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Full Length Research Paper

Rapid method for simultaneous determination of 11 chemical constituents in the traditional Chinese medicinal prescription Wu-Ji-San by reverse phase high-performance liquid chromatography coupled with diode array detection (RP-HPLC-DAD)

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In the present paper, a simple and sensitive high-performance liquid chromatography coupled with diode array detection (HPLC-DAD) method was investigated for simultaneous determination of 11 components in Wu-Ji-San (WJS). The 11 identified components were ephedrine hydrochloride, paeoniflorin, liquiritin, ferulic acid, naringin, hesperidin, cinnamaldehyde, imperatorin, glycyrrhizic acid, honokiol and magnolol. The method was established using an Inertsil ODS-2 (250 mm × 4.6 mm i.d. with 5.0 µm particle size, GL Sciences) column. The 11 components were separated in less than 75 min with gradient elution using acetonitrile and 0.35% phosphoric acid in water at a flow rate of 1 ml/min. All calibration curves showed good linear regression (r^2 > 0.9993) within the test ranges. The method was validated for specificity, accuracy, precision, and limits of detection. The proposed method not only enables in a single run the simultaneous identification and determination of the 11 multi-structural components from WJS which form the basis of its therapeutic effect for quality control, but can also be used as a reference for the other prescription.

Key words: Wu-Ji-San, traditional Chinese medicinal prescription, reverse phase high-performance liquid chromatography coupled with diode array detection (RP-HPLC-DAD), chemical constituents.

INTRODUCTION

Wu-Ji-San (WJS) has been used in China for approximately one thousand years, and originates from 1115 A.D. into the pharmacopoeia of China's first "Taiping Benevolent Dispensary side". The prescription has been developed and evolved by ancient Chinese medical experts of the Song, Yuan, Ming and Qing Dynasties. Now, WJS is widely used for exogenous cold or dietary inadvertently caused by headache, body pain, abdominal pain, vomiting, anorexia, and irregular menstruation embolism with high usability and efficacy. WJS has become a standard medicine in many Chinese families (Rao et al., 2009).

As a traditional Chinese medicine (TCM), WJS contains the following 15 traditional Chinese medicines: Herba ephedrae (*Ephedra sinica* Stapf), Radix Angelicae dahuricae (*Angelica dahurica* (Fisch. ex Hoffm.) Benth. et Hook.f.), Ramulus Cinnamomi (*Cinnamomum cassia* Presl), Rhiaoma Zingiberis (*Zingiber officinale* Rosc.), Poria (*Poria cocos* (Schw.) Wolf), Radix Angelicae sinensis (*Angelica sinensis* (Oliv.) Diels), Radix Paeoniae alba (*Paeonia lactiflora* Pall.), Radix Platycodi (*Platycodon grandiflorum* (Jacq.) A.DC.), Rhizoma

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Atractylodis (*Atractylodes lancea* (Thunb.) DC.), Cortex Magnoliae officinalis (*Magnolia officinalis* Rehd. et Wils.), Pericarpium Citri Reticulatae (*Citrus reticulata* Blanco), Fructus Aurantii (*Citrus aurantium* L.), Rhizoma Pinelliae Praeparatum (*Pinellia ternata* (Thunb.) Breit.), Radix Glycyrrhizae (*Glycyrrhiza uralensis* Fisch.) and Rhizoma Ligusticum (*Ligusticum chuanxiong* Hort.).

