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Journal of Pharmaceutical and Biomedical Analysis 39 (2005) 1031–1035
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Determination of limonin in rat plasma by liquid chromatography–electrospray mass spectrometry

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Received 1 March 2005; received in revised form 27 May 2005; accepted 8 June 2005
Available online 27 July 2005

Abstract

A simple, sensitive and selective liquid chromatography coupled with electrospray ionization mass spectrometry (LC/ESI/MS) method for determination of limonin (LM) in rat plasma has been developed and validated. The method had advantages of a single liquid–liquid extraction with ether and high sensitivity. Analyses were conducted at a flow rate of 0.2 ml/min by a gradient elution. The detection utilized selected ion monitoring in the negative ion mode at *m*/*z* 460.00 and 423.15 for the deprotonated molecular ions of LM and the internal standard, respectively. The quantitation limit for LM in rat plasma was 1.0 ng/ml. The linearity was also excellent over the concentration range of 1.9–500 ng/ml of LM. The intra- and inter-day precision (relative standard deviation (R.S.D.%)) was lower than 10% and accuracy ranged from 90 to 110%, showing a good reproducibility. This developed method was successfully applied to analysis of LM in biological fluids.

Keywords: Limonin; LC/ESI/MS; Rat plasma

1. Introduction

Limonins represent a group of chemically related triterpenes found in the Rutaceae and Meliaceae families, which include citrus fruits regularly consumed by humans such as orange, grapefruit, mandarin, lemon and lime [1–3]. Limonin (LM; for structure, see Fig. 1) and nomilin are the most prevalent of the citrus limonoids. LM has been shown to possess anti-carcinogenic, anti-bacterial, anti-viral, anti-feedant, anti-nociceptive and anti-inflammatory activities [4–6]. It also has been proved to inhibit buccal pouch, forestomach, colon, lung and skin carcinogenesis in rodents. Lam et al. reported that LM enhanced glutathione *S*-transferase (GST) activity in various organs of mice [7].

There have been several HPLC methods, using either UV or DAD detection, reported for the determination of limonins in citrus juices [8]. However, there are no reports of a bioanalytical method for LM. Since LM lacks strong UV absorption, detection at the more permissive wavelength of 204 nm required the use of large volumes (0.5–1.0 ml) of plasma. This may be suitable for human pharmacokinetic studies but not for preclinical PK studies in rodents since the collection of multiple large volume blood samples from each animal would be prohibited.

According to the result from a pilot study, plasma concentrations could be expected to be between 2 and 400 ng/ml. The objective of this study was to develop a fast, sensitive and reliable liquid chromatography coupled with electrospray ionization mass spectrometry (LC/ESI/MS) method for direct quantification of LM in the concentration range of 1.9–500.0 ng/ml in rat plasma.

2. Experimental

2.1. Material and reagents

Limonin was supplied by Chemistry Institute of China Pharmaceutical University and Ginkgolide B (internal standard) was supplied by National Institute for the Control of Pharmaceutical and Biological Produces (Beijing,

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doi:10.1016/j.jpba.2005.06.012

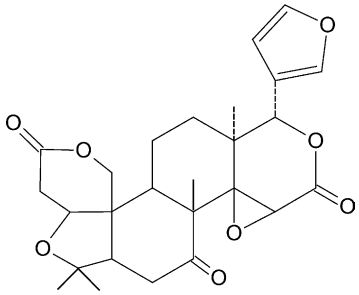

Fig. 1. Molecular structure of LM.

China). The other reagents were commercially available. Water was purified using Milli-Q Labo (Millipore, USA). Sprague–Dawley rats, weighing 180–220 g, were supplied by Experimental Animal Center of China Pharmaceutical University. The rats fasted, but were given free access to water 12 h prior to the experiment.

