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A sensitive and efficient method for simultaneous trace detection and identification of triterpene acids and its application to pharmacokinetic study

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ABSTRACT

A sensitive and efficient method for simultaneous trace detection of seven triterpene acids was developed and validated for analysis of rat plasma samples. The required micro-sampling of only 20 μL blood reduced the difficulty in blood collection and the injury to animal. The whole pretreatment procedure was more conveniently finished within 26 min through the application of the semi-automated derivatization extraction method to biological samples. Seven analytes were rapidly separated within 30 min on reversed-phase Akasil-C18 column and quantified by fluorescence detector. Online ion trap MS with atmospheric pressure chemical ionization (APCI) source was used for further identification. The novel application of artificial neural network (ANN) combined with genetic algorithm (GA) to optimization of derivatization was performed and compared with the classical response surface methodology (RSM). Optimal derivatization condition was validated by multi-criteria and nonparametric tests and used successfully to achieve the rather high sensitivity (limit of detection: 0.67–1.08 ng/mL). The limit of reactant concentration (LORC) special for derivatization was studied and the lower values (2.53–4.03 ng/mL) ensured the trace detection. Results of validation demonstrated the advantages for pharmacokinetic study, such as higher sensitivity, better accuracy, easier pretreatment and shorter run-time. Pharmacokinetic study of triterpene acids after oral administration of *Salvia miltiorrhiza* extract to mice was conducted for the first time. The present method provided more sensitive and efficient alternative for the medical detection of bioactive constituents from herbal extract in the biological liquid.

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1. Introduction

As one group of extracts from natural medical herb, triterpene acids play vital role in treatment of liver diseases, neoplastic diseases and cardiovascular diseases and fungal diseases, which has aroused researcher's great interests recently [1–4]. And even in treatment of severe diseases, triterpene acids and their derivatives constitute a promising group of anti-inflammation drugs, for example, oleanolic acid and ursolic acid have shown the ability to inhibit the tumor growth [5], some derivatives of betulinic acid, oleanolic acid and ursolic acid have been synthesized to be potent anti-HIV agent with novel resistant HIV strains mechanism [6]. Despite the significant activity, the mechanisms of medical herb are rather complex to be well understood [7]. The concentrations of active components in vivo are critical to explore mechanism, and are useful to achieve important pharmacokinetics parameters such as half-life, bioavailability etc. Therefore the pharmacokinetic study of the triterpene acids is necessary and helpful to better understand the pharmacological and clinical effects of medicinal herb. But the usual methods like UV and fluorescence cannot be directly utilized because of the non-chromophores structure of the analytes, especially for the triterpene acids such as ursolic acid, tormentic acid, oleanolic acid, maslinic acid, betulinic acid, corosolic acid, betulonic acid (structures in Fig. 1). To the best of our knowledge, so far there have been no published analytical methods for the simultaneous determination of these seven triterpene acids in biological liquid. Moreover, the concentrations of components in vivo after oral administration to animal are usually rather low (ng/mL), which bring about the challenge for detecting the trace content of triterpene acids in biological samples.

Many methods for the detection of triterpene acids are established, such as high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [8–12], HPLC with mass spectrometry (MS) [13–15], nuclear magnetic resonance (NMR)

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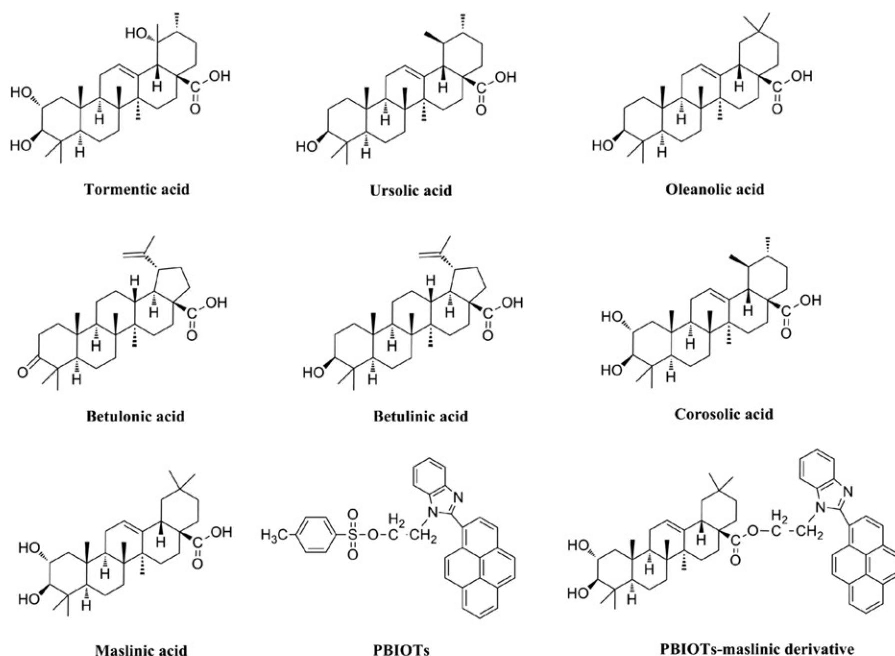

Fig. 1. Structures of seven triterpene acids (tormentic acid, ursolic acid, oleanolic acid, betulonic acid, betulinic acid, corosolic acid, maslinic acid), reagent PBIOTs and PBIOTs-maslinic acid derivative.

method [16], capillary electrophoresis (CE) with UV detection [17–19], HPLC with evaporative light scattering detection (ELSD) [20]. These methods show the limit of detection (LOD) ranged from 0.2 to 300 μg/mL, which are not sensitive enough for the trace detection in pharmacokinetic study. In addition, some matrix interferences from coexisted compounds in biological liquid usually result in the difficulties to the selective analysis. Accordingly, it is essential to develop the sensitive and efficient method for detection of triterpene acids in pharmacokinetic study. To improve the sensitivity, we focused exclusively on the pre-column derivatization techniques and optimization methods for optimal response of detection. Many fluorescent reagents were synthesized to label acid functional groups including fatty acids [21,22], bile acids [23,24], amino acids [25] and triterpene acids [26–28]. But none of them was employed to detect analytes in biological liquid samples like rat plasma. Although the methods for detection of triterpene acids showed the satisfactory sensitivity, they were not suitable for efficiently evaluating the pharmacokinetics in pre-clinical or clinical studies. Reagent 2-(2-(pyren-1-yl)-1*H*-benzo[d]imidazol-1-yl)-ethyl-*p*-toluenesulfonate (PBIOTs) was one of the developed labeling reagents. Comparing with the reported reagents for triterpene acids, PBIOTs had larger conjugated substructures and more nitrogen atoms which were helpful to produce the higher fluorescence sensitivity and the more intense ion current signals [29]. Therefore, PBIOTs was most likely to be suitable for the analysis of triterpene acids in biological liquid and thus was chosen for this work. Meanwhile, the efforts for optimal derivatization conditions should be made, because the satisfactory sensitivity would not be achieved unless the derivatization reaction was finished under the most appropriate conditions. So, in addition to the single variable experiments, the multivariate optimization methods were introduced to investigate the further interactive effects of the major factors on the derivatization reaction. The classical multivariate calibration method response surface methodology (RSM) [30] and the artificial neural network (ANN) [31] has been increasingly used to predict the optimal condition. Especially, ANN can intelligently learn the regularity from experimental data through the interconnected neurons in the architecture inspired by cerebral network and establish a more approximate dependence relationship than regression models [32]. Nevertheless, so far there have been no reports of application of ANN to optimization of the derivatization conditions. In this work, the Box–Behnken design (BBD) of RSM and ANN combined with BBD design (BBD-ANN) were applied and compared, since ANN combined with design of RSM usually provided more efficient procedure [30,33,34].

In pharmacokinetic study, the volume of one single rat blood sample is generally larger than 200 μL and the jugular-vein cannula or other injurious methods are always needed, which are inconvenient to manipulate [35]. Contrarily, with the highly sensitive method, the micro-sampling (less than 50 μL) of rat blood at a time point can meet the requirement of analysis. Thus the pharmacokinetic experiment can be finished in a convenient and simple way keeping the rat alive, which is also the added benefit of this work. In addition, since large batches of samples are always needed to be analyzed in pre-clinical or clinical pharmaceutical studies, the usual pretreatment method might be cumbersome and time-consuming [36,37]. Consequently, the highly sensitive and efficient analytical method for triterpene acids in biological liquid is urgently needed.

