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Short communication

Determination of oleanolic acid in human plasma and study of its pharmacokinetics in Chinese healthy male volunteers by HPLC tandem mass spectrometry

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Abstract

A highly selective and sensitive HPLC–ESI–MS–MS method was developed for the determination of oleanolic acid in human plasma. The oleanolic acid and glycyrrhetinic acid (internal standard) were recovered from plasma with ethyl acetate liquid–liquid extraction. The organic extracts were dried under a stream of warm nitrogen, reconstituted in mobile phase and injected into a Zorbax-Extend ODS analytical column (150 mm × 4.6 mm i.d., 5 μm), with the mobile phase consisting of methanol–ammonium acetate (32.5 mM) (85:15, v/v) pumped at a flow rate of 1.0 ml/min, and 30% of the eluent was split into a MS system with electrospray ionization tandem mass (ESI–MS–MS) detection in negative ion mode. The tandem mass detection was performed on a Finnigan Surveyor LC-TSQ Quantum Ultra AM tandem mass spectrometer operated in selected reaction monitoring mode. The parent to product ion combinations of m/z 455.4 → 455.4 and 469.3 → 425.2 at 38 V 1.5 mTorr Ar CID were used to quantify oleanolic acid and glycyrrhetinic acid, respectively. The assay was validated in the concentration range of 0.02–30.0 ng/ml for oleacolic acid when 0.5 ml of plasma was processed. The precision of the assay (expressed as relative standard deviation, R.S.D.%) was less than 15% at all concentrations levels within the tested range and adequate accuracy, and the limit of quantification was 0.02 ng/ml. The established method was applied for the pharmacokinetics study of oleanolic acid capsules in 18 healthy male Chinese volunteers with the mean values of C_{max}, T_{max}, AUC_{0-48}, $AUC_{0-\infty}$, $t_{1/2,}$ CL/F, and V/F of oleanolic acid after p.o. a single 40 mg dose obtained were 12.12 ± 6.84 ng/ml, 5.2 ± 2.9 h, 114.34 ± 74.87 ng h/ml, 124.29 ± 106.77 ng h/ml, 8.73 ± 6.11 h, 555.3 ± 347.7 L/h, and 3371.1 ± 1990.1 L, respectively.

Keywords: Oleanolic acid; Pharmacokinetics; HPLC–ESI–MS–MS

1. Introduction

Oleanolic acid [**a**, (3β)-3-hydroxyolean-12-en-28-oic acid (Fig. 1)] is one of the best known bioactive pentacyclic triterpenoids, that exists widely in medicinal herbs and plants, in the form of free acid or aglycones for triterpenoid saponins [1]. The traditional uses of plants containing oleanolic acid in folk medicines are multiple, in terms of anti-inflammatory [2–3], hepato-protection, analgesia, cardiotonic, glucose-lowering [4], tonic effects and enhancement of the body defense systems, etc. It is now marketed in China as an oral drug for human liver disorders.

Approaches for measuring of oleanolic acid in rabbit plasma has been described by using UV–vis spectrophotometry [5]. But the reported method is not suitable for the pharmacokinetics study of oleanolic acid in human. There is not any reported pharmacokinetics data of oleanolic acid in human up until now. Therefore, a sensitive HPLC–ESI–MS–MS method was established for the determination of oleanolic acid in human plasma and the study of its pharmacokinetics.

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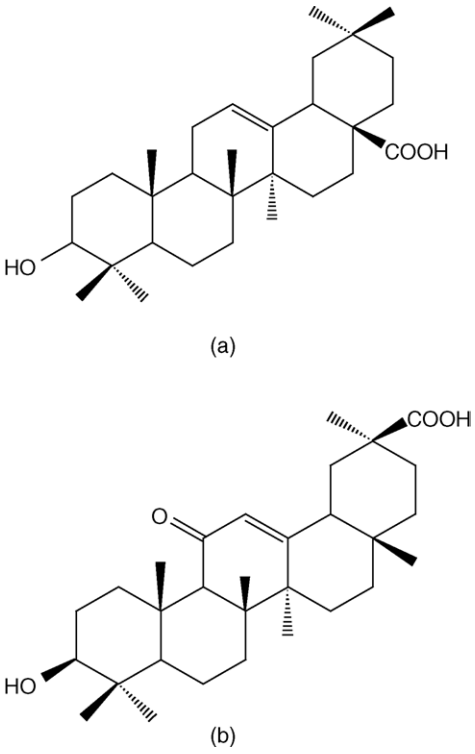

Fig. 1. Chemical structures of oleanolic acid (a) and glycyrrhetinic acid (b, internal standard).

