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Short communication

Simultaneous determination of jujuboside A, B and betulinic acid in semen Ziziphi spinosae by high performance liquid chromatography-evaporative light scattering detection

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ABSTRACT

A reverse phase high performance liquid chromatographic (HPLC) method with evaporative light scattering detection (ELSD) was developed for simultaneous determination of jujuboside A, B and betulinic acid in semen Ziziphi spinosae. The analysis was performed by gradient elution, using an aqueous mobile phase (containing 0.1% acetic acid) modified by acetonitrile. The evaporator tube temperature of ELSD was set at 45 °C, and with the nebulizing gas flow-rate of 1.8 l/min. The method was validated for accuracy, reproducibility, precision and limits of detection and quantification. Quantification of the three active compounds in semen Ziziphi spinosae from different locations was performed by this method, which provides a new tool for quality assessment of semen Ziziphi spinosae.

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1. Introduction

Semen Ziziphi spinosae**,** the seeds of *Ziziphus jujuba* Mill var. *spinosa* (Bunge) Hu ex. H.F. Chou (Rhamnaceae) has been used as a sedative medicine in China for over one thousand years. Modern pharmacological studies showed that semen Ziziphi spinosae possesses multiple activities such as hypnotic-sedative, hypotensive, antihypoxia, antihyperlipidemia, and hypothermic effects [1].

Extensive chemical studies revealed that semen Ziziphi spinosae contains many bioactive saponins and flavonoids along with fatty acids, in which jujuboside A and jujuboside B are the main bioactive compounds with sedative activity [2]. On the other hand, as an important triterpenoid in semen Ziziphi spinosae, betulinic acid has been the subject of intense studies because of its biological properties, especially its remarkable anti-melanoma, cytotoxic and anti-HIV activities [3,4,5].

Although the isolation and identification of various compounds from semen Ziziphi spinosae have been reported by several groups [6,7], few analytical methods have been reported on the quality assessment of semen Ziziphi spinosae [8].

Because the evaporative light scattering detector (ELSD) response does not depend on the samples' optical characteristics, it is capable of detecting most non-volatile compounds [9]. As a result, it has been successfully applied to the analysis of these compounds such as sugars [10], saponins [11,12], fatty acid esters [13] and steroidal alkaloids [14]. Moreover, as a standard method, it has been accepted to assay astragaloside IV and ginkgolide in Chinese Pharmacopoeia [15].

In this study, a simple HPLC-ELSD method was developed for simultaneous determination of jujuboside A, jujuboside B and betulinic acid (Fig. 1) in semen Ziziphi spinosae.

2. Experimental

2.1. Apparatus

The HPLC equipment used was Agilent HP-1100 system (Agilent, USA) including a HP-1100 quaternary pump, a degasser, and HP ChemStation for LC 3D software. Detector was an Alltech 3300 ELSD (Alltech Associates, USA). The column was a hypersil C_{18} (250 mm × 4.6 mm i.d., 5 μm, Dalian Elite Analytical Instruments, China).

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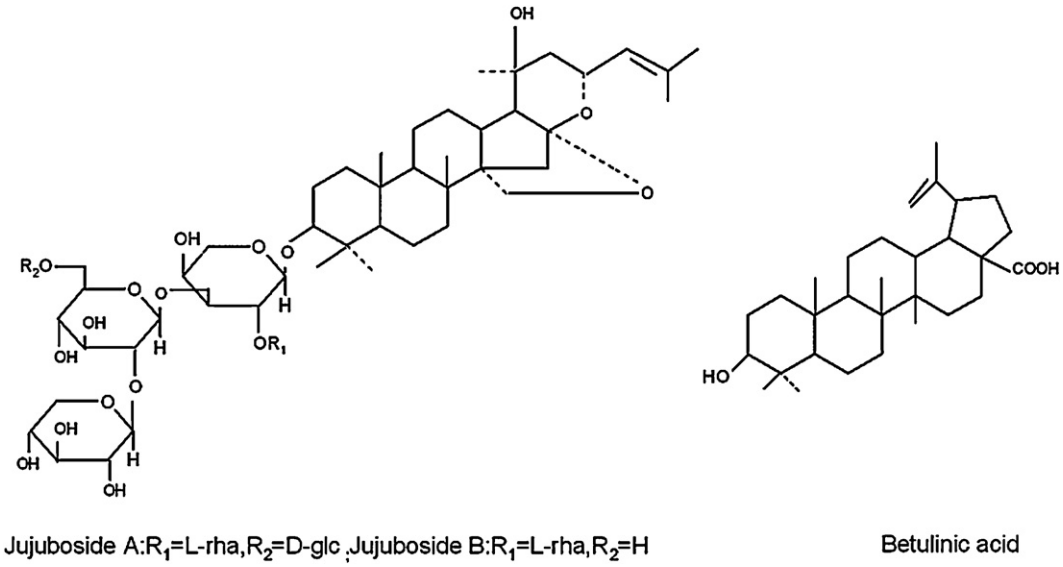

Fig. 1. Structures of three bioactive markers.

