four female and four male). The study was approved by the Medical Ethics Committee of the Affiliated Hospital of Nanjing University of TCM. All volunteers gave written informed consent to participate in the study according to the principles of the Declaration of Helsinki. The average age was 24.6 years (s = 2.1) with an average body mass index of 21.6 (s = 1.6). Eight healthy volunteers were divided into four groups of two healthy male and female subjects administered four doses of limonin capsules (15, 30, 60, 120 mg).

2.6.2. Urine samples

To minimize the possible appearance of dietary limonoids in the blood, the 8 subjects avoided consumption of citrus fruit or juices for 3 days prior to and after the limonoid dose tests. Blank urine samples were collected pre-dose after a period of 48 h. Urine samples were collected over the intervals of 0–4, 4–12, 12–24, and 24–36 h post-dose. The exact volume of urine was recorded and stored at −70 °C before analysis.

3. Results and discussion

3.1. LC–MS optimization

Under the electrospray ionization conditions chosen, greater sensitivity and selectivity was achieved for limonin in MRM mode than SIR mode. Addition of 1% formic acid to the mobile phase was found to be an important factor for acquiring the high sensitivity. Fig. 1 shows that the deprotonated molecular ion m/z 471.3 for limonin and m/z 397.2 for podophyllotoxin were truly predominant in positive mode. The collision energy gave the most abundant production at m/z 161.2 and 425.1 for limonin. The mass spectrometric parameters were optimized to obtain the higher signal for the selected ion 425.1, but which showed more internal interference. The response to MRM mode of transitions of m/z 471.3 → 161.2 was more stable and linearity was better than of m/z 471.3 → 425.1 for limonin. Therefore, the precursor to product transition was assigned in MRM mode as follows: m/z 471.3 → 161.2 for limonin, and m/z 397.2 → 313.1 for podophyllotoxin.

Table 1
Accuracy, precision, matrix effect and recovery of the method. Data are expressed in percentage ($n = 5$).

Concentration (ng/mL)	Intra-day		Inter-day		Matrix effect	Recovery
	Accuracy	R.S.D.	Accuracy	R.S.D.		
0.1	99.8	6.4	99.9	5.9	96.2	82.1
1	97.4	4.7	101.1	7.4	91.4	76.7
8	100.3	3.3	100.0	3.2	92.9	78.7

Table 2
Stability of limonin ($n = 3$).

	AL nominal conc. (ng/mL)		
	0.1	1	8
Room temperature (4 h)			
Measured conc. (ng/mL)	0.103 ± 0.003	0.975 ± 0.056	8.113 ± 0.200
Accuracy (%)	103.3 ± 3.1	97.5 ± 5.6	101.4 ± 2.5
Three freeze/thaw cycles			
Measured conc. (ng/mL)	0.098 ± 0.008	0.982 ± 0.086	8.220 ± 0.338
Accuracy (%)	97.9 ± 7.7	98.2 ± 8.8	102.8 ± 4.1
Autosampler rack for 24 h			
Measured conc. (ng/mL)	0.099 ± 0.006	0.997 ± 0.090	8.147 ± 0.306
Accuracy (%)	99.3 ± 5.7	99.7 ± 9.1	101.8 ± 3.8
Stored at −20 °C for 1 month			
Measured conc. (ng/mL)	0.101 ± 0.007	0.957 ± 0.047	8.100 ± 0.291
Accuracy (%)	101.3 ± 6.4	95.7 ± 4.9	101.3 ± 3.6

3.2. Method validation

3.2.1. Specificity

The specificity of the method was evaluated by analyzing individual blank urine samples. All samples were found to have no interferences from enhumanenous substances at the retention time of either limonin ($t_R = 3.0$ min) or the IS ($t_R = 3.5$ min). Representative chromatograms of a human blank urine, a human urine sample spiked with limonin and IS, and a urine sample from humans after oral administration are shown in Fig. 2.

3.2.2. Linearity and lower limits of quantification

The calibration curve for limonin was linear well within the range 0.0783–10 ng/mL. The mean value of regression equation was $Y = (0.931 \pm 0.087)X + (0.0721 \pm 0.0052)$ ($n = 5$) with a correlation coefficient over 0.996, where Y is the peak-area ratio of limonin to IS and X is the urine concentration of limonin. The LLOQ of limonin was 0.0783 ng/mL with 4.4% of the intra-day precision and 97.5% of the accuracy, which was more sensitive than the previously reported methods [16,17].

3.2.3. Recovery and matrix effect

The recoveries of limonin determined at 0.1, 1 and 8 ng/mL were 82.1%, 76.7%, and 78.7%, respectively. The mean matrix effect values obtained for limonin were 96.2%, 91.4% and 92.9% at low, medium and high QC level, respectively (Table 1).

3.2.4. Accuracy and precision

The results of intra- and inter-day were as shown in Table 1. The intra-day accuracy for limonin ranged from 97.4% to 100.3% within the testing concentrations with the precision (R.S.D.) between 3.3% and 6.4%, and the inter-day accuracy for limonin ranged from 99.9% to 101.1% with the precision (R.S.D.) between 3.2% and 7.4%. These results indicated that the present method was accurate, reliable and reproducible.

3.2.5. Stability

The stability data for limonin are represented in Table 2. Limonin is stable in human urine for at least three freeze/thaw stability and at ambient temperatures up to 24 h. Besides, limonin is stable in human urine for up to 1 month at −20 °C.

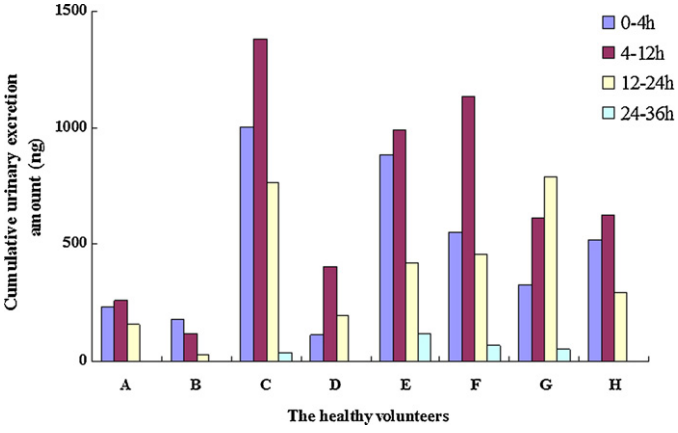

Fig. 3. The mean cumulative urinary excretion amount–time curve of limonin in 8 healthy volunteers after a single administration of limonin ($n = 8$).

3.3. Application to clinical studies

The method described above had been applied successfully to the urinary excretion study of limonin in healthy volunteers. This analytical method was able to measure the concentration of limonin up to 36 h. Fig. 3 is the mean cumulative urinary excretion amount–time curve of limonin in 8 healthy volunteers after a single administration of limonin. In the healthy volunteers who oral administered limonin, the total amount of unchanged limonin excreted in urine was less than 0.1% ($n = 8$).

4. Conclusion

A sensitive, rapid, and selective LC–MS/MS method combined with the solid-phase extraction has been developed for determination of limonin in human urine in this study. Meanwhile, a very low limit of quantitation was obtained, with LLOQ of 0.0783 ng/mL for limonin. This new method has been successfully applied to the evaluation of limonin in human urine after oral administration. This

study would be useful for further characterizations in pharmacokinetics, pharmacy and toxicity of limonin as a new drug.

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