2. Experimental

2.1. Materials

The reference substances of oleanolic acid and glycyrrhetinic acid were purchased from the National Institute for the Control of Pharmaceutical and Biological Products, Tiantanxili No. 2, Beijing, China. All chemicals and reagents used were of HPLC grade or analytical grade from the Nanjing Chemical Reagent Company, Yanyao Rd., Nanjing, China. The water was prepared with double distillation.

The oleanolic acid capsules (lot no: 20030301) were supplied by Zhuhai Rundu Pharmaceutical Co., Guangdong, China. The preparation had the strength of 20 mg of oleanolic acid.

2.2. Chromatography and tandem mass spectrometry

The HPLC–MS system consists of a Surveyor LC pump, a Surveyor auto-sampler, and a TSQ Quantum Ultra AM triple-quadrupole tandem mass spectrometer with an ion max source, Xcalibur 2.1 software for data acquisition and analysis (Thermo Finnigan, San Jose, CA, USA).

The HPLC separation was performed on a Zorbax-Extend ODS analytical column (150 mm × 4.6 mm i.d., 5.0 μm, Agilent, CA, USA) with a mobile phase of methanol–ammonium acetate (32.5 mM) (85:15, v/v) delivered at 1.0 ml/min and 30% of the eluent after column was injected onto the MS system using an electrospray ionization (ESI) source.

The mass spectrometer was operated in the negative ion detection mode with the spray voltage set at 4.5 kV. Nitrogen sheath gas was set at 35 psi and auxiliary gas at 5 psi. The heated capillary temperature was set at 350 °C. The collision energy of 38 V was used with argon at a pressure of 1.5 mTorr for collision-induced dissociation (CID). Quantification was performed with multiple selected reaction monitoring (MRM) of the transitions of m/z 455.4 → 455.4 for oleanolic acid (Fig. 2a) and 469.3 → 425.2 for glycyrrhetinic acid (internal standard, Fig. 2b) with a scan time of 0.2 s per transition.

2.3. Sample preparation

The plasma sample of 0.5 ml was spiked with glycyrrhetinic acid as internal standard (20 μL of 2.0 μg/ml methanol solution) and mixed briefly, then extracted with 5.0 ml of ethyl acetate through vigorous vortex mixing for 3 min and centrifugation at 3500 × g force for 10 min. Four milliliter of the supernatant ethyl acetate layer was separated and evaporated under a gentle stream of nitrogen gas at 40 °C. The residue was reconstituted in 150 μL of the mobile phase. An aliquot of 20 μL was injected onto the HPLC column.

2.4. Calibration

The stock (100.0 μg/ml) and working solutions (2.50, 50.0, and 500 ng/ml) of oleanolic acid were prepared by dissolving an accurately weighed quantity of oleanolic acid in methanol and serial dilution with the same solvent. And the stock (20.0 μg/ml) and working solution (2.00 μg/ml) of glycyrrhetinic acid were prepared in the same way.

Calibration standards were then constructed with the plasma concentrations of 0.02, 0.05, 0.10, 0.50, 1.00, 2.00, 5.00, 10.0, 20.0, and 30.0 ng/ml of oleanolic acid, respectively, and each containing 80 ng/ml of glycyrrhetinic acid by adding a proper volume of the working solutions in centrifugation tubes and dried before mixing with 0.5 ml of blank human plasma.

The within-day and between-day accuracy and precision were evaluated at three concentration levels (0.05, 1.00, and 10.0 ng/ml) based on five measurements carried out in a single day and over five days of validation period, respectively. The accuracy was expressed as bias, obtained by calculating the percentage of difference between the measured and spiked concentration over that of the spiked value, whereas the precision was denoted by using the relative standard deviation (R.S.D.%). The absolute recovery of the extraction was determined by comparing the peak area obtained from the plasma sample with peak areas obtained by the direct injection of pure oleanolic acid standard solutions in the mobile phase at three different concentration levels. The quantification of the chromatogram was performed by using peak area ratios of oleanolic acid to internal standard.