In this work, a more sensitive and efficient method for simultaneous quantification and pharmacokinetic study of seven triterpene acids was developed and validated. The seven triterpene acids were derivatized with PBIOTs for the first time. The multivariate optimization method ANN with GA was employed to optimize the derivatization extraction of triterpene acids in biological liquids, and compared with RSM. The separated PBIOTs-triterpene acid derivatives were monitored by fluorescence detection and further identified by online mass spectrometry. The new concept limit of reactant concentration (LORC) special for the derivatization was studied to ensure trace detection of analytes with the fewer amounts of biological samples (20 μL) required. With the superior method combining micro-sampling, derivatization extraction and HPLC-FLD-MS for isolation, quantitative determination as well as qualitative identification, the pharmacokinetic parameters with different doses of extract from the famous medicinal herb *Salvia miltiorrhiza* were conveniently obtained, which could be helpful for usage and pharmacology of medicinal herbs.

2. Experimental

2.1. Materials and chemicals

Salvia miltiorrhiza was collected from Yushu County, Qinghai province (elevation 4400 m, China). Its root and aerial part were dried at 40 °C under a stream of nitrogen, and milled to particle sizes of 60 μm and stored at −20 °C until use. Seven standard triterpene acids (ursolic acid, tormentic acid, oleanolic acid, maslinic acid, betulinic acid, corosolic acid, betulonic acid) were purchased from National institute for the control of pharmaceutical and biological products (Beijing, China). The derivatization reagent 2-(2-(pyren-1-yl)-1*H*-benzo-[d]imidazol-1-yl)-ethyl-*p*-toluenesulfonate (PBIOTs) was synthesized as described in our previous work [29]. The analytical grade Ethylenediamine tetraacetic acid disodium salt (EDTA-Na_2) was bought as anticoagulant from Tianjin Damao Chemical Reagent Factory. HPLC grade acetonitrile (ACN) was from Yucheng Chemical Reagent (Shandong Province, China). *N*,*N*-Dimethylformamide (DMF), potassium carbonate (K_2CO_3) and ethanol were of analytical grade obtained from Shanghai Chemical Reagent Co. (Shanghai, China). Water was prepared by Milli-Q ultrapure water system.

2.2. Instrumentation and conditions

Quantitative determination and identification were performed using Agilent 1100 Series high performance liquid chromatography with fluorescence detector and mass spectrometry, where the LC system was equipped with online vacuum degasser (G1322A), quaternary pump (G1311A), auto-sampler (G1329A), thermostated column compartment (G1316A) and fluorescence detector (FLD, G1321A), and the mass spectrometer 1100 Series LC-MSD Trap-SL (ion trap) from Bruker Daltonik (Bremen, Germany) was equipped with atmospheric pressure chemical ionization (APCI) source. Reversed-phase Akasil-C18 column (5 μm, 4.6 × 250 mm, Angela Technologies Inc. USA) was used to separate the derivatives. The semi-preparative HPLC column Sun Fire C18 column (10 μm, 10 × 150 mm) was applied to prepare the single PBIOTs-triterpene acid derivatives. The mobile phase was filtered through a 0.2 mm nylon membrane filter (Alltech, Deerfiled, IL, USA); F7000 fluorescence spectrophotometer (Hitachi, Japan); Ultrasonic instrument (SB-5200DTD, 40 kHz, Xinzhi Biotech Co., Ningbo, China). The syringe (1 mL), polymer filter (0.22 μm) and gastric needle were bought from Jiangyan Kangtai medical equipment company. Nitrogen blowing instrument (NBI, BF-2000M) was bought from Beijing Bafangshiji technology Co., Ltd.

Elution conditions were as follows: 0–15 min, 90% A and 10% B; 10–35 min, 100% A and 0% B (where, eluent A and B were water and 100% acetonitrile, respectively). The flow rate was set as 1.0 mL/min. The column was equilibrated by the initial mobile phase for 10 min before each injection and column temperature was set at 30 °C. The fluorescence excitation and emission wavelengths were set at 350 nm and 402 nm, respectively. Atmospheric pressure chemical ionization (APCI) source was used and operated in the positive-ion detection mode, nebulizer pressure 60 psi; dry gas temperature, 350 °C; dry gas flow, 5.0 L/min. APCI Vap temperature, 350 °C; corona current (nA) 4000 (pos); capillary voltage 3500 V.

2.3. Preparation of analytes and labeling reagent

2.3.1. Preparation of standard solutions and quality control samples

Standard solutions at a concentration of 10^{-3} mol/mL for each triterpene acid were prepared by dissolving appropriate amounts of triterpene acid standard in 10 mL of DMF, respectively. Working solutions of each triterpene acid were prepared by diluting the stock solutions of each triterpene acid with acetonitrile. The quality control (QC) samples containing ursolic acid (21.43, 67.25 and 112.37 ng/mL), tormentic acid (38.02, 164.84 and 308.56 ng/mL), oleanolic acid (48.21, 210.75 and 424.69 ng/mL), maslinic acid (43.45, 384.35 and 729.49 ng/mL), betulinic acid (170.53, 1394.76 and 2563.91 ng/mL), corosolic acid (19.89, 76.43 and 123.26 ng/mL) and betulonic acid (19.76, 82.48 and 149.65 ng/mL) were prepared at three concentration levels according to the contents of the seven triterpene acids in extract. To prepare the standards samples and quality control samples, the standard solutions were spiked into 20 μL of the blank bloods, respectively, and the resultant solutions were pretreated following the procedure of Section 2.5. The solution of PBIOTs (10^{-2} mol/mL) was prepared by dissolving 51.5 mg of 2-(2-(pyren-1-yl)-1*H*-benzo[d]imidazol-1-yl)-ethyl-*p*-toluenesulfonate in 10 mL of acetonitrile and the corresponding low concentration solutions were diluted by acetonitrile. All standards were stored at 4 °C until analysis.

2.3.2. Preparation of single PBIOTs-triterpene acid derivatives

To investigate the fluorescence spectra, single PBIOTs-triterpene acid derivative was prepared by the reaction of PBIOTs (10.0 mL, 1.0×10^{-5} mol/mL) with an excess amount of triterpene acid. The structure of representative derivative (PBIOTs-maslinic acid derivatives) was shown in Fig. 1. PBIOTs-maslinic acid derivative fraction was separated by the semi-preparative HPLC system in conjunction with a gradient elution using a mixed solvent acetonitrile–water (30:70, v/v) as initial mobile phase and changed to 100% acetonitrile within 20 min at a flow rate of 2 mL/min. The collected PBIOTs-maslinic acid derivative fraction was dried under a stream of nitrogen gas to give a yellow product (37.3 mg). The weighed PBIOTs-maslinic acid derivative (20 mg) was dissolved by 10 mL of dimethylformamide (DMF) to get the solution (2.4×10^{-6} mol/mL), which was then diluted by some mixed solvent of acetonitrile and water before fluorescence scan experiment. The volume ratio of acetonitrile to water in mixed solvents within the range (from 10:90, 20:80… to 80:20, 90:10, 100:0, v/v).

2.4. Preparation of Salvia miltiorrhiza extract

To 5 g of each powdered raw herb (2 g of root and 3 g of aerial parts of *Salvia miltiorrhiza*) in a narrow neck round-bottom flask (25 mL), 10 mL of ethanol was added. The mixture was placed in an ultrasonic cleaning instrument and treated for 1 h at room temperature. The extraction procedure was performed thrice for each sample. Ethanol extracts were pooled and filtered through analytical filter paper to remove fine particles; the extracts were then dried under vacuum. The residues were stored at −4 °C until analysis.

2.5. Preparation of plasma samples

The semi-automated derivatization extraction was designed for the preparation of plasma samples. 20 μL of blood were collected via tail vein puncture and drawn into the modified micro-syringe containing 10 μL of EDTA-Na_2 solution (4×10^{-6} mol/mL), 70 μL of potassium carbonate solution (3.0 mol/L), 100 μL of ACN and some appropriate amount of PBIOTs at the optimal molar ratio. The syringe was designed to be embedded in the tube with circulating water filled, which provided the heat for derivatization reaction. The proteineous material was removed by addition 200 μL of ACN and filtered by polymer filter (0.22 μm). 200 μL of the supernatant was transferred to sampling vials to be injected onto the HPLC-FLD-MS/MS.