Modern chemistry and pharmacology studies have shown that there are five scattered plot of 11 main active ingredients, respectively, with the relaxation of bronchial smooth muscle, blood vessels and uterine contraction (Tong and Eisenach, 1992; Bilcíková et al., 1987), anti-virus (Hirabayashi et al., 1995; Chan et al., 1995; Lee, 1999; Bae, 2000; Kim et al., 2000; Pompei et al., 1979), anti-bacterial (Tsou et al., 2000; Chang et al., 2001; Widelski et al., 2009; Rosselli et al., 2007; Clark et al., 1981), anti-inflammatory (Lee et al., 2009; Jayaprakasam et al., 2009; Shi and Wei, 2004), antioxidant (Kim et al., 2009; Deon et al., 2002; Lo et al., 1994; Bao et al., 2004; Yao et al., 2009), sedative (Zhang et al., 2009), anti-thrombosis (Teng et al., 1988), anti-tumor and other pharmacological effects (Teng et al., 1988; Moon et al., 1983). Keeping in view the current reports, it is believed that the main components of WJS are primarily responsible for its anti-viral, anti-bacterial, anti-inflammatory, analgesic and other pharmacological effects.

The currently available assay procedure for WJS detects only a single compound or a few marker compounds. In the present study, a simple, rapid and accurate HPLC-DAD coupled method was successfully developed for the simultaneous determination of 11 compounds in WJS. The developed method, provided grounds for its use in quality control studies on WJS, and it also added to characterize chemical constituents responsible for the therapeutic effect of WJS.

MATERIALS AND METHODS

The fifteen Chinese herbs that comprise WJS were supplied by a TCM dispensary store in the China Hospital (Hunan, China) and identified. Voucher specimens (No. 200505) were deposited at the Laboratory of Ethnopharmacology in Hunan Medical University No. 1 Affiliated Hospital. Authentic standards of geniposide, puerarin, paeoniflorin, ferulic acid, liquiritin, hesperidin, naringin, paeonol, daidzein, glycyrrhizic acid, honokiol and magnolol were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade acetonitrile was purchased from Merck (Darmstadt, Germany). Phosphoric acid (analytical grade) was purchased from Gaojing Chemical Industry Company (Hangzhou, China). Other reagents were of analytical grade.

Instrumentation and analytical conditions

The high performance liquid chromatography (HPLC) system 1200 series (Agilent Technologies, Palo Alto, CA, USA) was equipped with ChemStation software (Agilent Technologies) and comprised of a quaternary solvent delivery pump, an online vacuum degasser, an autosampler, a thermostated compartment and a diode array detector. All separations were carried out on an Inertsil ODS-2 column (250 mm × 4.6 mm i.d. with 5.0 µm particle size, GL Sciences, Tokyo Japan) from Hanbang Science and Technology (Hunan, China).

Mobile phase A was 0.35% (v/v) phosphoric acid aqueous solution. Phase B was pure acetonitrile. The elution was performed using a linear gradient of (0 to 5 min, 7 to 18% A; 15 to 38 min, 18 to 30% A; 38 to 42 min, 30 to 41% A; 42 to 45 min, 41 to 55% A; 45 to 65 min, 55 to 62% A; 65 to 75 min, 62 to 75%). The flow-rate was 1.0 $ml \cdot min^{-1}$, column temperature was maintained at 30°C. The effluent was monitored at 200 to 400 nm, and the injection volume was 5 µl. The peak identification was based on the retention time and comparison with the DAD spectrum of the standard.

Preparation of standard solutions

Standard stock solutions of the 11 reference standards (geniposide, puerarin, and others) were directly prepared by accurately weighing appropriate amounts of the standard compounds and dissolving them in methanol. They were then diluted to six concentrations for construction of calibration plots. The standard stock solutions were all prepared in dark brown calibrated flasks and stored at 4°C in a refrigerator till used. Empower software was used to prepare the standard curves from the peak area of each compound. The contents of these constituents in the test samples were calculated using the regression parameters obtained from the standard curves.