2.5. Subjects and pharmacokinetics study protocol

Eighteen healthy male Chinese volunteers (aged 20–24 years, body weight 65 ± 5 kg) checked perfect for this study were selected as subjects. All subjects gave written consents to their participation after having been informed by the medical supervisor about the aim, course and possible risks of the study. The study protocols were approved by the relevant Ethical Review Committee in accordance with the principles of the Declaration of Helsinki, and the recommendations of the State Food and Drug administration of China. The volunteers participated in a single dose pharmacokinetics study. Subjects fasted 10 h before until 4 h after drug administration. Venous blood samples about 3.5 ml were collected in heparinized tubes at pre-dose (0 h) and 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 15.0, 24.0, and 48 h p.o. administration of the capsules of 40 mg oleanolic acid. The plasma samples were separated by centrifugation at $3000 \times g$ for 10 min and stored at $-20\,^{\circ}$C until analysis.

2.6. Pharmacokinetics

The maximum plasma concentrations (C_{max}) and their time of occurrence (T_{max}) of oleanolic acid were obtained directly from the observed data. The area under the plasma concentration–time curve (AUC) from the time zero to the last

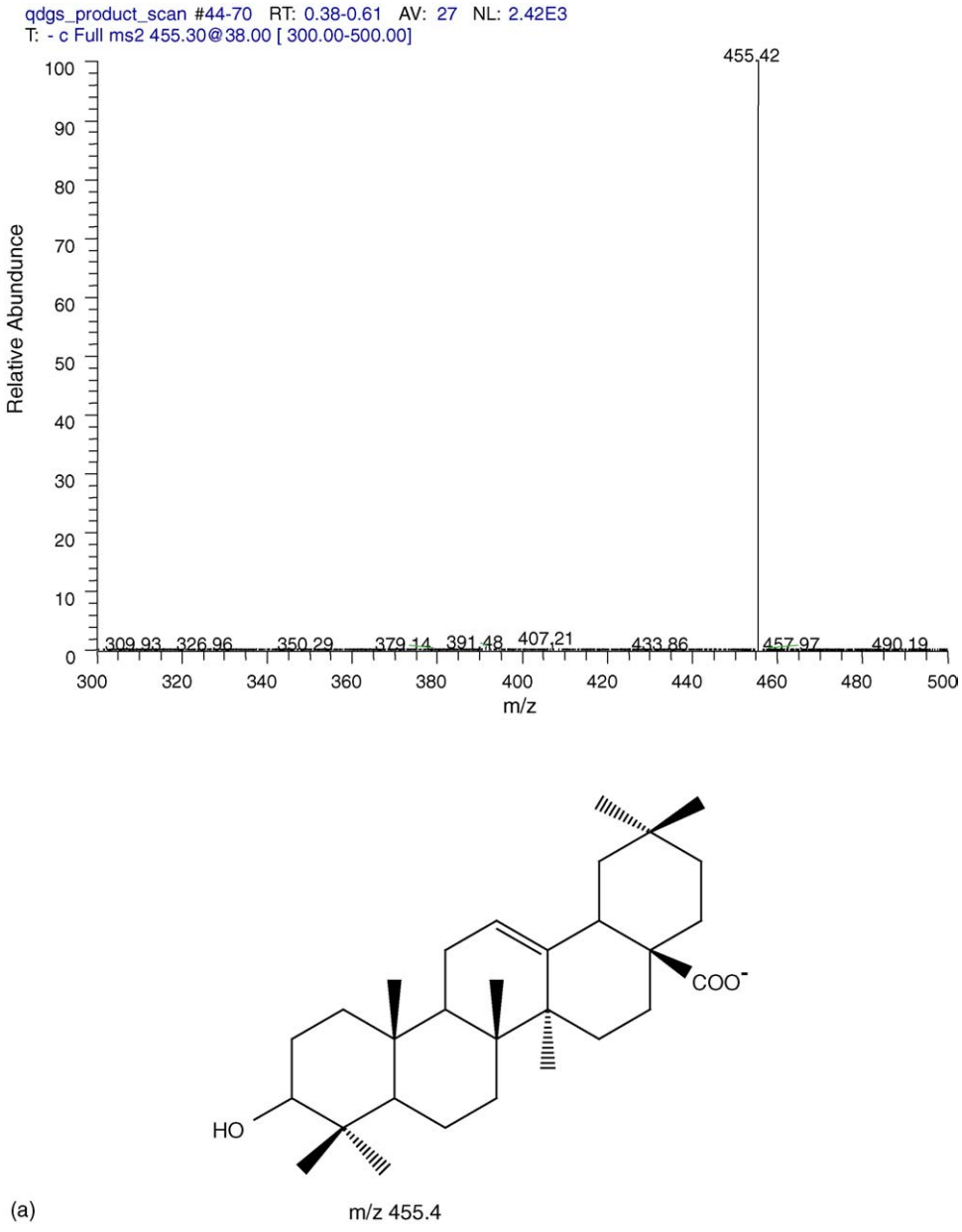

Fig. 2. Mass spectra of [M−H]− of oleanolic acid (a) and glycyrrhetinic acid (b, internal standard).