2.6. Optimization of derivatization condition

2.6.1. Single variable experiments

Derivatization conditions such as cosolvent, pH value, catalyst, molar ratio of reagent to analytes, temperature and reaction time have the important influences on the efficiency of derivatization reaction that whether the produced derivatives can be detected with the highest sensitivity. Another factor put forward by us is the limit of reactant concentration (LORC) defined as the lowest concentration of reactants to produce quantifiable derivative whose concentration was equal to the concentration at the limit of quantification (LOQ) level. The value of LORC to a great extent decides whether the produced derivatives can be accurately quantified especially for the detection of trace amount of analytes in biological liquid with derivatization technique. The single-factor experiments were performed with betulinic acid as the representative and the variables were set as: molar ratios from 3 to 7, temperature from 85 to 100 °C, reaction time from 5 to 25 min. All the above single variable experiments were performed at three low concentration levels (8.76 ng/mL, 19.25 ng/mL and 28.17 ng/mL). To investigate the effect of concentration on response, the experiments were performed from 5 ng/mL to 170 ng/mL, with oleanolic acid, corosolic acid and betulinic acid as representatives.

2.6.2. Multivariate optimization

Three interactive variables: molar ratio of PBIOTs to total triterpene acids (X_1), temperature (X_2, °C) and time (X_3, min) were designed by BBD from RSM and listed in Table 1 and the average peak area reflecting the response was analyzed by the software Design-Expert 7.1.3 Trial. The results were fitted to a second-order polynomial equation. The optima were derived with the regression of the non-linear relationship. Artificial neural network in this work includes input layer, hidden layer and output layer [38]. The operating of ANN is to adjust the neuron parameters to acquire the regularity of the system just like the action of their biological counterparts. In this work, the variables combinations (X_1: molar ratio of reagent to total triterpene acids, X_2: temperature and X_3: time) were delivered to a back-propagation ANN with one hidden layer containing eight neurons. To improve the reliability and generalization ability of the ANN, the input data were normalized between the values −1 and 1, before they were numbered in random order, 70% of which were used for ANN training, 15% of which were used for ANN testing, and other 15% of which were used for ANN validation. The ANN was constructed by Logistic Sigmoid and purelin transfer functions, with the Levenberg–Marquardt algorithm as training tool. To search the optimum, results from ANN were passed to GA algorithm program, where the collection and individual of predicted responses were, respectively, termed chromosome and genes. The fitness value (optimum) of each chromosome was evaluated and selected to generate a new set of child chromosome through the crossover and mutation procedures, in which the diversity was introduced into the child chromosomes while preserving the information carried by the parent chromosome. Such an iterative evolutionary process in search of the chromosome with the highest fitness value terminated when the epoch of generations was finished or chromosome with a fixed level of fitness was found [39]. The ANN training and GA optimization were accomplished with mathematical program Matlab R2010a. Since Box–Behnken design (BBD) was thought to be efficient, the data from BBD were used to be inputs of ANN (i.e., BBD-ANN) to improve the efficiency. The results from BBD and BBD-ANN were compared with each other, and the optimal variables combination was applied as the derivatization condition.

2.7. Method validation

2.7.1. Selectivity and sensitivity

The method were validated in accordance with United States Food and Drug Administration (FDA) guidelines [40] and some researches on the development of HPLC [8]. The selectivity of the method was assessed by anlyzing blank and spiked plasma samples. The potential endogenous interference from the extraction procedure and HPLC condition could be tested by comparing the chromatograms. Method sensitivity was determined by limit of detection (LOD) and limit of quantification (LOQ) based on a signal-to-noise ratio of 3:1 and 10:1, respectively. For further validation of the sensitivity of the method based on derivatization reaction, LORC was investigated by decreasing the concentration of reactants under the optimized derivatization condition.

Table 1
Designs of multivariate methods, validation by multi-criteria and nonparametric tests and optima.

Run[a]	Variables[b]			Response[c]			Validation[d]		
	X_1	X_2	X_3	Exp.	BBD	BBD-ANN		BBD	BBD-ANN
1	3	90	25	937.75	933.06	937.75	Multi-criteria	–	–
2	7	90	5	767.62	772.39	767.62	AME	8.65	10.33
3	3	90	5	698.43	689.85	698.43	CE	0.9997	0.9991
4	3	100	15	679.02	686.04	689.35	MAE	0.04	−1.14
5	7	100	15	769.58	763.25	769.58	RMSE	5.09	9.22
6	7	80	15	419.23	412.29	419.23	MRE (%)	0.05	−0.09
7	5	90	15	1128.25	1131.76	1135.60	R^2	0.9996	0.9989
8	5	90	15	1136.54	1131.76	1135.60	Nonparametric tests	–	–
9	5	80	5	399.08	401.35	399.08	p-value	0.6790	0.6241
10	5	100	25	1020.65	1018.45	997.31	–	–	–
11	5	80	25	743.65	742.03	743.65	Optima	–	–
12	5	90	15	1129.84	1131.76	1135.60	X_1	5.20	5.41
13	5	90	15	1134.65	1131.76	1135.60	X_2	92.09	92.31
14	5	100	5	853.42	855.11	853.42	X_3	23.14	25.00
15	5	90	15	1129.34	1131.76	1135.60	Response	1242.31	1271.57
16	7	90	25	1024.55	1033.20	998.74	Exp.	1231.68	1237.51
17	3	80	15	300.42	306.82	300.42	–	–	

[a] The 17 runs from the Box–Behnken design were given by the software Design-Expert 7.1.3 Trial.
[b] Variables were X_1: ratio of PBIOTs to analytes, X_2: temperature and X_3: time.
[c] Experimental and predicted peak area of the representative PBIOTs-betulonic acid derivative.
[d] Validation: multi-criteria (AME: absolute maximum error, CE: coefficient of efficiency, MAE: mean absolute error, RMSE: root Mean squared error, MRE: mean relative error, R^2: correlation of determination) and nonparametric tests (p-value with Wilcoxon rank sum method).

2.7.2. Linearity

The standard calibration curves were established using blank plasma samples spiked with triterpene acids within the concentration ranges: ursolic acid (5 to 200 ng/mL), tormentic acid (5 to 300 ng/mL), oleanolic acid (5 to 500 ng/mL), maslinic acid (5 to 800 ng/mL), betulinic acid (5 to 3000 ng/mL), corosolic acid (5 to 150 ng/mL) and betulonic acid (5 to 150 ng/mL), and each level sample was prepared and assayed in duplicate on separate 3 day. The linear equation was got from regression of the peak area (Y) of each compound, versus the concentration (x). The concentration of the analytes in samples was determined by interpolation from the calibration curve.

2.7.3. Accuracy and precision

Three batches of samples, each one of which consisted of six replicates of QC samples at three concentration levels, were analyzed on three consecutive validation days. The relative error percentage (RE%) and the relative standard deviations percentage (RSD%) were measured to evaluate the accuracy and precision (intra-day, inter-day).

2.7.4. Extraction recovery and matrix effect

The extraction recovery of analytes was conducted by analyzing the samples spiked before extraction and the equivalent samples spiked after extraction using the same assay run conditions. The ratio percentage was calculated following the equation: recovery (%)$=S_b/S_a\times100\%$ to indicate the recovery, where S_b and S_a are the peak area values of each spiked concentration before and after extraction, respectively. The matrix effect was evaluated by analyzing the spiked post-extracted sample and the standard solution sample evaporated and redissolved in mobile phase, following the equation: matrix effect (%)$=S_p/S_s\times100\%$, where, S_p is the peak area value of sample spiked after extraction and S_s is the peak area of the standard sample reconstituted.

2.7.5. Stability

The stability of triterpene acids in rat plasma was evaluated by assaying six aliquots of QC samples at three concentrations exposed to practical experimental conditions. Stability of stock solution was evaluated at room temperature for a week. Freeze-thaw stability was evaluated after two cycles of freeze (-20 °C)-thaw (room temperature, spontaneously) performance. The short-term stability was determined by analyzing the QC samples at room temperature for 3 h that exceeded the usual time of samples preparation. Long-term stability was determined by analyzing the QC samples kept at the storage temperature (-20 °C) for 2 weeks. Post-preparative stability was assessed by analyzing QC samples at room temperature for 12 h. The evaluations for the stability of the analytes in rat plasma were the ratio percentage of the detected concentration and the concentration of QC samples. In addition, the stability of PBIOTs-triterpene acid derivatives was investigated by analyzing the derivatives in acetonitrile and aqueous acetonitrile (50%, v/v).