2.2. Chromatographic system and conditions

Analysis was carried out using the Shimadzu LCMS-2010A (Shimadzu Technologies, Japan) composed of the following units: two solvent delivery pumps, an automatic sample injector (140 vials capacity), a controller module, a column oven and a Model series 2010A single quadrupole mass spectrometer with an electrospray ionization interface. Data acquisition and processing were accomplished using Shimadzu LCMS Solution Software for LCMS-2010A system.

A stainless-steel column (C_{18} Shim-pack 5 μm 250 mm × 2.0 mm i.d. Shimadzu) was used and maintained at 40 °C. mobile phase A consisted of 0.02 mmol/l ammonium acetate and 0.004‰ (v/v) triethylamine in water (pH 7.2); Mobile phase B was acetonitrile. Each mobile phase was filtered through a 0.45 μm membrane and degassed under reduced pressure. Linear gradient elution was employed with a 10 min run time; its sequence was as follows: A–B (40:60) held for 4.5 min after injection, 10:90 at 5.5 min and held up to 7.0 min, and thereafter 40:60. Analyses were conducted at a flow rate of 0.2 ml/min.

The mobile phase pH was set at 7.2 because this was a good pH for the ionization of LM and it was relatively easy to maintain using triethylamine and ammonium acetate, a volatile, MS friendly buffer. Changes in the mobile phase constituents had the expected results; increasing the organic phase content resulted in less retention at the expense of peak separation and increasing the aqueous phase allowed for better resolution of the peaks with lower peak height and less sensitivity.

2.3. Mass spectrometric conditions

The LC–MS system was operated using an electrospray ionization probe in the negative ion mode with the capillary voltage set at 4.5 kV; the detector voltage was maintained 1.60 kV. Nitrogen was used as the nebulizer gas and drying gas at a flow rate of 1.5 and 2.0 l/min, respectively. The eluent from the HPLC column was transferred directly into ESI probe. Analysis in the mass spectrometer was performed in the SIM model (*m/z* 469.00 for LM and 423.15 for Ginkgolide B). The ratios of peak area between LM and Ginkgolide B were plotted versus concentrations using unweighted linear regression. From the calibration line obtained, concentrations of unknown samples were calculated.

2.4. Preparation of standard and quality control samples

A stock solution of LM was prepared in acetonitrile (1.0 mg/ml) and stored at 4 °C. The stock solution was diluted with acetonitrile–water (50:50, v/v) to prepare working solutions at the final concentrations of 10, 5, 2.5, 1.25, 0.63, 0.31, 0.156, 0.078 and 0.039 μg/ml. Internal standard stock solution (500.0 μg/ml) was also prepared in acetonitrile, with further dilutions in water for a working solution (10.0 μg/ml). The calibration curve consisted of nine plasma standards: 1.9, 3.9, 7.8, 15.6, 31.3, 62.5, 125.0, 250.0 and 500.0 ng/ml.

2.5. Sample extraction procedures

To 0.2 ml drug-free rat plasma were added 10 μl of LM working solutions, 10 μl of the working I.S. solution and 4.0 ml ether in a 15-ml polypropylene tube. The tubes were vortex-mixed 5 min at 900 rpm and centrifuged for 5 min at 3500 rpm. The organic layer (3.0 ml) was transferred to another disposable glass tube and evaporated to dryness under N_2. The residue was dissolved in 200 μl acetonitrile, and 5 μl supernatant was injected into the LC–MS system.

2.6. Plasma samples

Twenty rats were randomly assigned to two groups of five male and five female rats in each. After given LM 3.6 mg/kg by intravenous injection (i.v.), 0.5 ml blood samples were obtained through postoccular vein under ether anesthesia at 5, 10, 20, 30, 45 and 60 min with a heparinized capillary tube from the first group and at 90, 120, 180, 240, 360, 480 and 720 min, 0.5 ml blood samples were obtained from the second group. Just the same method was used for oral administration by gavage (i.g.) of 18 mg/kg LM in rats, Serial blood samples were normally collected at 0, 15, 30, 45, 60 and 90 min from one group, and 0.5 ml blood samples were pooled at 120, 180, 240, 360, 480 and 720 min from the other group following drug administration.

