Pharmaceutical Analytical Chemistry: Open Access

Zhang et al., Pharm Anal Chem Open Access 2016, 2:2
DOI: 10.4172/2471-2698.1000115

Research Article Open Access

Simultaneous Determination of Four Index Polar Constitutes by HILIC-ESI-MS/MS for the Quality Control of Guizhi Fuling Wan

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Abstract

A rapid and accurate method was developed and validated for the analysis of four index polar constitutes in Chinese medicinal preparation Gui-zhi-fu-ling Wan by microwave-assisted extraction coupled with hydrophilic interaction liquid chromatography-tandem mass spectrometry (MAE-HILIC -MS/MS). The chromatographic separation was performed on a Waters HILIC column (100 mm × 1.7 mm, 2.1 μm) with gradient elution using 0.1% formic acid aqueous solution (A) and acetonitrile (B) at a flow rate of 0.2 mL/min. All the compounds showed good linearity. The method provided good accuracy with perfect recoveries and precision, which has been successfully applied to the simultaneous identification and determination of 4 index compounds in a single run for the quality control of Gui-zhi-fu-ling Wan.

Keywords: HILIC-UHPLC-MS/MS; Traditional chinese medicine; MAE; Guizhi fuling wan; Quality control

Introduction

Gui-zhi-fu-ling Wan (GFW) has been used in China for centuries. It is a traditional Chinese medical (Kampo) formulation that composed of five kinds of medicinal plants, Cinnamomum cassia BLUME *(Cinnamomi Cortex)*, Paeonia lactiflora PALL *(Peonies Radix)*, Paeonia suffruticosa ANDREWS *(Moutan Cortex)*, Prunus persica BATSCH *(Persicae Semen)*, and Poria cocos WOLF *(Hoelen)* [1]. GFW, prescript for treating blood disorders was compiled by Zhang Zhongjing, an outstanding physician in the Eastern Han Dynasty (150-219 AD) [2,3]. Based on a large amount of pharmacological researches, the polar active constituents of pachymic acid, albiflorin, amygdalin and cinnamic acid, were found to be responsible for the biological activities of treating gynecological diseases, uterine fibroids, pelvic inflammatory disease, ovarian cyst, dysmenorrhea [4], endometriosis [5], cervical cancer [1], hot flashes [6], improvement of blood circulation [7-9]. In addition, GFW was reported to prevent the progression of atherosclerosis in cholesterol-fed rabbits *in vivo* by limiting oxidative LDL modification [10,11].

Only a few analytical methods has been reported to determine the active components in GFW, including Reverse phase high-performance liquid chromatography (RP-HPLP) [12-14] and gas phase chromatography coupled with mass spectrometry (GC-MS) [15,16], high-performance liquid chromatography-diode array detection-tandem mass spectrometry (HPLC–DAD–MS/MS). However, up to now, HILIC-MS/MS coupled with MAE has rarely been employed for the determination of these four polar active constituents contained in TCM samples.

Experimental

Chemicals and reagents

The standards of albiflorin, amygdalin and cinnamic acid were obtained from Chengdu Must Bio-technology Co., Ltd. (Sichuan, China), while pachymic acid was isolated and purified in our laboratory, and the structure was characterized by comparing the chemical and spectroscopic (UV, NMR and MS) data. GFW was purchased from ShanXi zheng yuan sheng bang pharmaceutical Co., Ltd. (Shanxi, China). Acetonitrile (HPLC-grade) was purchased from Tedia Company Inc., Formic acid (HPLC-grade) was obtained from DIMA Technology Inc., (USA). Acetonitrile and methanol of HPLC grade were purchased from Tedia Company Inc., (Fairfield, USA). Formic acid of HPLC grade was purchased from Dikma (Richmond Hill, NY, USA). All other reagents were of analytical grade.