Preparation of sample solutions

A total of 4 g of Herba ephedrae, Radix Angelicae dahuricae, Ramulus Cinnamomi, Rhizoma Zingiberis, Poria, Radix Angelicae sinensis, Radix Paeoniae alba, Radix Platycodi, Rhizoma Atractylodis, Cortex Magnoliae officinalis, Pericarpium Citri Reticulatae, Fructus Aurantii, Rhizoma Pinelliae Praeparatum, Radix Glycyrrhizae and Rhizoma Ligusticum at a weight ratio of 0.4: 0.3: 0.5: 0.3: 0.3: 0.3: 0.3: 0.6: 0.8: 0.4: 0.5: 0.5: 0.3: 0.3: 0.2 were crushed into small pieces. The mixture was placed in a refrigerator at 4°C in 50 ml pure methanol, soaked overnight and passed through a filter paper. The final extraction was equal to 0.04 g crude drug/ml methanol. The methanolic extract was sealed in sterile bottles and retained in a refrigerator (4°C) until used. The sample injection volume for HPLC analysis was 5 µl. The samples were filtered through a 0.45 µm filter before HPLC analysis.

RESULTS AND DISCUSSION

Optimization of extraction procedure

Various extraction methods, solvents and times duration for extraction, were evaluated to obtain the best possible extraction efficiency. The results showed that soaking overnight at low temperature was better than using ultrasonic extraction. Therefore, in all further experiments, the procedure of soaking overnight at low temperature was employed. Various solvents including water, methanol-water (50:50, v/v) methanol and ethanol were screened. Methanol exhibited complete extraction of all the major constituents. It is a convenient and economical method that the sample was placed in the refrigerator overnight for cold soak extraction.