Blood was then centrifuged at 3000 rpm for 20 min, 0.2 ml plasma sample was used for determining the concentration of LM. Sodium heparin was used as anticoagulant. The obtained plasma was pooled and stored frozen at −70 °C until use.

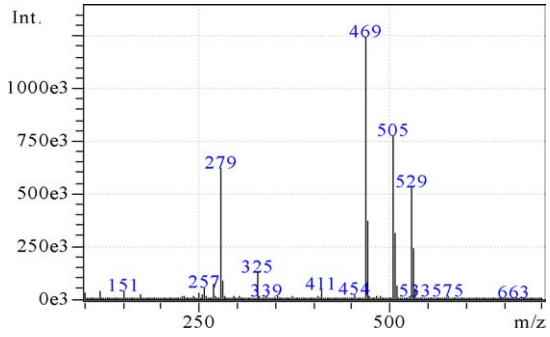

Fig. 2. Negative ion electrospray mass spectrum obtained after injection of 50 ng of LM, scanning range from *m*/*z* 100 to 700.

3. Results and discussion

Fig. 2 shows typical mass spectra obtained for LM using the electrospray technique in the negative ion mode. Deprotonated molecules (*m*/*z* 469.0) were detected as the base peaks for LM.

3.1. Chromatography

Fig. 3 shows four examples of chromatograms: (A) one obtained from injecting standard directly; (B) one obtained from blank rats plasma; (C) one obtained from rats plasma spiked with LM at 50 ng/ml; (D) a sample from a dosed rat ($t = 2$ h).

It is necessary to use an I.S. to get high accuracy to deal with sample matrix effects when a mass spectrometer is used as the HPLC detector. A stable isotope labeled analyte has always been used as an internal standard. Since such internal standard is not commercially available, an alternative approach has been adopted. Internal standard substance should match the chromatographic retention, recovery, matrix effects and ionization properties with LM. Ginkgolide B (Fig. 4) was found to fulfill these criteria sufficiently since it is a fragment that is being used which may vary in intensity depending on instrument conditions. So, Ginkgolide B has been chosen as internal standard in the quantitative assay for LM.

Retention times of LM and I.S. were 5.8 and 5.0 min, respectively. The lower limit of quantitation (LOQ) of LM was 1.0 ng/ml. At this concentration, no interference in blank plasma was detected, and the response was greater than 10 times baseline noise.

Results obtained showed that no interference from rat plasma components was observed in this study.

3.2. Method validation

3.2.1. Calibration linearity

The calibration graph was obtained by plotting the peak area ratio (*y*) of LM to the I.S. against the concentration (*x*) of LM, using unweighted linear regression. The calibration curve for LM was linear within the range 1.9–500 ng/ml examined using 0.2 ml samples, and could be expressed by the equation: $y = 0.0048x + 0.0065$ ($r^2 = 0.9999$).