2.7.6. Application and pharmacokinetic study

The method was applied to detect the concentrations of seven triterpene acids in plasma samples after the oral administration of the *Salvia miltiorrhiza* extract. To calculate the administered dose, the contents of seven triterpene acids in *Salvia miltiorrhiza* extract were determined in advance with this method. The contents of seven triterpene acids in the extract were 7.23 (ursolic acid), 13.57 (tormentic), 18.57 (oleanolic acid), 30.82 (maslinic acid), 99.58 (betulinic acid), 101.48 (Corosolic acid) and 123.85 (betulonic acid) μg/g. The mice (Mus musculus, 40–50 g) were bought from Shandong Lukang Pharmaceutical Co., Ltd and were treated following the guideline approved by the institutional animal care and use committee of the National Health Research Institutes. Blood samples were collected at 30, 60, 90, 120, 180, 240, 300 and 360 min from 6 healthy mice after oral administration of *Salvia miltiorrhiza* extract with different doses (1 g/kg and 1.5 g/kg). Non-compartmental pharmacokinetic analysis of concentration-time data was performed using the software Winnonlin 5.2, and the parameters were obtained including elimination rate constants (k_{el}), elimination half-life ($T_{1/2}$), peak time (t_{max}), peak concentration (C_{max}), the area under the plasma concentration-time curve up to the last time (t) (AUC_{0-t}), $AUC_{0-\infty}$(area under curve from 0 to infinite time), clearance (CL), mean residence time from 0 to last time (MRT_{0-t}), area under curve from 0 to infinite time ($MRT_{0-\infty}$).

3. Results and discussion

3.1. Fluorescence spectra of PBIOTs-triterpene acid derivatives

The fluorescence scan spectra of representative PBIOTs-maslinic acid derivative in Fig. 2 exhibited an excitation maximum at 350 nm and an emission maximum at 402 nm in acetonitrile. As can be seen, no obvious blue- or red-shift of the spectra with the decreasing solvent polarity was observed. The fluorescence intensity of excitation and emission increased with the increasing of the volume ratio of acetonitrile in the mixed solvents. The emission intensity in pure acetonitrile was 9 times higher than the value obtained in the mixed solvents containing 10% of acetonitrile. The main reason of fluorescence quenching in aqueous acetonitrile for PBIOTs-maslinic acid derivative should be attributed to the fact that the nitrogen atom in molecular core of PBIOTs reacted with H_2O to form the protonated ion [28]. The results revealed that the acetonitrile was appropriate to highly sensitive FLD and the wavelength of maximum excitation and emission kept almost unchanged.

3.2. Pretreatment procedure development

It usually takes some time for the pharmacokinetic study to pretreat the blood samples, especially for the derivatization method. More efficient pretreatment procedure will save the total run time of the study. During the preparation of sample, less amount of blood (20 μL) was collected via tail vein puncture into the modified syringe containing appropriate anticoagulant, PBIOTs, catalyst and solvent, with the heated water circulating. After derivatization, ACN was drawn into the syringe and precipitate of protein was formed immediately, and the derivatives were transferred into the organic phase. The supernatant solution was filtered into sampling vials with polymer filter. Excessive ACN were continuously added to the obtained solution, no more precipitate or suspended substance was observed, indicating that plasma samples would not produce precipitate when they were eluted by the mobile phase consisting of ACN and water in HPLC-FLD-MS. Thus the pretreatment procedure including blood collection, derivatization and injection into via was more conveniently and efficiently finished than usual derivatization procedure [21–27,36,37]. Large batches of samples in pre-clinical or clinical studies could be prepared with this semi-automated derivatization extraction method.

3.3. Optimization of derivatization condition

3.3.1. Single variable optimization

Before the multivariate optimization, single factor experiments should be thoroughly conducted to get the optimal ranges for highly sensitive detection. The usual solvents including acetonitrile (ACN),

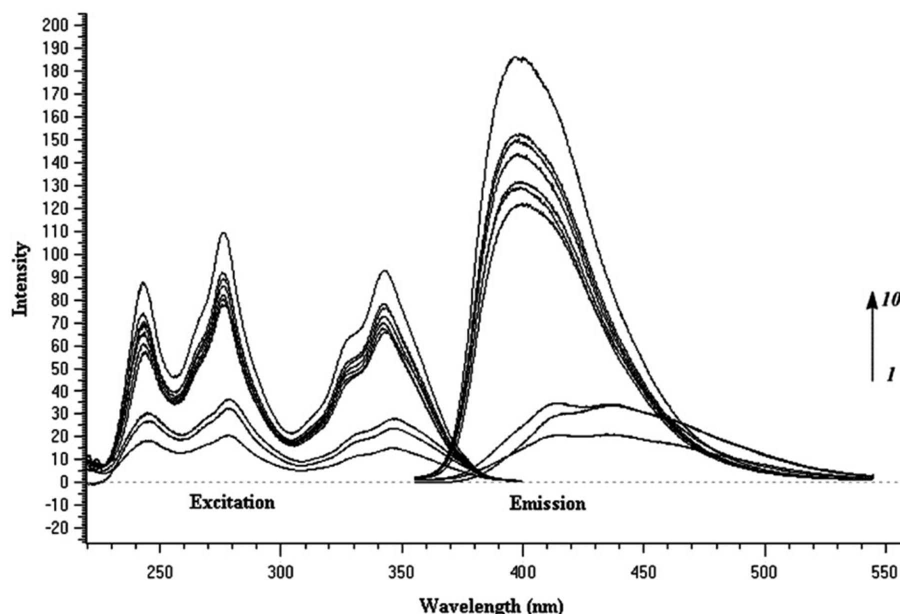

Fig. 2. Fluorescence spectra for the derivative (PBIOTs-maslinic acid) in different mixed solvents acetonitrile–water within the volume ratio range (1 to 10 were the ratio of ACN to water: from 10:90, 20:80… to 80:20, 90:10, 100:0 v/v).