Apparatus and chromatographic conditions

ACQUITY Ultra Performance LCTM UPLC was gifts from Waters (USA). The separation was carried out on a Waters HILIC column (100 mm × 1.7 mm, 2.1 μm). The mobile phase consisted of aqueous solution (A) and 0.1% formic acid acetonitrile (B). A gradient programmer was used according the following profile: 0-3 min, linear gradient 3% A; 3-5 min, linear gradient 3-90% A; 5-8 min, the system returned to initial condition. The flow rate was 0.2 mL/min, and the injection volume was 20 μL.

Mass spectrometry condition

The detector used was a Quattro Micro API mass spectrometer (Waters Corporation, Milford, MA, USA) equipped with an ESI working in either positive (ESI+) or negative (ESI−) ion mode. The MS parameters were manually optimized by the direct infusion of the

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Received June 29, 2016; **Accepted** July 18, 2016; **Published** July 21, 2016

Citation: Zhang Z, Wang J, Yao W, Men J, Wang X, et al. (2016) Simultaneous Determination of Four Index Polar Constitutes by HILIC-ESI-MS/MS for the Quality Control of Guizhi Fuling Wan. Pharm Anal Chem Open Access 2: 115. doi:10.4172/2471-2698.1000115

compounds (20 μL) into the MS interface. The mass spectrometer was tuned as follows in the final methods. The capillary voltage was set to 3 kV in negative ion mode and 1 kV in positive ion mode. The source and desolvation temperatures, desolvation gas flow were 110 and 400°C, 450 L/h, respectively. The cone gas flow was 30 L/h. The RF Lens was set to 0.3 V in negative ion mode and 0.1 V in positive ion mode. Nitrogen was used as the nebulizer, desolvation and cone gas. Argon gas was used for collision induced dissociation. Data acquisition was performed in MRM mode, and the dwell time was 0.2 s. The system was controlled by Mass Lynx™ NT 4.1 software with QuanLynx™ program (Waters Corp., Milford, MA, USA).

Preparation of standard solutions

Appropriate amount of paeoniflorin, cinnamic acid, amygdalin, pachymic acid, accurately weighed, dissolved with 70% methanol in a 25 mL volumetric flask to produce a reference standard stock solution. A precision volume of 0.1, 2.0, 4.0, 6.0, 8.0, 10.0 ml of the mixture standard stock solution were transferred to a 10 mL volumetric flask, diluted with acetonitrile to produce mixed reference solution with series concentration. All solutions above were prepared in volumetric flasks and stored at 4°C.

Preparation of sample solutions

GFW was powdered to a homogeneous size in mortar. Accurately weighed powder 0.25 g, extracted with 25 mL 70% (v/v) methanol aqueous for 5 min in microwave-assisted extraction. The extract was centrifuged for 10 min at 13000 rpm to get supernatant. The solution was filtered through 0.22 μm membrane before UHPLC/MS analysis.

Results and Discussion

Optimization of chromatographic conditions

The optimization of chromatographic conditions was guided by the requirement of obtaining chromatograms with better resolution of adjacent peaks within a short analysis time. Different mobile phase compositions were assessed and different additives (formic acid, formic acid-ammonium acetate) and column temperature (20°C, 30°C and 40°C) were examined, respectively. It was found that acetonitrile-0.1% formic acid solution by gradient elution at a flow rate of 0.2 mL/min with the column temperature of 20°C lead to a significant improvement on the better resolution of adjacent peaks within a short analysis time (5 min) of the different component in GFW.

Optimizing MAE conditions

Single-factor experiment shows that the extraction of GFW was deeply affected by extracting agent concentration, extraction time, extraction temperature, microwave power. On the basic of single-factor experiment, we selected the four factors three levels orthogonal test, choose methanol volume fraction (A), extraction time (B), extraction temperature (C), solid to solvent ratio (D) as four factors, each factor in three levels to find suitable extraction conditions. Orthogonal factors and levels were shown in Table 1.

Based on the above analysis, the final optimal extraction conditions were: 25 mL 70% methanol-water (v/v) extracting 0.25 g GFW for 5 min at 80°C. The results of validated experiments suggested this extraction procedure was feasible (Table 1).