2.2. Reagents and solutions

Jujuboside A was purchased from the Chinese Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Jujuboside B and betulinic acid were purchased from Shunbo Biotech Co. Ltd. (Shanghai, China). Four samples of semen Ziziphi spinosae were obtained from different areas of Tianjin, Hebei, Shandong, Beijing in China. They were all identified by Doctor Junbo Xie. The voucher specimens were deposited in the department of pharmaceutical engineering, Tianjin University of Commerce, China. Chromatographic grade acetonitrile was purchased from Dima Technology Inc. USA, and other solvents from Deen Chemical Company (Tianjin, China) were of analytical grade. All aqueous solutions were made up in deionized water.

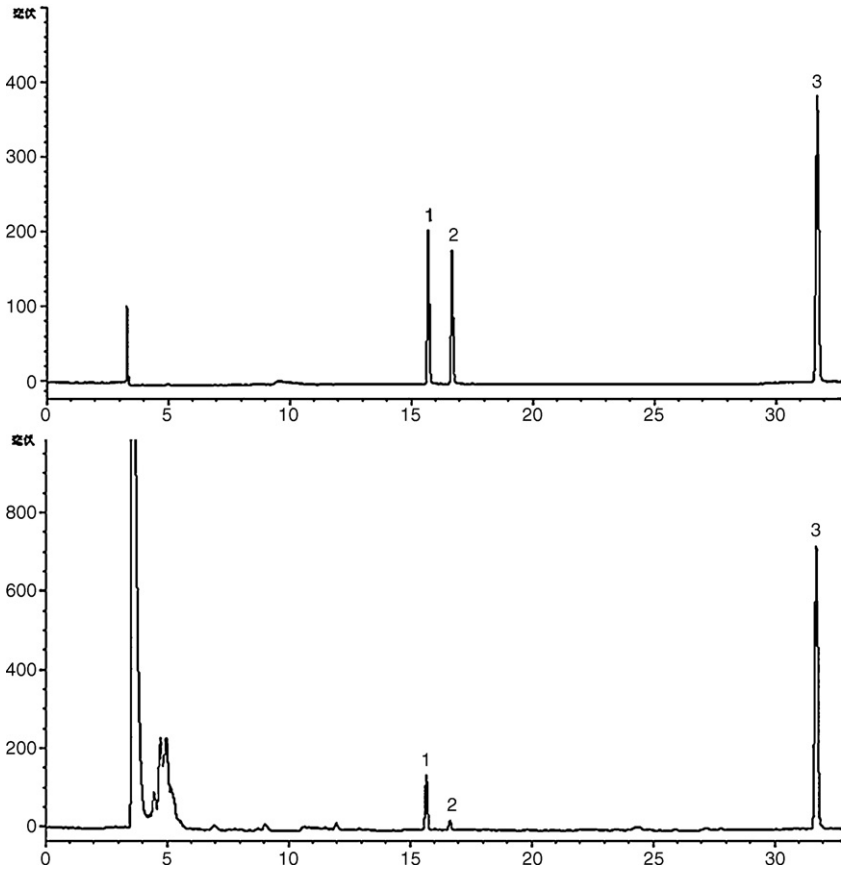

Fig. 2. Chromatographic profiles of standards and semen Ziziphi spinosae samples. 1 jujuboside A 2 jujuboside B 3 betulinic acid.

2.3. Preparation of sample solutions

The crude material of semen Ziziphi spinosae (100 g) were milled into powder and dried at 80 °C for 4 h before determination. Approximately 1.2 g of dried herb were accurately weighed and transferred into Soxhlet's apparatus. After degreased with 50 ml of petroleum ether (30–60 °C), the samples were extracted with 50 ml 95% ethanol for 6 h. The solution was evaporated to dryness in a rotary evaporator, and residue was dissolved with methanol in a 25 ml volumetric flask. Before being injected into the HPLC system, all solutions were filtered through 0.45 μm membrane filters.

2.4. Preparation of standard solutions

Standard stock solution of Jujuboside A (0.154 mg/ml), Jujuboside B (0.105 mg/ml), and betulinic acid (0.280 mg/ml) was prepared in methanol. The standard stock solution was further diluted with methanol to make six different concentrations including 1, 4/5, 1/2, 3/10, 1/5 and 1/10 of the original concentration.

2.5. Liquid chromatographic conditions

HPLC analysis was carried out by gradient elution beginning with a mobile phase of acetonitrile–aqueous phase (containing 0.1% acetic acid) at a flow-rate of 1.0 ml/min. The percentage of acetonitrile in the mobile phase was programmed as follows: 20% (0 min)–20% (5 min)–70% (20 min)–90% (32 min). Then the column was re-equilibrated for another 10 min, using a mobile phase composition of 20:80 (acetonitrile–aqueous phase) before the next injection. The elution was carried out at ambient temperature. The injection volume is 20 μl.

2.6. Validation of HPLC method

Stock solution containing three reference compounds was diluted to a series of appropriate concentrations with methanol. The limits of detection (LOD) and quantification (LOQ) were determined at a signal-to-noise (S/N) ratio of 3 and 10, respectively.