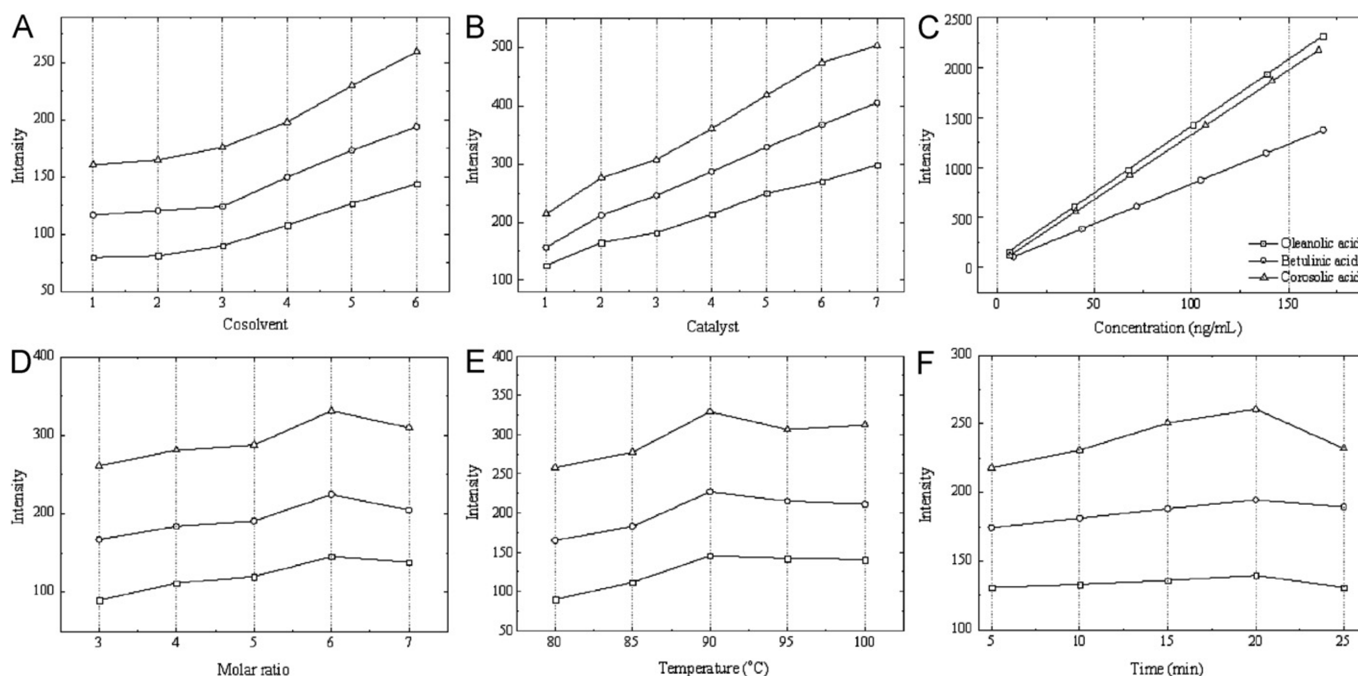

Fig. 3. Single variable optimization for the derivatization reaction condition: (A) fluorescence response figure of analyte at three concentration levels in usual solvents (1: DCM, 2: CHF, 3: DMSO, 4: DMF, 5: EA, 6: ACN); (B) response figure of analyte at three concentration levels with different catalysts (1: Py, 2: TEA, 3: DMAP, 4: $K_2C_2O_4$, 5: $(CH_3)_4NCO_3$, 6: Na_2CO_3, 7: K_2CO_3); (C) response of representative analytes (oleanolic acid, corosolic acid and betulinic acid) with variation of the concentration (from 5 to 170 ng/mL); (D) molar ratio of PBIOTs to total triterpene acids (from 3 to 7); (E) responses at the temperatures from 80 to 100 °C; (F) responses with increasing reaction time from 5 to 25 min.

N,*N*-dimethylformamide (DMF), dimethylsulfoxide (DMSO), dichloromethane (DCM), ethyl acetate (EA) and chloroform (CHF) were investigated to get a more appropriate cosolvent. Fluorescence responses of derivatives in DCM and CHF were observed to be weaker than that in other solvents (Fig. 3A), which was caused by the lower solubilities of reagent and derivatives in the former two. DMF and DMSO had the higher solubilities for reactants and derivatives, but they resulted in the weaker fluorescent response and larger changes of maximum wavelength in FLD. The fluorescence responses in EA and ACN were stronger, but EA showed low solubility in the mobile phase. Thus the application of ACN as the cosolvent was helpful to the higher sensitivity for the derivatization. To get the higher derivatization response and reaction rate, several basic catalysts including pyridine (Py), triethylamine (TEA), 4-dimethylaminopyridine (DMAP), $K_2C_2O_4$, $(CH_3)_4NCO_3$, Na_2CO_3 and K_2CO_3 were evaluated for the derivatization reaction of carboxyl group with reagents (Fig. 3B). Results showed that the added K_2CO_3 solution gave the highest detection responses. The effect of initial concentration of reactant on the response was studied with oleanolic acid, corosolic acid and betulinic acid as representatives

(Fig. 3C). With the concentration decreasing, the LORC values were obtained (see Section 3.5.1). Therefore, the initial concentrations of reactants in the following experiments were kept higher than the corresponding LORC values. The molar ratio of PBIOTs to triterpene acids from 3 to 7 were investigated in Fig. 3D. The fluorescence intensity of PBIOTs derivatives increased with the increasing molar ratio but the maximum fluorescence intensity was achieved at the 6-fold molar ratio. The effect of temperature on derivatization reaction was investigated from 80 to 100 °C (Fig. 3E). The results indicated that the highest detection responses appeared at 90 °C. The reaction times from 5 to 25 min were investigated in Fig. 3F and the maximum detection responses appeared at 20 min of reaction time.

3.3.2. Multivariate optimization

With the optimal ranges of variables, the multivariate optimization methods were performed to find the optimal combination of variables. Among the six variables discussed above, co-solvent, catalyst and concentration of reactants were the relatively independent variables, while the other three variables including molar ratio, reaction temperature and time were affected interactively by each other. Therefore the multivariate optimization methods were employed to get the optimal combination of the three variables (X_1: ratio of PBIOTs to analytes, X_2: temperature and X_3: time). The designs, validation and optima of multivariate methods were listed in Table 1, where Multi-criteria and nonparametric tests were used to reflect the accuracy of the model and to indicate the best model [41]. The two models showed good correlation (R^2 greater than 0.9) and coefficient of efficiency (CE approximately to 1) (see Table 1). The values of MRE were within 1.5%, which indicated that the fitting or learning processes of the two models were fully operational and the predicted responses could be correlated with the experimental responses [42]. The two p-values from nonparametric tests with Wilcoxon rank sum method were greater than 0.05, which demonstrated that the differences between the experimental and predicted value were not statistically significant and the two models could simulate the statistic characteristic of predicted responses at the 95% confidence level [41]. Thus, the two models proved to be able to produce predicted values correlating well with experimental data. Optima were achieved by regression of BBD model (1242.31) and iterative evolutionary optimization with GA method (1271.57). The corresponding combinations of variables were applied to obtain similar experimental responses 1231.68 and 1237.51 (see Table 1). Comparison of the two models revealed that BBD method got the better validation with the more favorable results such as MAE, RMSE, MRE, R^2 and p-value. BBD-ANN method obtained the larger RMSE, which probably attribute to the fact that the ANN model was convergent not well enough with the limited input data from the efficient BBD in this work. Larger amounts of data might be needed to establish better artificial neural network model, but that might be more time-consuming. As a result, the variables combination (X_1: 5.21, X_2: 92.09 and X_3: 23.14) from BBD method was selected to keep the higher accuracy.

3.4. Detection and identification method development

3.4.1. HPLC separation and fluorescence detection

To get better peak shape with a short retention time for the separation of PBIOTs-triterpene acid derivatives, several chromatographic columns, such as Spherisorb C18 (200 mm × 4.6 mm, 5 μm), Hypersil C18 (200 mm × 4.6 mm, 5 μm) and Akasil-C18 column (4.6 × 250 mm, 5 μm) were evaluated for the optimal separation. The results indicated that the Akasil-C18 column gave the best resolution and then was selected for all experiments. With the column operating temperature at 30 °C, acetonitrile and water were used as eluent A and B, respectively, to provide an efficient separation in conjunction with the gradient elution mentioned in experimental section. The complete separation of seven triterpene acid derivatives could be obtained within 30 min (Fig. 4). The separation time was obviously reduced relative to that of the reported method [9,11,14–16,20]. Thus the HPLC-FLD after pre-column derivatization was capable of separating and detecting the seven triterpene acids in rat plasma efficiently.

3.4.2. Mass spectrometry identification

The ionization and fragmentation of triterpene acid derivatives were investigated by atmospheric chemical ionization mass spectrometry (APCI/MS) in positive-ion detection mode. The derivatization produced intense molecular ion peaks at m/z $[M+H]^+$ and the collision-induced dissociation spectra of triterpene acid derivative produced MS/MS fragmentation. The positive-ion mass spectra, corresponding to the separated components, are quite complex. MS spectra for the representative maslinic acid are shown in Fig. 5 (A: MS, B: MS/MS). In most cases, the collision-induced dissociation spectra of m/z $[M+H]^+$ produced a relatively weak fragment ion by losing one water molecule as follows: ursolic acid, m/z 801.1 $[M+H]^+$; tormentic acid, m/z 833.1 $[M+H]^+$; oleanolic acid m/z 801.1 $[M+H]^+$; maslinic acid, m/z 817.1 $[M+H]^+$, m/z 799.1 $[M+H-H_2O]^+$; m/z 782.7 $[M+H-H_2O]^+$; betulinic acid, m/z 801.1 $[M+H]^+$, m/z 781.8 $[M+H-H_2O]^+$; corosolic acid m/z 817.1 $[M+H]^+$; betulonic acid, m/z 799.1 $[M+H]^+$, m/z 780.9 $[M+H-H_2O]^+$ (see Fig. 5). The specific fragment ion at m/z 499.0 was from the cleavage of CO–C ester bond of the N-linked side chain, corresponding to the protonated maslinic acid-H_2O moiety. The selected reaction monitoring, based on the m/z $[M+H]^+ \rightarrow m/z$

