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Short communication

Determination of andrographolide in human plasma by high-performance liquid chromatography/mass spectrometry

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Abstract

In this paper, a rapid method based on high-performance liquid chromatography/electrospray–mass spectrometry (HPLC/ESI-MS) method for the quantitative determination of andrographolide (AND) in human plasma has been developed and validated. A liquid–liquid extraction (LLE) procedure was selected to isolate AND from biological matrixes. Isosorbide-5-mononitrate (IS-5-MN) was selected as the internal standard (IS). The correlation coefficient of the calibration curve was 0.998, in the range of 9.9–320.0 ng/mL. The validated method may be used to assess the bioavailability and pharmacokinetics of the drug.

Keywords: Andrographolide; HPLC/ESI-MS; Human plasma

1. Introduction

Andrographis paniculata (Burm. F.) Nees is one of most important medicinal herbs to treat different diseases. This herb has been used traditionally for several applications such as antimicrobial [1], anticancer [2], and against HIV [3]. The typical medicinal component is andrographolide (AND), the structure of which is shown in Fig. 1.

Several methods have been reported for the determination of AND extracted from medicinal plants, including spectrometry [4], HPLC and HPTLC [5,6], LC [7], and micellar electrokinetic capillary (MEKC) [8].

However, for supporting clinical pharmacokinetic program, the determination of AND in biological matrix, especially in human plasma, is necessary. In this respect, the method of HPLC, GC-MS, and capillary electrophoresis (CE) [9] has been reported. Because the expected concentration of AND in the blood of humans after the uptake of a therapeutic dose was in the range of 50–300 ng/mL, which is less than the limit of detection of AND using HPLC, the method of GC-MS was developed [9]. But this method involved solid-phase extraction in sample preparation and hence was time-consuming and complicated. In this paper, we developed a simpler high-performance liquid chromatography/electrospray–mass spectrometry (HPLC/ESI-MS) method based on one-step liquid–liquid extraction (LLE) for the determination of AND in human plasma. This method has not yet been reported and has sufficient sensitivity for pharmacokinetic study of AND.

2. Experimental

2.1. Chemicals and reagents

AND and internal standard isosorbide-5-mononitrate (IS-5-MN) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol and ethyl acetate were of HPLC grade, purchased from Tedia Company, Inc. (Fairfield, Ohio, USA) and Tianjin Kermel Chemical Reagents Development Center, respectively.

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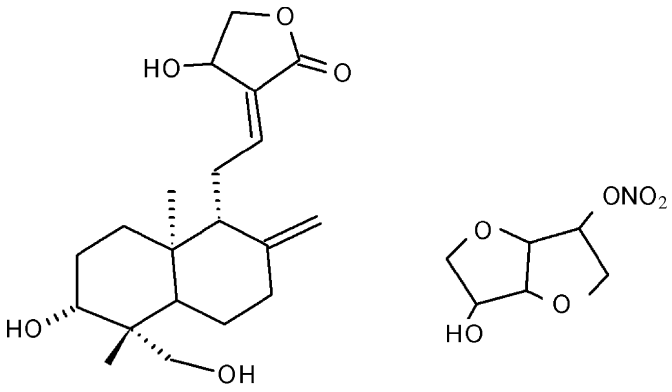

Andrographolide IS-5-MN

Fig. 1. Chemical structures of AND and internal standard IS-5-MN.

2.2. LC-MS instruments and conditions

A Waters (Milford, MA, USA) Alliance 2695 liquid chromatographic system was equipped with a Johnson Spherigel C_{18} column (200 mm × 4.6 mm, 5 μm). Separation was performed at 30 °C. The mobile phase consisted of methanol and aqueous solution (70:30 v/v).

Mass spectrometric detection was performed on a Micromass ZQ 2000 ESI mass spectrometer and a Masslynx^{TM} 4.0 data system. The mass spectrometer was operated in the negative ion mode over a range of *m/z* 160–400, and selective ion monitors (SIM) were at *m/z* 331 ($[M\text{-}H_2O\text{-}H]^-$) for AND and 190 ($[M\text{-}H]^-$) for internal standard (IS), respectively. The outlet of the column was split, and only 0.2 mL/min was delivered into ESI source. Nitrogen was used as desolvation gas and cone gas, which were at a flow rate of 200 and 50 L/h, respectively. The desolvation temperature was 200 °C. Capillary and cone voltages were 3500 and 25 V, respectively. The ionization source worked at 105 °C.

2.3. Calibration and quality control samples

The stock solution of AND was prepared by dissolving 5 mg in 50 mL of mobile phase. Working solutions for calibration and controls were prepared from the stock solution by appropriate diluting. The proper volumes of working solutions were added to drug-free plasma to obtain AND concentration levels from 9.9 to 320.0 ng/mL. Quality control (QC) samples were prepared at three different concentrations: 20.0 ng/mL (low), 80.0 ng/mL (medium), and 160.0 ng/mL (high). The stock solutions were all stored in refrigeration at −4 °C.