Intra- and inter-day variations were determined for precision of the developed method. Certain concentrations of standard and sample were tested. To confirm the reproducibility, five different working solutions prepared from the 1# sample were analyzed. Variations were expressed by relative standard deviations (R.S.D.). Recovery test was used to evaluate the accuracy of this method. Accurate amounts of reference compounds were added to 1# sample separately, and then extracted and analyzed according to previously described procedure. The average recoveries were calculated by the formula: recovery (%) = (amount found − original amount)/amount spiked × 100%, and R.S.D. (%) = (S.D./mean) × 100%.

3. Results and discussion

3.1. Optimization of the chromatographic conditions

Due to the very broad range of polarity of the analytes gradient elution was carried out to separate jujuboside A, B and betulinic acid in semen Ziziphi spinosae. Various compositions of mobile phase including methanol–water and acetonitrile–water were tried. Good separation was not achieved although gradient elution method was employed. Considering the presence of acidic ingredients in the extraction, a little amount of acetic acid was added to the mobile phase attributed to the lesser extent of ionization and polarity of these compounds. Finally, it was found that 0.1% (v/v) aqueous acetic acid–acetonitrile system gave the best separation of these compounds (Fig. 2).

Table 1
Regression data and LODs for the three bioactive markers in HPLC.

Analyte	Calibration curve	r	Linear range (μg/ml)	LOD (ng)
Jujuboside A	$Y = 1.8369X - 0.1881$	0.9991	15.4–154	28.7
Jujuboside B	$Y = 1.5503X - 0.0104$	0.9992	10.5–105	22.5
Betulinic acid	$Y = 1.3453X + 0.7118$	0.9992	28.0–280	31.4

Table 2
Precision and reproducibility of the three bioactive markers.

Analyte	Concentration (μg/ml)	Precision (R.S.D (%))		Reproducibility ($n = 5$) (R.S.D (%))
		Intra-day ($n = 3$)	Inter-day ($n = 3$)	
Jujuboside A	30.83	2.02	1.39	3.33
Jujuboside B	45.61	1.43	0.69	2.57
Betulinic acid	56.09	1.57	2.11	2.48

3.2. Optimization of ELSD parameters

The quantitation of investigated compounds was achieved by using an Alltech 3300 ELSD (Alltech, USA). The parameters of ELSD including evaporator tube temperature and nebulizing gas flow-rate were optimized to obtain the best signals, and signal to noise (S/N) ratio. Betulinic acid was selected as a model compound for optimizing ELSD conditions. Temperature and flow rate of the gas for the detector was evaluated systematically from 30 to 50 °C, and from 1.5 to 2.0 l/min, respectively. Although the highest signal was obtained at drift tube temperature 35 °C, in the present study the baseline was unstable and noise was high. Finally, the optimized parameters of ELSD were 45 °C for evaporator tube temperature and 1.8 l/min for nebulizing gas flow-rate.

3.3. Method validation for quantitative determination of the bioactive markers

The biomarkers in semen Ziziphi spinosae were identified by comparing the retention time of the reference standards. For determination of the bioactive markers, a calibration curve for each marker was constructed and tested for linearity. The linearity was determined by triplicate analyses of standard solutions. For cali-

Table 3
Recovery of the three bioactive markers.

Analyte	Added mean (mg)	Recorded mean (mg)	Recovery mean (%)	R.S.D (%) ($n = 5$)
Jujuboside A	0.352	0.344	97.76	1.69
Jujuboside B	0.181	0.175	96.50	1.58
Betulinic acid	2.210	2.068	93.58	3.16

Table 4
The mean contents of three bioactive markers in semen Ziziphi spinosae samples ($n = 3$). "–" under limit of detection.

Contents (mg/g)	1#	2#	3#	4#
Jujuboside A	0.582	0.485	–	–
Jujuboside B	0.451	0.459	0.118	0.278
Betulinic acid	4.562	4.497	3.190	2.987

bration, the log–log plots for the peak area versus concentration were drawn to obtain linearity, because the peak area varies exponentially with the mass of analyte [12]. As shown in Table 1, all calibration curves showed good linear regression ($\gamma \geq 0.999$). LOD and LOQ for each compound were also shown in Table 1. The results of precision, reproducibility and accuracy were shown in Tables 2 and 3.

3.4. Sample analysis

The method was subsequently applied to a simultaneous determination of the three bioactive markers in semen Ziziphi spinosae. The assay results are shown in Table 4.

Due to the different growth environment of the medical plant there were remarkable differences between the samples, in terms of concentrations of the three bioactive markers. 3# and 4# samples were prepared semen Ziziphi spinosae, which was fried with the special method to modify its efficacy according to the theory of Chinese medicine. Compared with 1# and 2# samples, the content of the three bioactive markers was obviously lower in 3# and 4# samples.

The content of betulinic acid in semen Ziziphi spinosae was up to about 5 mg/g, as a result, semen Ziziphi spinosae could be an important natural resource of betulinic acid to substitute for white birch bark [16].

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