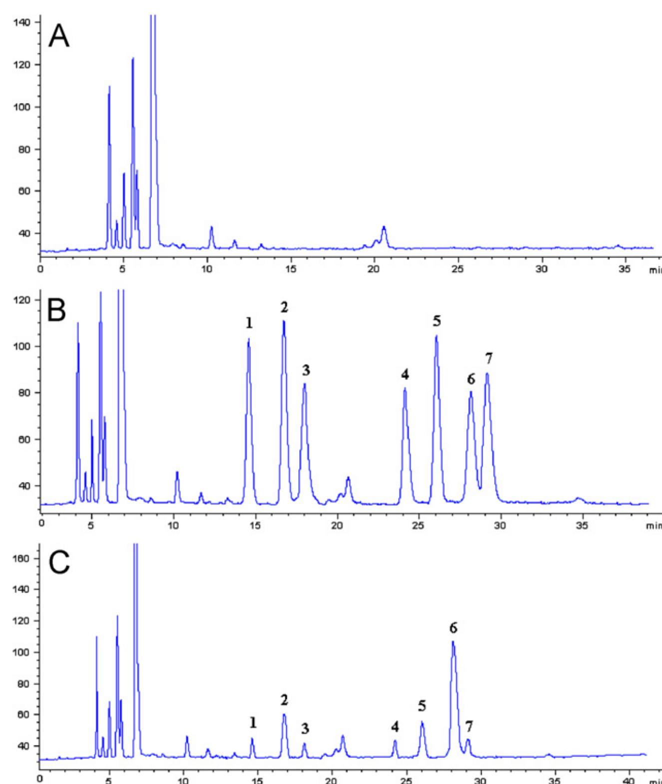

Fig. 4. The representative chromatograms for (A) blank plasma, (B) blank spiked with the seven triterpene acids (1: tormentic acid, 2: maslinic acid, 3: corosolic acid, 4: ursolic acid, 5: oleanolic acid, 6: betulinic acid, 7: betulonic acid) at 100 ng/mL and (C) 30 min sample plasma after a single oral administration of *Salvia miltiorrhiza* extract.

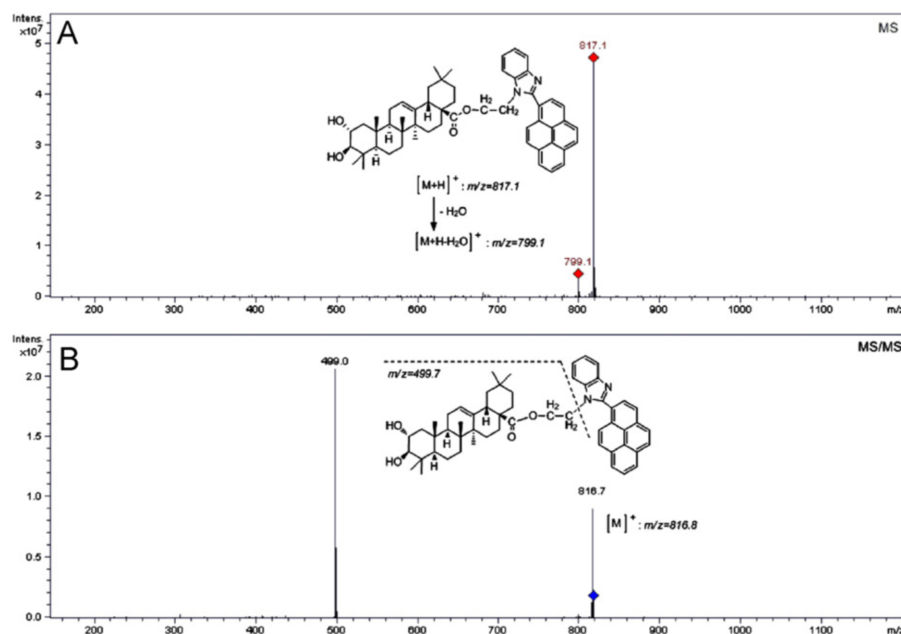

Fig. 5. The MS, MS/MS spectra and cleavage mode for representative derivative PBIOTs-maslinic acid.

799.1 $[MH-H_2O]^+$ and m/z 499.0 transitions, was specific for the identification of maslinic acid derivative.

3.4.3. Comparison with the existing procedures

Comparison with the existing pretreatment procedures of blood samples for detection of triterpene acids was made. Remarkably, sample amounts (moun μL) in the reported methods [35,37,43,44] were much greater than that (20 μL) in this work, which practically brought the more difficulty to blood collection and the more injury to animal. Moreover, the pretreatment procedures for blood samples including multiple centrifugation, vortex, evaporation and reconstitution, were complicated and low-efficient as the routine operation, which were not suitable for large batches of blood samples analysis in pre-clinical or clinical studies. The corresponding run time for pretreatment in the existing procedures were presumably longer than that (within 26 min) in this work, even though some of the run time mentioned explicitly in the complicated procedures seemed to be shorter (see Supplementary Table) [37,44]. In addition, the LOD values in reported methods were much greater than those (0.67–1.08 ng/mL) in this work. Although the LOD value from the HPLC-MS method [35] was lower than the LOD in this work, the complicated operation, the larger sample amount and the longer run time for the pretreatment procedure showed its disadvantages, which was incompetent to the medical research with larger batches of blood samples. Consequently, micro-sampling amount, the convenient and efficient operation, and the lower LOD justified the superiority of the present method.

3.5. Method validation

3.5.1. Selectivity and sensitivity

By comparing the chromatograms of the blank sample, spiked sample and sample collected after administration of *Salvia miltiorrhiza* extract, the selectivity of the method was validated. The clear peaks of the seven PBIOTs-triterpene acid derivatives were observed at the relatively fixed retention time and some peaks besides analytes on chromatograms were the peaks of other unconcerned components (Fig. 4). No endogenous interference was found, due to the good specificity of the method with the proposed pretreatment procedure and HPLC conditions. Sensitivity of this method was determined by LOD and LOQ. As expected, the method gave the higher sensitivity with the lower LOD (from 0.67 to 1.08 ng/mL) than those reported in previous works [8–20,26,27]. The excellent selectivity and sensitivity of the method were attributed to the selective labeling by appropriate fluorescent reagent under the optimal reaction condition. To validate the trace detection based on derivatization method, the limit of reactant concentration (LORC) values of seven triterpene acids in plasma samples were achieved: 2.54 (ursolic acid), 2.95 (tormentic acid), 2.53 (oleanolic acid), 2.61 (maslinic acid), 4.03 (betulinic acid), 3.33 (corosolic acid) and 3.98 ng/mL (betulonic acid). The produced derivatives from this pre-column derivatization method could be sensitively detected on the condition that the concentrations of the triterpene acids were greater than the corresponding values of LORC. Moreover, the lower LORC value ensured the trace detection of blood samples by pre-column derivatization method.

3.5.2. Linearity

The calibration curves were prepared daily and showed good linearity in corresponding ranges. The linearity was evaluated by using the regression analysis of the working curve depicted by the injected concentration and the peak area after the six replicates of experiments. The correlation coefficients (r^2) were greater than 0.9987 and the RSD of the retention time and peak area were lower than 0.04% and 5%, respectively.

3.5.3. Accuracy and precision

The accuracy and precision (intra-day and inter-day) were, respectively, investigated by spiking each of triterpene acid at three different concentrations into samples. The results were shown in Table 2. Values presented at each concentration were the mean of six independently prepared samples. The intra-day accuracy ranged from 94.31% to 105.32%, while the inter-day accuracy ranged from 94.87% to 104.28%. Intra-day and inter-day precision were found to be in the range of 3.23–6.87% and 3.96–6.82%, respectively. The results demonstrated that the assay was reproducible and reliable

Table 2
Intra- and inter-day precision and accuracy of seven triterpene acids in rat plasma at low, medium and high concentration levels ($n=6$).

Compound spiked conc (ng/mL)	Intra-day ($n=6$)			Inter-day ($n=6$)		
	Mean	Accuracy	Precision	Mean	Accuracy	Precision
Ursolic acid						
21.43	20.90	97.54	3.23	20.33	94.87	3.96
67.25	68.06	101.20	2.98	65.59	97.53	4.27
112.37	111.59	99.31	3.01	109.19	97.17	3.85
Tormentic acid						
38.02	36.84	96.89	3.69	37.58	98.85	4.98
164.84	172.69	104.76	2.14	160.60	97.43	5.45
308.56	324.98	105.32	6.87	297.76	96.50	3.32
Oleanolic acid						
48.21	46.56	96.57	3.99	46.78	97.04	4.96
210.75	204.13	96.86	4.75	203.04	96.34	4.87
424.69	435.65	102.58	4.76	439.81	103.56	5.15
Maslinic acid						
43.45	45.53	104.79	4.98	41.18	94.78	5.32
384.35	372.97	97.04	5.21	361.06	93.94	4.83
729.49	707.46	96.98	4.39	750.57	102.89	4.34
Betulinic acid						
170.53	166.64	97.72	5.02	168.40	98.75	2.19
1394.76	1337.16	95.87	3.62	1318.47	94.53	3.35
2563.91	2528.78	98.63	3.98	2444.94	95.36	4.91
Corosolic acid						
19.89	19.26	96.81	5.39	19.10	96.03	6.82
76.43	74.55	97.54	4.46	77.71	101.68	4.93
123.26	116.25	94.31	5.06	128.54	104.28	2.54
Betulonic acid						
19.76	18.93	95.82	2.43	18.77	94.97	2.64
82.48	80.53	97.63	2.94	80.71	97.86	2.08
149.65	144.83	96.78	3.66	144.50	96.56	3.27

for the quantification of the seven triterpene acids in rat plasma samples.