2.4. Sample preparation

Drug-free and drug-containing heparinized plasma was obtained from volunteers and patients, respectively. Plasma was stored at −40 °C until used for analysis.

A 5-μL aliquot of internal stock solution was added to 0.8 mL of plasma sample and then 3.0 mL of ethyl acetate was added immediately. The mixture was centrifuged at 12,000 rpm for 10 min after vortex-mixed for 2 min, and 2.4 mL of the supernatant was transferred to a clean centrifuge tube. The organic extract was evaporated to dryness under a stream of nitrogen at 38 °C, the residue was reconstituted in 0.1 mL mobile phase, and 20 μL aliquot was injected into HPLC system.

3. Results and discussion

3.1. Method development

In the experiment, the different percentages of mobile phase consisting of water–methanol were investigated to improve HPLC separation and enhance sensitivity. We found that the maximum response of AND reached when the percentage of methanol was 65%, but considering the separation of AND and IS, 70% methanol was selected. Ammonia aqueous, formic acid, acetic acid, and trifluoroacetic acid as mobile phase modifier were investigated separately, the peak shape and sensitivity were not obviously improved. IS-5-MN was selected as IS and proved that it could meet the acceptable accuracy and precision of the method.

All parameters of MS and ionization mode were optimized by flow injection analysis. The optimum response of MS for AND was obtained using the negative mode. The optimum capillary voltage was 3.5 kV. The intensity of compound mainly depends on collision-induced dissociation fragmentation voltage, so the cone voltage was investigated as the key factor. Cone voltage was optimized by switching from 5 to 30 V in scan mode. The maximum response of MS was achieved when it was 25 V. The extractor voltage is also an essential factor affecting the sensitivity of MS. The response began to decrease when it was more than 4 V and the molecular-ion peak vanished when it was more than 6 V. So the best extractor voltage was 4 V. The peak area at *m/z* 331 (fragmentation ion) was 5.73 times more than that at *m/z* 348.8 (molecular ion) in selected-ion current profiles under the above conditions. So we adopted the fragmentation at *m/z* 331 for quantitative analysis of target compound. The mass spectrum shows in Fig. 2C.

3.2. Specificity

The specificity was assessed by comparing the signal for blank human plasma extract (Fig. 2A) with the response of an extract spiked with AND and IS (Fig. 2B). As shown in Fig. 2, no significant endogenous interferences were observed at the retention time of the target compound and IS in selected ion monitoring (SIM) current profiles. These typical SIM current profiles indicated that the assay adopted was applicable.

3.3. Calibration curves

Calibration standards were prepared at six different concentrations: 9.9, 19.6, 39.6, 80.6, 160.0, and 320 ng/mL for AND; every calibration standard was injected in triples. The calibration curve was obtained by weighted ($1/x^2$) least-squares linear regression analysis of the data. The regression equation

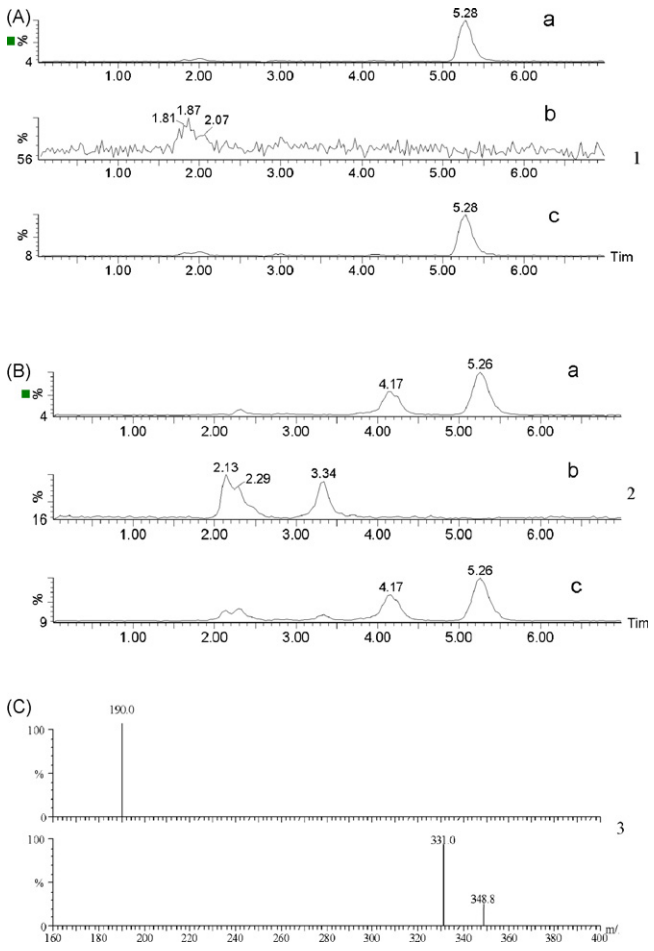

Fig. 2. Representative HPLC-ESI-MS chromatograms. (A) A blank plasma sample. (B) A spiked plasma sample containing 50 ng/mL AND and 0.5 μg/mL IS-5-MN with the retention time at 4.17 and 3.34, respectively. (C) The corresponding MS spectra of spiked sample at 4.17 min, 3.34 min for AND and IS-5-MN, respectively. (a) Mass fragmentogram at *m/z* 331 for AND; (b) mass fragmentogram at *m/z* 190 for IS-5-MN; (c) total ion current profile.