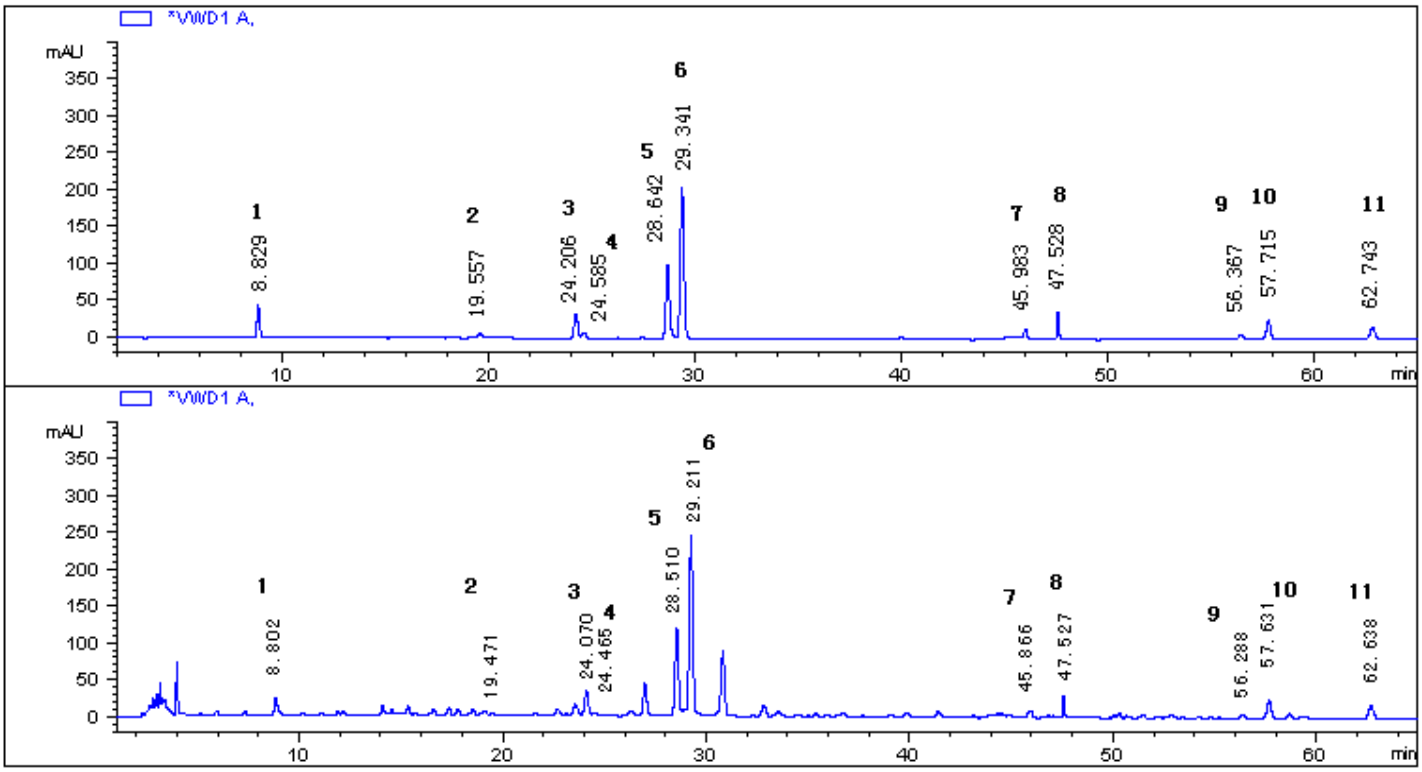

Figure 1. Typical chromatograms of the standard mixture (A) and Wu-Ji-San (B) at different detection wavelengths. (1) Ephedrine hydrochloride; (2) Paeoniflorin; (3) Liquiritin; (4) Ferulic acid; (5) Naringin; (6) Hesperidin; (7) Cinnamaldehyde; (8) Glycyrrhizic acid; (9) Imperatorin; (10) Honokiol; (11) Magnolol.

Optimization of chromatographic conditions

A common limitation of multiple component analysis is the low sensitivity of detection for some analytes at the selected single monitoring wavelength. Hence in the current study diode array detection (DAD) was used providing the facility of using multi wave-length monitoring facilities. Different detection wavelengths used to monitor different compounds simultaneously in a single run were as follows: λ 207 nm (for 1, 2, 3, 4, 5); 237 nm (for 6 and 8), 300 nm (for 9), 294 nm (for 7, 10 and 11) to provide sufficient sensitivity for each analyte.

With the DAD, the ultraviolet (UV) spectra of the bioactive constituents could be compared with those of the authentic standards. The desired compound from WJS was identified by comparing both the retention times and UV spectra with those of the authentic standard. The identity of each analyte was further confirmed by spiking the actual sample with the standard. The excellent agreement between the standard and sample spectra found in all analyzed samples of WJS indicated that under the proposed analytical conditions, the 11 marker constituents were sufficiently resolved. The peak for each compound was separated successfully by gradient elution in less than 75 min, and there was no interference by one or the other components in the matrix. Typical chromatograms of the authentic standards and WJS recorded at different detection wavelengths are depicted in Figure 1.

Method validation

Standard stock solutions containing 11 analytes were prepared and diluted to appropriate concentrations for plotting the calibration curves. The assay linearity was determined by the analysis of six different concentrations of the standard solutions. Table 1 shows the regression data and LODs (S/N = 3) of the components. All calibration curves showed good linear regression (r^2 > 0.9994) within the test ranges.

The relative standard deviation (R.S.D.) was considered to be a measurement of precision and accuracy. The intra- and inter-day precisions were determined by analyzing working solutions at three concentrations in 5 replicates during a single day and by duplicating the experiments on 3 successive days. As shown in Table 2, the overall intra- and inter-day variations was less than 5% for all 11 analytes. These results demonstrated that the developed method is reproducible with good precision.

The stability test was performed with sample solutions

Table 1. Regression data and LODs for the 11 components determined (n = 6).

Components	Regression equation	Correlation coefficient (r^2)	Linear range (μg/ml)	LOD (μg/ml)
Ephedrine hydrochloride	y = 2018.6x + 46.011	0.9994	2.52-75.11	0.19
Paeoniflorin	y = 1.332x + 12.813	0.9993	3.43-102.16	0.52
Liquiritin	y = 3564x - 34.343	0.9994	3.15-93.37	0.74
Ferulic acid	y = 378.21x - 3.285	0.9995	3.85-114.72	0.79
Naringin	y = 3264.6x - 65.26	0.9998	8.56-255.28	0.76
Hesperidin	y = 3493.2x + 61.495	0.9998	13.54-405.62	0.15
Cinnaldehydum	y = 3083x - 9.238	0.9995	0.69-20.74	0.43
Glycyrrhizic acid	y = 549.05x - 23.338	0.9998	9.96-298.80	0.40
Ammidin	y = 5503.4x + 3.7606	0.9996	0.26-7.82	0.17
Honokiol	y = 2307.1x + 1.5637	0.9999	2.95-88.55	0.15
Magnolol	y = 1499.5x + 86.085	0.9999	3.27-96.21	0.17