3.5.4. *Recovery evaluation and matrix effect*

The recoveries of the seven triterpene acids in rat plasma at three examined concentrations levels were in the range from 96.99% to 110.36% with RSD less than 9%, and the matrix effects values ranged from 98.69% to 106.91% with RSD less than 8%. The results indicated that the recoveries and matrix effects of samples were acceptable. Moreover, the peak shape and resolution of the seven analytes in rat plasma were sharp and clear, which proved that there were no significant matrix interference from the concomitants in the examined samples. The results were attributable to the intense fluorescence response that made the interfering signal insignificant. And that, other potential interferences might be removed by the sample pretreatment process. Therefore the method offered accurate detection with satisfactory recoveries and insignificant matrix effect, and was suitable for detecting the trace content of the seven triterpene acids in rat plasma.

3.5.5. *Stability*

The stability of seven triterpene acids in stock solution, freeze and thaw stability, short-term stability, long-term stability and post-preparative stability were investigated at low, medium and high concentration levels with QC samples. The results for stability validation were listed in Table 3. The percent relative error values were within 15% for all the QC samples, which indicated that the working solutions proved to be stable for a week and the analytes were stable enough to be handled in the experiments. The relative stabilities of PBIOTs-triterpene acid derivatives in acetonitrile and aqueous acetonitrile (50%, v/v)

Table 3
Stability of seven triterpene acids in rat plasma at three QC levels ($n=6$).

Compound spiked conc (ng/mL)	Stock solution			Freeze–thaw			Short-term			Long-term			Post-preparative		
	Mean	SD(±)	RE%	Mean	SD(±)	RE%	Mean	SD(±)	RE%	Mean	SD(±)	RE%	Mean	SD(±)	RE%
Ursolic acid															
21.43	21.86	2.35	2.00	21.95	1.30	2.43	20.46	2.15	−4.53	20.52	2.36	−4.25	21.87	3.24	2.05
67.25	65.23	3.62	−3.00	69.89	2.93	3.93	67.94	5.05	1.03	65.25	3.74	−2.97	66.76	4.37	−0.73
112.37	116.86	6.53	4.00	118.78	4.75	5.70	107.38	9.68	−4.44	103.94	9.66	−7.50	116.29	8.27	3.49
Tormentic acid															
38.02	36.54	2.69	−3.89	36.36	1.09	−4.37	36.44	4.82	−4.16	36.43	2.74	−4.18	37.42	3.01	−1.58
164.84	172.67	5.93	4.75	168.02	4.63	1.93	174.28	10.11	5.73	160.27	8.17	−2.77	169.57	6.25	2.87
308.56	312.14	24.87	1.16	321.42	10.88	4.17	316.85	29.57	2.69	311.57	24.83	0.98	315.36	26.55	2.20
Oleanolic acid															
48.21	44.99	3.56	−6.68	49.15	5.25	1.95	50.64	6.36	5.04	47.48	4.59	−1.51	45.90	4.71	−4.79
210.75	224.35	27.58	6.45	217.94	17.19	3.41	197.01	23.59	−6.52	200.42	26.89	−4.90	225.58	28.26	7.04
424.69	442.67	19.83	4.23	439.27	27.59	3.43	428.61	33.19	0.92	429.39	38.21	1.11	439.71	17.29	3.54
Maslinic acid															
43.45	45.83	2.67	5.48	44.86	4.27	3.25	44.95	5.64	3.45	42.57	6.81	−2.03	41.37	3.75	−4.79
384.35	393.57	29.67	2.40	398.72	24.12	3.74	388.72	31.47	1.14	378.63	34.77	−1.49	402.61	30.79	4.75
729.49	778.60	54.79	6.73	759.46	50.66	4.11	759.26	61.77	4.08	749.9	63.48	2.80	765.56	55.69	4.94
Betulinic acid															
170.53	160.68	10.55	−5.78	163.25	9.34	−4.27	165.78	10.38	−2.79	171.81	10.51	0.75	173.28	8.93	1.61
1394.76	1459.72	86.61	4.66	1451.88	79.51	4.10	1431.79	105.46	2.65	1410.4	92.79	1.12	1461.26	91.65	4.77
2563.91	2759.36	137.95	7.62	2639.81	117.43	2.96	2652.41	154.84	3.45	2611.6	147.7	1.86	2749.85	138.54	7.25
Corosolic acid															
19.89	21.08	0.68	5.98	18.85	1.26	−5.23	19.44	4.81	−2.26	20.51	5.85	3.12	20.37	1.79	2.41
76.43	82.39	8.36	7.80	82.37	2.79	7.77	79.25	6.27	3.69	81.79	10.36	7.01	81.57	7.38	6.73
123.26	129.85	10.45	5.35	130.42	8.59	5.81	129.22	9.83	4.84	131.78	10.96	6.91	128.64	9.26	4.36
Betulonic acid															
19.76	20.34	1.39	2.94	20.38	3.79	3.14	19.99	1.87	1.16	18.86	2.31	−4.55	20.12	1.35	1.82
82.48	84.14	5.67	2.01	78.69	12.52	−4.60	86.98	13.35	5.46	82.94	13.39	0.56	80.37	3.59	−2.56
149.65	144.79	9.84	−3.25	154.39	11.33	3.17	141.27	11.38	−5.60	146.57	11.47	−2.06	151.46	10.93	1.21

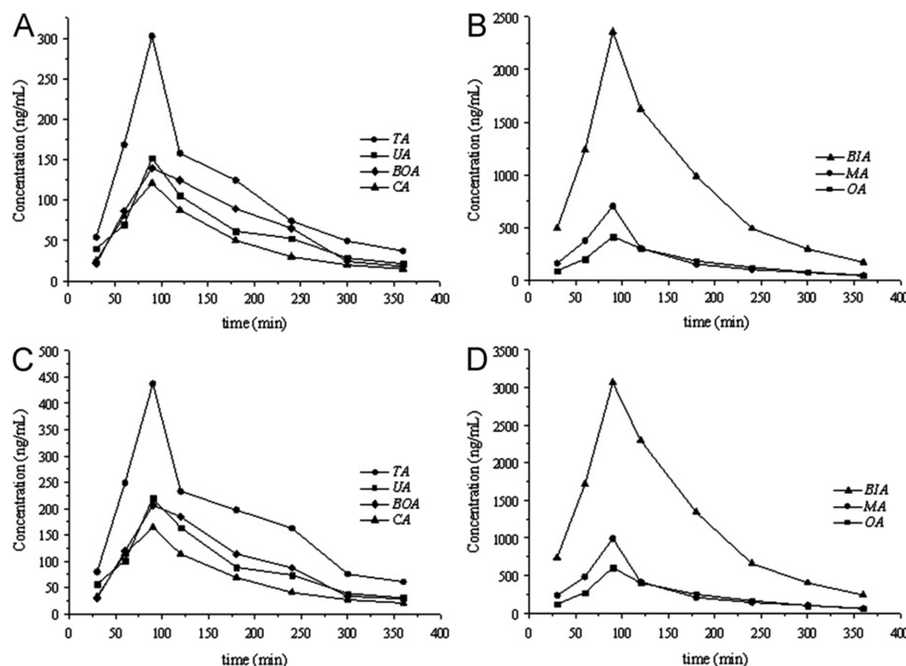

Fig. 6. The mean plasma concentration–time profile of triterpene acids (**UA**: ursolic acid, **TA**: tormentic acid, **OA**: oleanolic acid, **MA**: maslinic acid, **BIA**: betulinic acid, **CA**: corosolic acid, **BOA**: betulonic acid) after oral administration of *Salvia miltiorrhiza* extract (**A** and **B**: dose of 1 g/kg; **C** and **D**: dose of 1.5 g/kg).