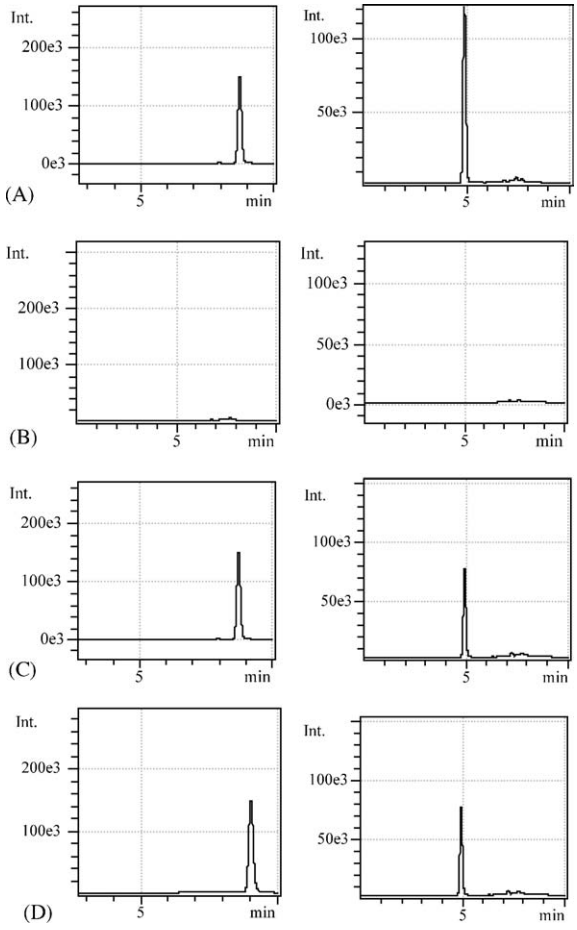

Fig. 3. Chromatograms of: (A) standard; (B) blank plasma; (C) blank plasma spiked with LM at 50 ng/ml; (D) rat plasma of 2 h after i.g. 18 mg/kg LM (LM, $t_R = 5.8$ min; Ginkgolide B, $t_R = 4.9$ min).

3.2.2. Recoveries from rat plasma

Absolute recoveries were calculated by comparing LC–MS results from samples prepared in organic solvent with ones obtained from spiked extracted plasma samples. The mean extraction recovery was between 80.7 and 84.5%

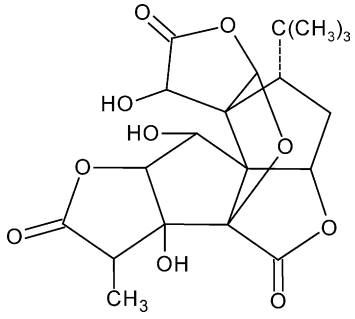

Fig. 4. Molecular structure of Ginkgolide B (internal standard).

Table 1
Observed recoveries of LM in plasma

LM concentrations (ng/ml)	Recovery (%)					Mean	R.S.D.%
3.9	80.99	75.79	75.08	80.09	91.95	80.78	6.76
31.3	86.55	92.55	80.87	80.41	82.07	84.49	5.12
250.0	82.08	93.63	79.84	87.4	78.08	84.21	6.33

Table 2
Reproducibility and accuracy of LM assay

Spiked value (ng/ml)	Within-day			Between-day		
Assayed value (ng/ml)	3.9	31.3	250.0	3.9	31.3	250.0
	4.24	31.30	232.88	3.63	32.60	238.13
	3.93	29.45	238.69	3.45	29.47	264.31
	3.89	33.35	250.73	4.10	29.11	242.50
	3.62	33.14	252.84	3.96	29.38	216.87
	3.71	33.92	255.39	3.60	33.35	236.20
Mean	3.88	32.23	246.11	3.75	30.78	239.60
S.D.	0.24	1.84	9.78	0.27	2.02	16.95
R.S.D.%	6.17	5.71	3.98	7.23	6.58	7.07

for LM and 87.2% for internal standard, and there were negligible ion-suppression effects found for this established method (Table 1).

3.2.3. Accuracy and precision of intra- and inter-day measurements

Within-batch precision and accuracy of the method was evaluated by analyzing replicate samples each of blank rat plasma spiked at three concentration levels, 3.9, 31.3 and 250 ng/ml. Relative standard deviations (R.S.D.s) and percent differences between the measured and the nominal concentration were calculated for each concentration level and considered acceptable when the R.S.D.s and the percent differences were <15%. The result obtained for the within-batch accuracy and precision R.S.D.s were <10% for LM assay. The results are presented in Table 2 and indicate that the values are within acceptable range and that the method was accurate and precise.

3.2.4. Freeze–thaw stability of LM in rat plasma

Stability of the LM in rat plasma at −70 °C was performed. Spiked samples (three concentration levels) at 3.9, 62.5 and 500 ng/ml were frozen and stored at −70 °C for a week. They were then extracted and the results obtained were compared to freshly prepared and extracted material at the same level.