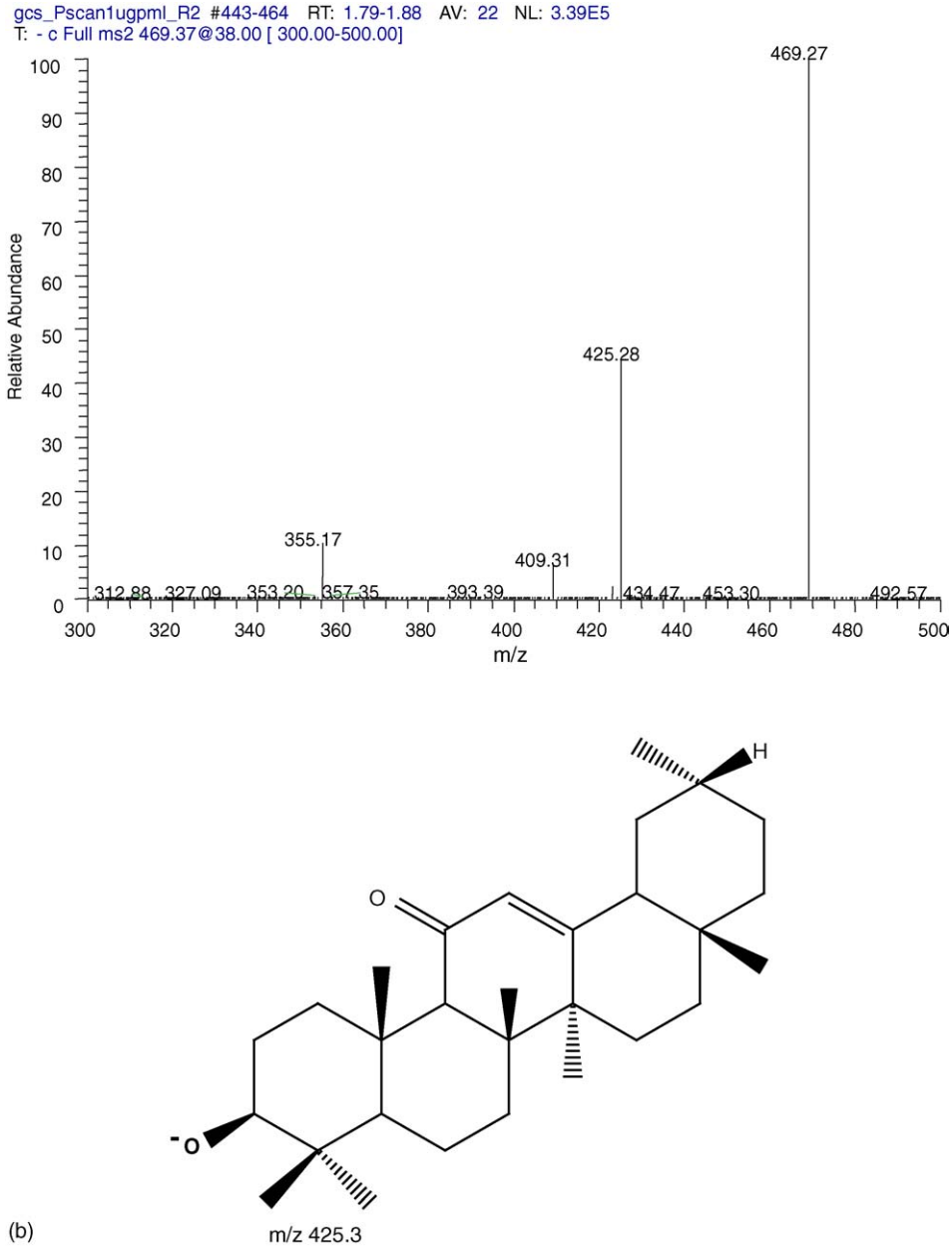

Fig. 2. (*Continued*).

measured concentration (AUC_{0-t}) was calculated according to the linear trapezoidal rule. The terminal elimination rate constant (λ_Z) was calculated by least-square regression of the logarithm scale concentrations to time for the last four measurable points, the terminal half-life was calculated with $t_{1/2} = 0.693/\lambda_Z$ accordingly, and the $AUC_{0-\infty}$ was the corresponding area extrapolated to infinity by $AUC_{0-t} + C_t/\lambda_Z$, where C_t is the last measurable drug concentration. And other major pharmacokinetic parameters calculated through the model fitting of the time–concentration curves of each subjects, including the clearance (CL/F, L/h) and the volume of distribution (V/F, L).

3. Results and discussion

3.1. Recovery, linearity, precision and accuracy

The Zorbax-Extend ODS analytical column and the mobile phase used for the determination gave a well-defined separation between the drug, internal standard and endogenous components. Typical chromatograms were shown in Fig. 3 with the retention time for glycyrrhetinic acid and oleanolic acid was about 3.0 and 6.3 min, respectively.

The absolute recoveries of both oleanolic acid and glycyrrhetinic acid from the plasma were more than 88% and

less than 100%, indicating that most of oleanolic acid in the plasma sample was extracted with no obvious interferences in the chromatogram.

The linear calibration curve was proven for oleanolic acid in the range from 0.02 to 30.0 ng/ml. The coefficient of correlation for all measured sequences was at least 0.998. The quantification limit for oleanolic acid was determined to be 0.02 ng/ml.

The within-day and between-day precision (R.S.D.%, $n = 5$) for the oleanolic acid spiked control samples at 0.05, 1.00, and 10.0 ng/ml levels varied between 3.8 and 11.6. The corresponding within-day and between-day