is $y = 0.0387x + 0.0072$, where y represents the peak area ratios of AND to IS and x represents the concentration of the calibration standard. The R.S.D. of intercept and slope were 2.98 and 2.56%, respectively. The mean correlation coefficient obtained was 0.998 (R.S.D. = 1.21%), indicating a good linearity. The limit of quantification (LOQ) was 9.9 ng/mL (*S/N* 10:1; R.S.D. 12.1%). As described above, the calibration curve was suitable for generation of acceptable data for the concentrations of the analyte during the determination.

3.4. Precision and accuracy

The precision of the method was evaluated by processing QC samples at three different concentrations of AND (20.0, 80.0, and 160.0 ng/mL). Every sample of the same concentration was injected five times. The accuracy of the method obtained by QC samples of the same with the above was assessed by calculating the mean recovery of the target compound by adding the known concentration to the drug-free plasma samples. The mean recovery was examined by comparing the determined concentrations with the nominal concentrations. We found that the repeatability was below 7.4% R.S.D. and the accuracy of the method was 97.9–100.3%. The results met the acceptable criteria [10].

3.5. Extraction recovery

The extraction recovery of AND and IS was measured by comparing the peak area ratios of extracted QC samples with standard solutions. The results demonstrated that the mean extraction recovery of AND and IS were 84.35 and 72.50%, respectively. The R.S.D. values were all below 9.3%.

3.6. Stability

The stability of AND in human plasma samples was investigated by determining the concentrations of QC samples stored for 24 h at room temperature, undergoing three freeze–thaw cycles, respectively. The stability of the stock solutions was also evaluated by storing in refrigeration at −4 °C for 1 month. The results are summarized in Table 1. The result showed that AND was stable during the determination.

3.7. Application

We hydrolyzed plasma samples using acidic hydrolysis by microwave and reflux according to the reported method [11], respectively. The results showed that there was no significant difference of detected concentration of AND in hydrolyzed and unhydrolyzed samples.

Although assays measuring only the free, unconjugated molecules are of limited value, the method described in this article is also significant. As the free fraction of AND may be significantly altered by many clinical conditions that affect protein binding, such as hypoalbuminemia, uremia, and hyperkinemia, free AND monitoring would be desirable in these situations.

It has been applied successfully to the determination of AND in human plasma samples for the purpose of the pharmacokinetic study. Plasma samples were collected after oral administration

Table 1
Stock solution stability at −4 °C (1 month) and the stability of AND in QC samples storing for 24 h at room temperature, undergoing three freeze–thaw cycles ($n = 5$)

Stability test	Nominal concentration		
	20.0 ng/mL	80.0 ng/mL	160.0 ng/mL
Stock solution stability (1 month)			
Mean ± S.D.	19.8 ± 0.83	79.8. ± 2.60	159.6 ± 4.76
R.S.D. (%)	4.20	3.26	2.98
Short-term stability (24 h)			
Mean ± S.D.	19.6 ± 1.02	79.4 ± 2.74	159.3 ± 5.09
R.S.D. (%)	5.20	3.45	3.19
Freeze–thaw stability (three cycles)			
Mean ± S.D.	19.2 ± 1.35	79.6 ± 2.59	159.8 ± 4.90
R.S.D. (%)	7.03	3.25	3.07

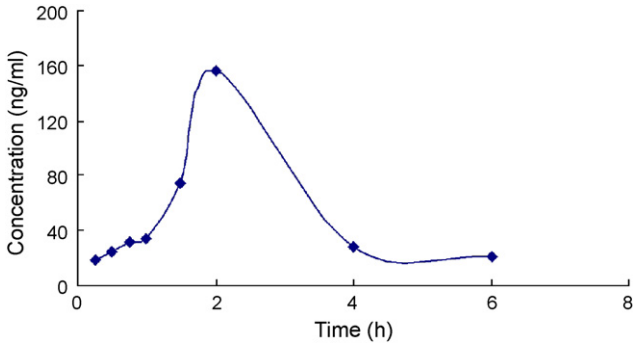

Fig. 3. Plasma concentration–time curve of one healthy subject after oral administration of 50 mg of AND capsule.

of 50 mg of AND capsule to 15 healthy male volunteers. The typical plasma concentration versus time profile is presented in Fig. 3. The plasma level of AND reached a maximum 2 h after the administration.

4. Conclusions

In summary, the method was developed for the determination of AND in human plasma by HPLC-ESI-MS. The precision, accuracy, and sensitivity of the method accord with FDA criteria [10]. Furthermore, simple sample pre-treatment and short runtime (6 min) were achieved in the method. The assay may be used to study the pharmacokinetics of AND.

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