Table 2. The precision data of the proposed HPLC method.

Components	Nominal concentration (μg/ml)	Precision			
		Intra-day (n = 5)		Inter-day (n = 3)	
		Mean±SD (μg/ml)	R.S.D (%)	Mean±SD (μg/ml)	R.S.D (%)
Ephedrine hydrochloride	9.53	9.25±0.25	2.66	9.34±0.23	2.50
	19.97	20.05±0.08	0.39	20.49±0.55	2.70
	34.14	33.96±0.45	1.32	34.76±0.66	1.91
Paeoniflorin	8.96	8.66±0.37	4.25	8.25±0.21	2.57
	17.58	17.85±0.21	1.16	18.12±0.32	1.78
	30.76	30.52±0.20	0.66	31.29±0.67	2.13
Liquiritin	16.89	16.23±0.70	4.31	15.56±0.51	3.29
	33.07	33.25±0.17	0.51	34.42±1.19	3.46
	56.76	57.09±0.26	0.45	58.58±1.58	2.69
Ferulic acid	11.27	10.45±0.79	4.55	9.76±0.68	4.92
	23.11	23.33±0.90	3.85	24.28±0.60	2.45
	44.34	44.71±1.81	4.04	46.96±3.03	4.45
Naringin	51.81	49.48±2.44	4.93	46.13±1.29	2.79
	98.62	98.79±0.21	0.21	101.65±3.18	3.13
	167.33	167.95±0.47	0.28	171.38±3.71	2.16
Hesperidin	83.19	79.93±3.77	4.72	74.32±1.59	2.14
	160.86	161.22±0.38	0.23	164.84±5.07	3.08
	276.09	276.25±0.61	0.22	281.54±5.42	1.93
Cinnamaldehyde	3.27	3.37±0.21	4.32	3.24±0.20	4.30
	6.92	6.77±0.39	4.75	6.72±0.29	4.26
	9.93	9.61±0.52	4.43	10.14±0.56	4.56
Glycyrrhizic acid	47.47	44.49±2.63	4.91	40.78±0.77	1.90
	87.11	87.32±0.30	0.35	89.72±2.80	3.12
	144.43	144.87±0.46	0.32	147.22±2.54	1.73

Table 2. Contd.

	1.10	1.56±0.07	4.69	1.50±0.06	4.19
Imperatorin	2.13	2.06±0.12	4.67	1.93±0.08	3.89
	2.43	2.47±0.03	1.42	2.52±0.04	1.49
	16.05	15.45±0.73	4.74	14.30±0.22	1.57
Honokiol	31.61	31.76±0.11	0.33	32.64±1.06	3.24
	55.19	55.33±0.14	0.26	56.29±1.06	1.88
	14.01	13.40±0.74	4.51	12.42±0.38	3.05
Magnolol	27.58	27.49±0.16	0.57	28.00±0.95	3.40
	48.01	47.96±0.25	0.51	48.97±1.00	2.05

Table 3. Statistic results of recovery for extraction of analytes in LDP.