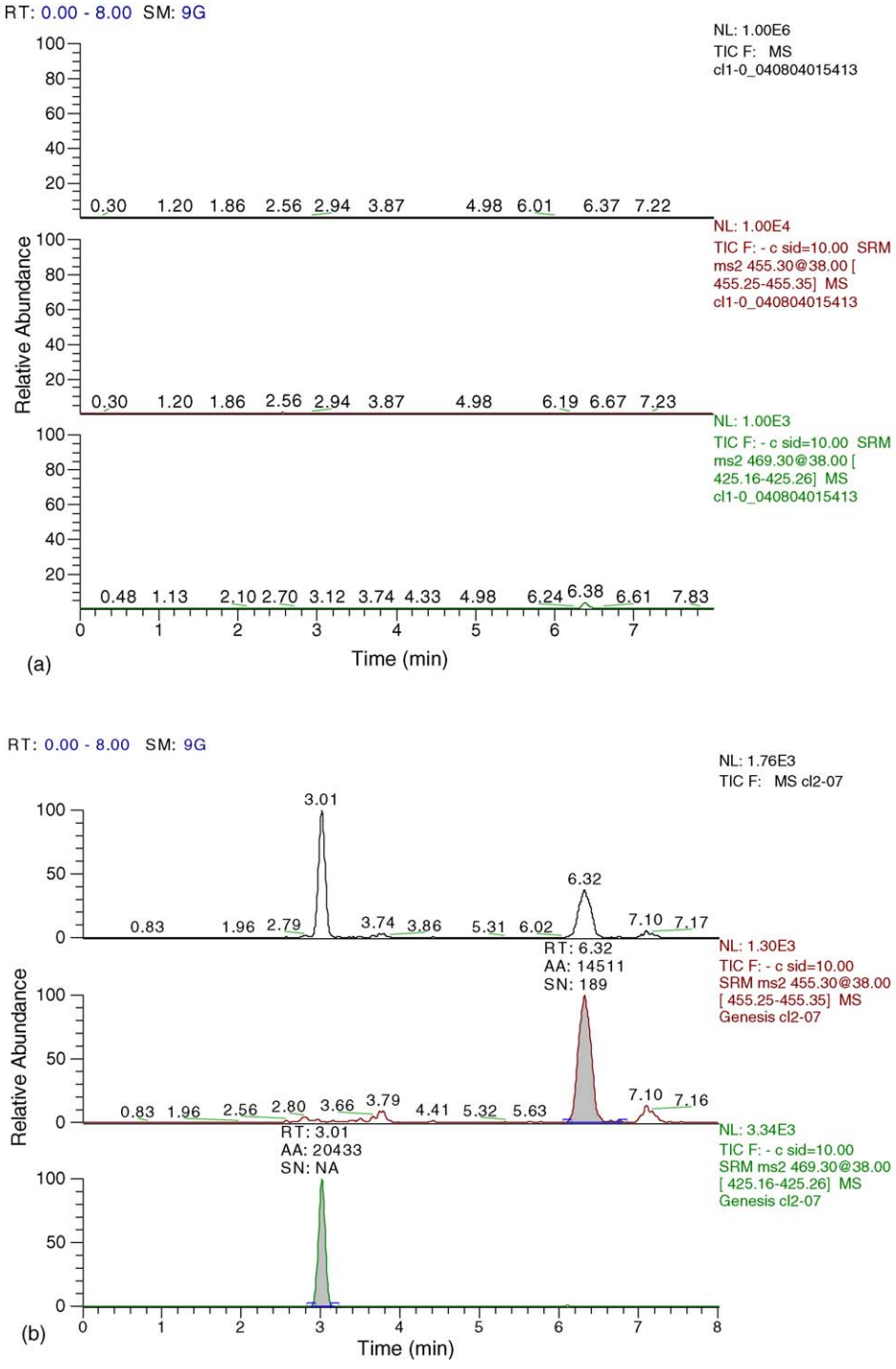

Fig. 3. Representative chromatograms: (a) blank pasma; (b) plasma spiked with 1.0 ng/ml oleanolic acid ($t_R = 6.3$ min) and 80 ng/ml glycyrrhetinic acid ($t_R = 3.0$ min); (c) plasma sample from a subject 12 h after administration of 40 mg oleanolic acid capsules, the concentration of oleanolic acid was found to be 6.7 ng/ml.