Method validation

Calibration curves and limit of detection: All calibration curves were constructed by plotting the peak area ratio of analyte/IS versus the analyte concentration of the corresponding working standard solution. The limit of quantification (LOQ) was determined as the concentration of diluted working standard solutions when the signal-to-noise ratio was about 10. The regression equations, correlation coefficients, linear ranges and LOQ for the analysis of the four index constituents are shown in Table 2.

Precision, accuracy and stability: The relative standard deviations (RSD) were determined as inter- and intra-day precisions and accuracy. The intra-day precision was investigated for a mixed standard solution of seven analytes using five replicates within one day, and inter-day precision was determined in duplicates over consecutive three days. The results indicated that the intra- and inter-day precision was less than 2.81% for all 4 analytes (Table 2). Recovery of all 4 tested bioactive constituents was within the range of 95.81-104.77%, with an RSD of between 0.65% and 2.81% (n=5). From the results of precision test and recovery test, it was known that the method manifested good precision and accuracy.

For stability test, the same sample solution was analyzed every 12 h in 3 days at the room temperature. The RSD values of the peak area and retention times were no more than 5.12% and 2.0%, respectively. The solution was therefore considered to be stable within 72 h.

Sample analysis

The newly established method has been applied to the determination of the 4 index constituents in GFW.

As shown in Figure 1 and Table 3 the 4 marker constituents in GFW can be sufficiently resolved and separated, which is suitable for the routine analysis and quality control of commercial GFW.

Conclusion

An accurate and reliable HPLC method to simultaneously determine multiple active components in traditional Chinese medicinal preparation GFW was developed. This is the first report for the simultaneous determination of 4 major active components in GFW by using HILIC-MS/MS. High extraction efficiency was achieved by an optimized MAE procedure, with shorter extraction time and less solvent consumption obtained. This method provides useful experience and shows promising perspective in analysis of other similar TCM samples, especially concerning the efficient extraction and convenient simultaneous determination of multiple target analytes contained in TCM samples.

Levels	A Methanol volume /%	B Extraction time /min	C Microwave temperature / °C	D solid to solvent ratio (w/v)
1	50	5	40	1:60
2	70	10	60	1:80
3	90	15	80	1:100

Table 1: Factors and levels for the orthogonal design.