Components	Original (mg)	Spiked (mg)	Detected (mg)	Calculated recovery (%)	R.S.D (%)
Ephedrine hydrochloride	0.249	0.113	0.364	102.19	4.21
	0.250	0.225	0.473	99.18	2.03
	0.251	0.450	0.688	97.11	1.98
Paeoniflorin	0.192	0.153	0.341	97.35	3.02
	0.193	0.306	0.501	100.89	1.59
	0.193	0.612	0.820	102.37	2.25
Liquiritin	0.425	0.140	0.563	99.04	2.79
	0.426	0.279	0.701	98.71	1.45
	0.427	0.558	0.974	97.95	0.89
Ferulic acid	0.133	0.171	0.311	103.85	4.87
	0.133	0.342	0.477	100.59	3.47
	0.134	0.684	0.798	97.12	2.66
Naringin	1.202	0.383	1.581	98.95	2.64
	1.205	0.765	1.994	103.14	1.90
	1.208	1.530	2.731	99.53	0.69
Hesperidin	2.012	0.608	2.618	99.73	2.45
	2.017	1.215	3.256	101.92	2.06
	2.022	2.430	4.364	96.35	1.20
Cinnamaldehyde	0.078	0.031	0.110	101.16	4.69
	0.079	0.062	0.140	98.26	3.77
	0.079	0.124	0.204	100.65	4.51
Glycyrrhizic acid	1.084	0.448	1.540	101.76	3.04
	1.087	0.896	1.955	96.86	2.52
	1.090	1.793	2.874	99.55	1.33
Imperatorin	0.013	0.012	0.024	96.56	4.38
	0.013	0.023	0.037	102.39	2.71
	0.013	0.047	0.060	101.23	2.48

Table 3. Contd.

Honokiol	0.400	0.133	0.531	98.42	3.27
	0.401	0.266	0.668	100.47	1.48
	0.402	0.531	0.948	102.76	2.36
Magnolol	0.345	0.144	0.485	97.04	3.72
	0.346	0.288	0.633	99.55	2.95
	0.347	0.576	0.919	99.23	1.47

Table 4. Amount of the 11 main components found in WJS (*n* = 3).

Components	Contents (ng/mg)	R.S.D (%)
Ephedrine hydrochloride	700 ± 13	1.86
Paeoniflorin	539 ± 18	2.34
Liquiritin	1193 ± 20	1.71
Ferulic acid	374 ±35	2.92
Naringin	3377 ± 71	2.11
Hesperidin	5652 ± 91	1.60
Cinnamaldehyde	220 ± 6	2.79
Glycyrrhizic acid	3045 ±44	1.44
Imperatorin	36 ± 1	1.49
Honokiol	1125 ±16	1.43
Magnolol	970± 16	1.66

placed at 4°C and these were analyzed at 0, 24, and 48 h. The R.S.D. values of the peak area and retention times were no more than 4.9 and 2.0%, respectively. The solution was therefore considered to be stable for at least 48 h at 4°C.

Recovery tests were carried out to further investigate the accuracy of the method by adding three concentration levels of the mixed standard solutions to known amounts of the working samples. The resultant samples were then extracted and analyzed using the described method. The average percentage recoveries were evaluated by calculating the ratio of detected amount versus added amount. The recovery of the method was in the range of 96.56 to 103.85%, with R.S.D. less than 4.87% as shown in Table 3. Considering the results, the method was deemed to be accurate.

Determination of 11 components in WJS

The developed assay was subsequently applied for the simultaneous determination of eleven major compounds in WJS samples. A representative chromatogram of the extracts is shown in Figure 1B and the quantity of each compound identified is summarized in Table 4. These data indicate that the proposed method is suitable for the simultaneous determination of 11 compounds in WJS. Therefore, this assay method provided the option to be used in the quality control of WJS and other drugs containing such active biological molecules.

Conclusion

This is the first report of accurate and reliable analytical method for the simultaneous determination of 11 major bioactive constituents in Chinese medicine WJS by using reverse phase high-performance liquid chromatography coupled with diode array detection (RP-HPLC-DAD). This method is validated for good accuracy, repeatability and precision. Hence, this method can be used as a reference to evaluate the quality of WJS and other related herbal drug products. It is therefore very significant for further experiments that focus on evaluating the ancient prescription with China and Japanese origin.

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