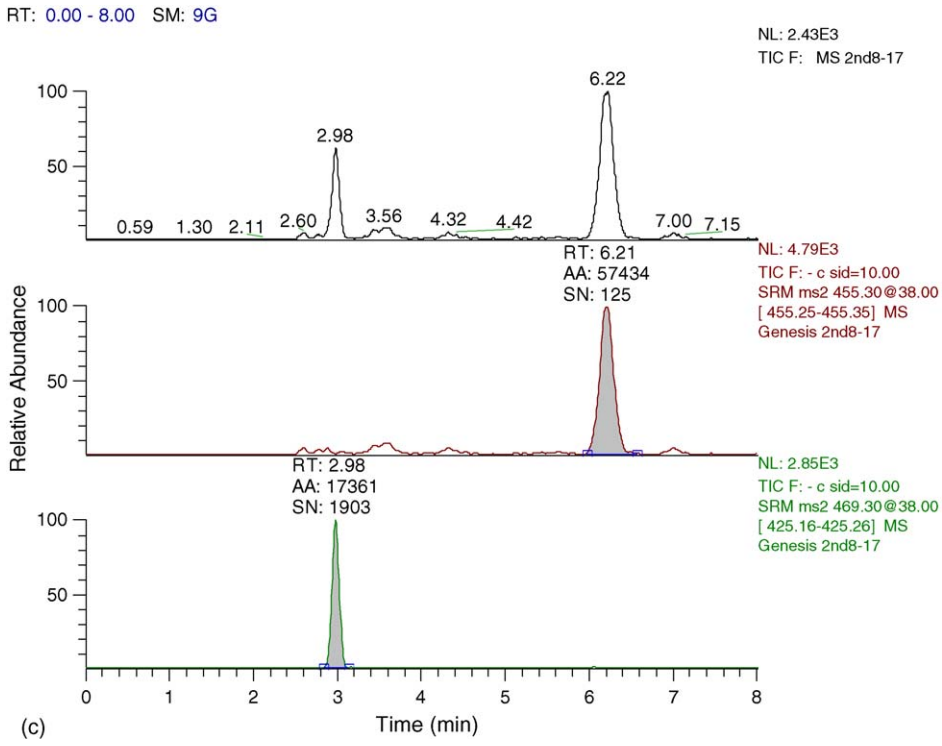

Fig. 3. (*Continued*).

accuracy (Bias%, $n = 5$) ranged between −7.6 and +7.8 (see Table 1).

3.2. Stability of oleanolic acid in plasma

The stability of oleanolic acid in plasma was tested by the analysis of the spiked quality control samples at 0.05, 1.00, and 10.0 ng/ml levels stored at −20 °C for a different period of days. Oleanolic acid was found to be stable in plasma at −20 °C for at least 30 days and three freeze–thaw cycles. And it was stable after sample preparation at room temperature.

3.3. Pharmacokinetics results

The mean plasma concentration–time curve of oleanolic acid capsule in 18 healthy male Chinese volunteers tested was shown in Fig. 4.

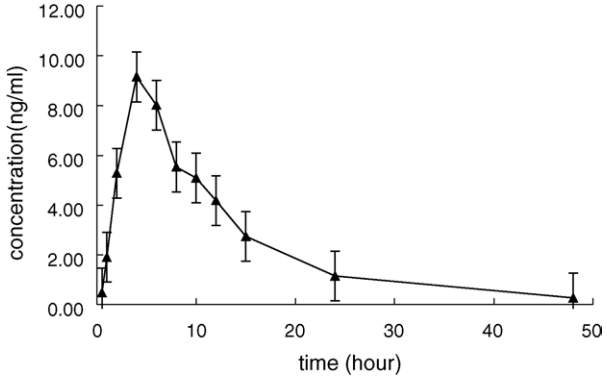

Fig. 4. Mean plasma oleanolic acid concentration–time profile following oral administration of 40 mg oleanolic acid in 18 healthy volunteers.

The plasma oleanolic acid concentration–time curves of most of the 18 subjects were best fitted with one-compartment models.

Table 1
The recovery, precision and accuracy ($n = 5$) of the assay method

Concentration (ng/ml)	Absolute recovery		Within-day		Between-day	
	Mean (%)	R.S.D. (%)	Precision (R.S.D.%)	Accuracy (bias%)	Precision (R.S.D.%)	Accuracy (bias%)