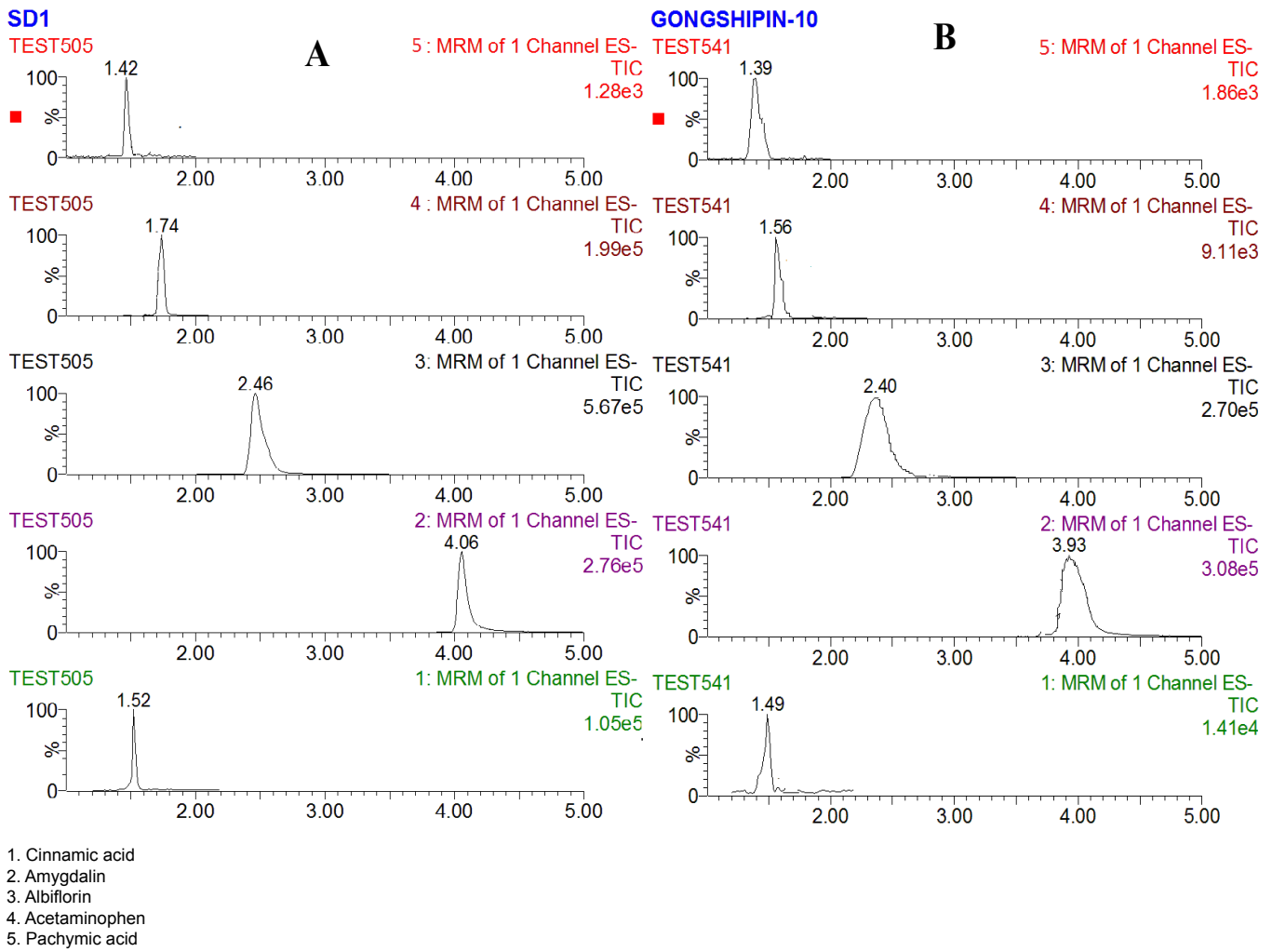

Figure 1: Typical chromatograms of the standard mixture (A) and Guizhifuling Wan (B).

Compound	Linear range (μg/ml)	Linearity equation a	r	Concentration (μg·mL^{-1})	Precision		Accuracy (%Re, n=6)	Stability (%RSD, n=6)
					Intra-day (%RSD, n=6)	Inter-day (%RSD, n=3)		
pachymic acid	0.05-0.6	y=0.1858x-0.0017	0.9995	0.1	0.65%	2.72%	104.8%	4.81%
				0.3	1.35%	2.18%	101.1%	3.77%
				0.5	1.62%	2.08%	100.6%	3.56%
albiflorin	0.5-6	y=4.6653x+2.1185	0.9984	1	1.30%	1.18%	102.7%	5.23%
				3	2.12%	0.50%	97.94%	3.67%
				5	1.93%	1.32%	101.1%	3.54%
amygdalin	1-12	y=1.3621x+0.3641	0.9989	2	2.57%	1.73%	97.30%	3.16%
				6	2.81%	1.17%	105.9%	2.35%
				10	1.76%	0.76%	105.1%	2.76%
cinnamic acid	0.5-6	y=0.1983x+0.1092	0.9993	1	1.27%	1.84%	95.81%	5.12%
				3	1.99%	2.32%	102.1%	3.43%
				5	1.74%	1.52%	103.3%	4.86%

Table 2: Summary of regression equation, linear range, accuracy, precision and stability of the 4 analytes in GFW.

Analytes	MAE
Amygdalin	2.351 ± 0.090
Albiflorin	1.118 ± 0.036
Cinnamic acid	0.206 ± 0.006
Pachymic acid	0.156 ± 0.005

Table 3: Quantitative analytical results for the target analytes in GFW (n=3, mg/g).

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