Table 4
The pharmacokinetic parameters (mean ± SD) after oral administration of *Salvia miltiorrhiza* extract with different doses (n=6).

Parameter[a]	Unit	Ursolic acid	Tormentic acid	Oleanolic acid	Maslinic acid	Betulinic acid	Corosolic acid	Betulonic acid
I[b]								
k_{el}	1/min	0.0071 ± 0.0009	0.0064 ± 0.0012	0.0081 ± 0.0011	0.0082 ± 0.0026	0.0089 ± 0.0021	0.0078 ± 0.0016	0.0081 ± 0.0018
$t_{1/2}$	min	98.09 ± 4.15	108.97 ± 9.26	86.74 ± 6.79	84.34 ± 8.32	78.14 ± 7.95	88.36 ± 6.84	87.03 ± 9.38
T_{max}	min	90 ± 3.63	90 ± 5.24	90 ± 5.81	90 ± 7.56	90 ± 6.24	90 ± 5.22	90 ± 6.34
C_{max}	ng/mL	152 ± 5.82	302.65 ± 12.67	412.58 ± 58.65	751.64 ± 88.74	2358.35 ± 379.11	121.23 ± 9.57	139.24 ± 11.54
AUC_{0-t}	min ng/mL	21823.24 ± 275.56	38887.24 ± 347.81	58290.58 ± 576.12	92328.53 ± 866.71	306941.2 ± 3354.21	17212.06 ± 210.31	24308.86 ± 416.54
$AUC_{0-\infty}$	min ng/mL	24848.61 ± 278.32	44727.84 ± 376.72	64047.48 ± 596.53	98476.79 ± 645.34	325942.1 ± 3167.52	19124.25 ± 295.54	26597.14 ± 378.66
CL	mL/min/kg	2012.18 ± 256.95	1117.87 ± 139.12	780.67 ± 46.95	507.73 ± 37.38	17294.22 ± 142.81	333292.62 ± 5464.73	236044.01 ± 4091.37
MRT_{0-t}	min	151.79 ± 12,68	147.34 ± 15.63	148.67 ± 17.49	132.10 ± 15.84	153.40 ± 16.89	2614.48 ± 335.79	1879.90 ± 254.68
$MRT_{0-\infty}$	min	194.37 ± 17.79	195.64 ± 21.56	178.91 ± 34.37	153.93 ± 30.69	138.85 ± 14.72	143.21 ± 19.69	154.857 ± 24.35
II[c]								
k_{el}	1/min	0.0073 ± 0.0009	0.0068 ± 0.0011	0.0077 ± 0.0012	0.0078 ± 0.0019	0.0085 ± 0.0018	0.0073 ± 0.0011	0.0079 ± 0.0012
$t_{1/2}$	min	94.41 ± 5.31	101.94 ± 11.76	90.12 ± 5.19	89.04 ± 8.92	81.84 ± 9.61	95.38 ± 7.53	87.85 ± 7.59
T_{max}	min	90 ± 4.17	90 ± 9.31	90 ± 6.74	90 ± 8.87	90 ± 9.79	90 ± 6.84	90 ± 8.97
C_{max}	ng/mL	219.66 ± 11.74	437.52 ± 17.41	605.45 ± 580.76	992.57 ± 759.40	3065.49 ± 298.21	164.28 ± 24.85	206.52 ± 19.74
AUC_{0-t}	min ng/mL	31685.7 ± 349.74	61461.39 ± 564.89	80506.52 ± 979.31	98742.54 ± 1043.76	419979.8 ± 3563.78	23265.99 ± 413.85	33910.80 ± 624.52
$AUC_{0-\infty}$	min ng/mL	35836.8 ± 379.51	70411.85 ± 615.46	88681.37 ± 1017.74	106043.44 ± 1123.61	448110.2 ± 3894.52	26106.80 ± 507.34	37519.84 ± 650.18
CL	mL/min/kg	2092.82 ± 179.47	1065.16 ± 110.24	845.72 ± 68.21	471.50 ± 57.31	167.36 ± 21.34	1915.20 ± 393.93	1998.94 ± 328.45
MRT_{0-t}	min	150.05 ± 14.62	153.83 ± 17.85	147.91 ± 11.58	128.99 ± 13.54	138.54 ± 19.51	142.96 ± 19.47	153.15 ± 21.86
$MRT_{0-\infty}$	Min	190.15 ± 15.12	198.73 ± 19.66	179.44 ± 19.67	153.74 ± 21.64	159.86 ± 17.38	181.56 ± 19.68	185.24 ± 24.95

[a] The parameters were k_{el} (first-order elimination rate constant), $t_{1/2}$ (half life), T_{max} (peak time), C_{max} (peak concentration), AUC_{0-t} (area under curve from 0 to last time), $AUC_{0-\infty}$(area under curve from 0 to infinite time), CL (clearance), MRT_{0-t} (mean residence time from 0 to last time) and $MRT_{0-\infty}$ (mean residence time from 0 to infinite time).

[b] **I** with dose of 1 g/kg.

[c] **II** with dose of 1.5 g/kg of *Salvia miltiorrhiza* extract.

were investigated over a period of one month. With pure acetonitrile as solvents, daylight had no effect on stabilities of PBIOTs-triterpene acid derivatives. When PBIOTs-triterpene acid derivatives were stored in aqueous acetonitrile (50%, v/v) and exposed to daylight for one week, a slight degradation (1.8%) was observed (data are not listed). In addition, derivatives were dissolved in the mixed solvent acetonitrile–water and stored at 4 °C in a refrigerator. Peak areas of derivatives were determined in a one-day interval for one week; no significant changes were observed. It indicated that the derivatives were sufficiently stable to be analyzed during the period of HPLC analysis. Thus the results indicated the method and procedure were competent for the simultaneous detection and the pharmacokinetic study of the seven triterpene acids in rat plasma samples.

3.5.6. Application and pharmacokinetic study

The extracts of triterpene acids from famous medicinal herb *Salvia miltiorrhiza* can be used as traditional pharmaceutical prescription herbs. The accurate determination of triterpene acids is valuable for the preparation of new dosage forms of traditional herbal medicine. The mean plasma concentration-time profiles of the analytes were illustrated in Fig. 6 and the pharmacokinetic parameters ($\pm$SD) were listed in Table 4. For the dose **I** (1 g/kg), the T_{max} values were at about 90 min and C_{max} ranged from 121.23 ± 9.57 to 2358.35 ± 379.11 ng/mL. The $t_{1/2}$ values ranged from 78.14 ± 7.95 to 108.97 ± 9.26 min and the k_{el} ranged accordingly from 0.0089 ± 0.0021 to 0.0064 ± 0.0012 1/min, which indicated the different metabolism rate in vivo. The C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ increased with the increasing dose (dose **II** 1.5 g/kg), and the k_{el} and CL seemed to decrease. There were no significant differences in $t_{1/2}$ and T_{max} values at the two dosage levels. The results might contribute to further studies on pharmacokinetic mechanism of the *Salvia miltiorrhiza* and development of medical herbs.

4. Conclusion

The novel method for simultaneous detection of seven triterpene acids (ursolic acid, tormentic acid, oleanolic acid, maslinic acid, betulinic acid, corosolic acid, betulonic acid) was developed and validated for the analysis of rat plasma samples. The microsample collection and pretreatment procedure were finished more efficiently and conveniently with the semi-automated derivatization extraction method than with the usual preparation of biological liquid samples. Seven analytes were rapidly separated with HPLC, selectively quantified with fluorescence detection and further identified with mass spectra with no significant matrix effect. The obtained higher sensitivity was attributable to the appropriate labeling reagent PBIOTs and derivatization condition optimized by multivariate methods BBD from RSM and ANN combined with GA. The BBD was proved to be more accurate in predicting the optimal conditions for this derivatization reaction by validation with multi-criteria and nonparametric tests. With the developed method, the pharmacokinetic study of seven triterpene acids after oral administration of *Salvia miltiorrhiza* extract to mice was conducted for the first time. The concentration of the triterpene acids were analyzed with acceptable performance of linearity, precision, repeatability and accuracy, and the pharmacokinetic parameters were successfully obtained. The method provided the more sensitive and efficient alternative for simultaneous analysis of large batches of biological liquid samples containing trace content of triterpene acids in preclinical or clinical studies.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.06.053.

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