0.05	97.8	9.0	5.3	5.7	11.6	−7.6
1.00	92.7	7.2	6.9	6.7	9.9	−5.9
10.0	88.2	6.1	3.8	7.8	9.5	3.0

Bias% = {(concentration added − concentration found)/concentration added} × 100.

Table 2
Pharmacokinetic parameters of oleanolic acid in healthy male Chinese volunteers p.o. a single 40 mg dose of the capsules

Parameters	Mean (±s)
C_{max} (ng/ml)	12.12 (6.84)
T_{max} (h)	5.2 (2.9)
$t_{1/2}$ (h)	8.73 (6.11)
AUC_{0-t} (ng h/ml)	114.34 (74.87)
$AUC_{0-\infty}$ (ng h/ml)	124.29 (106.77)
CL/F (L/h)	555.3 (347.7)
V/F (L)	3371.1 (1990.1)

The major pharmacokinetic parameters found were listed in Table 2. The estimated volume of distribution had a mean value of 3371.1 L, which was very large in comparison with 2.5 L of the average volume of total plasma of a man. This is probably caused by that oleanolic acid was distributed to great extent out of blood compartment, or amassed in some tissue.

4. Conclusions

In summary, a novel sensitive HPLC–ESI–MS–MS method for the determination of oleanolic acid in human plasma has been developed and validated over the concentration range from 0.02 to 30.0 ng/ml. The method has been successfully used for the pharmacokinetics study of oleanolic acid in Chinese healthy male volunteers.

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