The results showed that LM was stable for at least one week in plasma kept at −70 °C based on the residual fraction of 95.6–100.2% (Table 3).

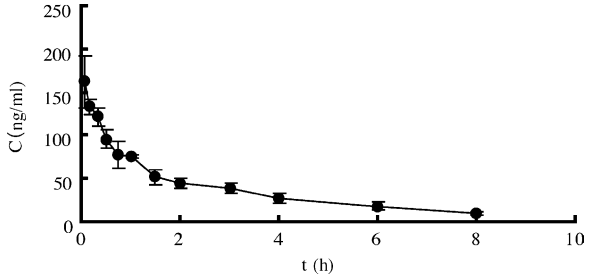

Fig. 5. Plasma concentration–time profile (mean ± standard deviation, $n = 10$) of LM after i.v. 3.6 mg/kg LM to rats.

3.3. Method application

The method described was successfully used to monitor LM in rat plasma. Typical concentration–time profiles of LM

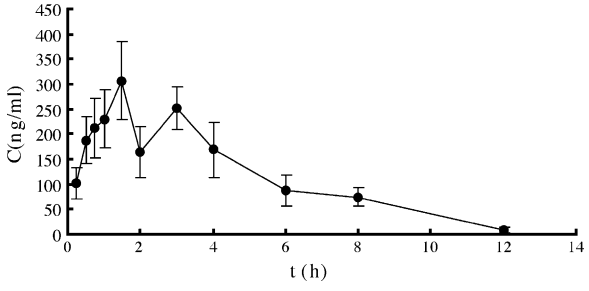

Fig. 6. Plasma concentration–time profile (mean ± standard deviation, $n = 10$) of LM after i.g. 18 mg/kg LM to rats.

Table 3
Stability of the LM in rat plasma after freezing for a week at −70 °C

Added (ng/ml)	Found (ng/ml)					Mean	Residual fraction (%)	R.S.D.%
3.9	3.72	4.14	3.74	3.83	3.81	3.85	98.67	4.41
31.3	31.87	31.15	31.92	30.01	29.91	30.97	98.95	3.14
250.0	252.76	247.32	252.27	247.81	247.36	249.50	99.79	2.76

following i.g. 18 mg/kg and i.v. 3.6 mg/kg in the rat are presented in Figs. 5 and 6.

4. Conclusion

A fast and sensitive LC–MS method for determination LM in rat plasma was developed. The unique features of the assay include a simple extraction procedure with regard to a single liquid–liquid extraction and the use of low sample volumes.

LC–MS with a single quadrupole analyzer was found to be suitable for quantification of LM based on good intra- and inter-day validation data and more than sufficient sensitivity to analyze study samples with limited volume. In particular, electrospray ionization mass spectrometry has the advantage that it can be easily coupled to LC as the ion source is at atmospheric pressure [9]. Since analyses were conducted at a flow rate of 0.2 ml/min, we could greatly reduce the consumption of mobile phase and nebulizing gas.

In this study, deprotonated molecules (m/z 469) were detected as the base peaks for LM. The spectrometer (m/z 505) issued from $M + Cl^-$ can also be detected as the base peaks for LM if we added a little NH_4Cl in the mobile phase. However, the amount of NH_4Cl needed is not easy to validate as too little may cause poor linearity while too much may contaminate the MS source.

The LOQ of LM was 1.0 ng/ml in rat plasma. The linearity was also excellent over the concentration range of 1.9–500 ng/ml of LM. The intra- and inter-day precision (R.S.D.%) was lower than 10% and accuracy ranged from 90 to 110%, showing good reproducibility. This developed method was successfully applied to analysis of LM in biological fluids. A more sensitive method utilizing LC/MS/MS can measure even lower concentrations of LM in plasma, but it is too expensive for routine use in preclinical laboratories.

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