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Simultaneous Determination of Cinnamaldehyde and Coumarin in *Oryeong-san* using HPLC with Photodiode Array Detector

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ABSTRACT

Objectives : To develop and validate High-performance liquid chromatography-photodiode array methods for simultaneous determination of two constituents in *Oryeong-san*(ORS).

Methods : Reverse-phase chromatography using a Gemini C18 column operating at 40°C, and photodiode array(PDA) detection at 280 nm, were used for quantification of the two marker components of ORS. The mobile phase using a gradient flow consisted of two solvent systems. Solvent A was H_2O and solvent B was acetonitrile.

Results : Calibration curves were acquired with correlation coefficient (r^2)>0.9999, and the relative standard deviation(RSD) values(%) for intra- and inter-day precision were not exceed 1.0%. The recovery rate of each compound was in the range of 93.01-104.16%, with an RSD less than 2.0%. The contents of two compounds in ORS were 1.10-3.72 mg/g.

Conclusions : The established HPLC method will be helpful to improve quality control of ORS.

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I. Introduction

Many kinds of traditional oriental medicines have been attracting more and more attentions for their complementary therapeutic effects to western medicines with few or no side effect[1,2]. However, although many traditional herbal medicines have been proven effective by modern pharmacological studies and clinical trials, their bioactive constituents and the remedial mechanism are still not well understood. Thus, traditional herbal medicines remain of considerable interest[3-5].

Oryeong-san(ORS, *Wuling-san* in Chinese; *Gorei-san* in Japanese) is one of traditional herbal medicine and was first described in the Han dynasty(China, about AD168)[6]. It is thirst and urine flow decrease, and it has been used widely for the treatment of headache.[7] A composite traditional Korean medicine, ORS consist of five herbs including *Alisma canaliculatum*, *Poria cocos*, *Atractylodes japonica*, *Polyporus umbellatus*, and *Cinnamomum cassia*(Table 1). Recently, results from pharmacological studies indicated that ORS has therapeutic potentials against liver injury and obesity, and also enhance diuretic action[8-12]. However, previous works have not been reported the simultaneous analysis about constituents of ORS by High-performance liquid chromatography (HPLC). Therefore, we needed to develop the simultaneous determination for quality control of traditional herbal medicine.

HPLC is a convenient, widely used, and effective method to control the quality of botanical extracts and plants important in traditional Chinese medicine due to its rapid separation and quantification[13,14]. Therefore, we here focused on quantitative determination of the main components of ORS, and used HPLC-PDA-coupled methods for simultaneous detection of two constituents(Fig. 1).

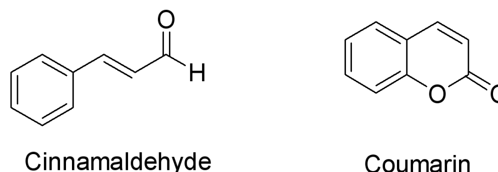

Fig. 1. Chemical structures of two constituents in *Oryeong-san*.

II. Materials and Methods

1. Chromatographic system

We analyzed using a Shimadzu LC-20A HPLC system(Shimadzu Co., Kyoto, Japan), consisting of a solvent delivery unit, an on-line degasser, a column oven, an autosampler, and a PDA detector. The data processor employed LCsolution software (Version 1.24). The analytical column was used a Gemini C18(250×4.6 mm; particle

size 5 μm; Phenomenex, Torrance, CA). The mobile phases were solvent A (H_2O) and solvent B (Acetonitrile). The gradient flow was as follows: (A)/(B)=75/25 (0 min) → (A)/(B)= 0/100 (30 min) → (A)/(B)= 75/25 (35 min; hold for 15 min). Column temperature was maintained at 40°C. The analysis was carried out at a flow rate of 1.0 mL/min with PDA detection at 280 nm. The injection volume was 10 μL.

2. Reagents and materials

The reference standards of cinnamaldehyde and coumarin were purchased from Wako(Osaka, Japan) and Sigma-Aldrich(St. Louis, CA, USA). The purity of all reference standards was above 98.0%. The HPLC-grade reagents methanol, acetonitrile, and water were obtained from J.T. Baker(Phillipsburg, NJ, USA). The materials forming ORS were purchased from Omniherb and HMAX. All ORS components were taxonomically confirmed by Professor Je-Hyun Lee, Dongguk University, Korea. A voucher specimen (2008-KE17-1 ~ 2008-KE17-5) has been deposited at the Herbal Medicine EBM Research Center, Korea Institute of Oriental Medicine.

3. Preparation of standard solutions and calibration curves

A methanol standard stock solution containing compounds cinnamaldehyde(1,050 μg/mL) and coumarin(1,000 μg/mL) was prepared and stored below 4°C. Working standard solutions were prepared by serial dilution of stock solutions with methanol. All calibration curves were obtained from assessment of peak areas of standard solutions in the concentration ranges: cinnamaldehyde, 1.64-105.00 μg/mL; and coumarin, 0.78-50.00 μg/mL.

4. Preparation of sample solutions

An ORS was prepared in our laboratory from a mixture of chopped crude herbs purchased from Omniherb(Yeongcheon, Korea) and HMAX(Chungbuk, Korea). ORS was prepared as described in Table 1 and extracted in distilled water at 100°C for 2 h. The extract was evaporated to dryness and freeze-dried(yield; 22.7%). Lyophilized ORS extract was weighed(200 mg) into a 20 mL flask and distilled water added to the volumetric mark, and then the mixture was filtered a 0.2 μm syringe filter. Injection volume for HPLC analysis was 10 μL.

Table 1. Composition of *Oryeong-san*

Scientific name	Amount (g)	Company of purchase	Source
Alisma canaliculatum	9.375	Omniherb	Imsil, Korea
Poria cocos	5.625	Omniherb	Yeongcheon, Korea
Atractylodes japonica	5.625	HMAX	China
Polyporus umbellatus	5.625	HMAX	China
Cinnamomum cassia	1.875	HMAX	Vietnam
Total amount	28.125		

5. Limits of detection (LODs) and quantification (LOQs)

Stock solutions of reference compounds were further diluted with methanol to assess LOD and LOQ values. The LOD and LOQ data obtained under the chromatographic conditions used in this report were determined using signal-to-noise(S/N)

ratios of 3 and 10, respectively.

6. Precision and accuracy

Repeatability was assessed by analysis of seven independently prepared standard solutions. The relative standard deviation(RSD) of peak areas of analytes, and peak retention times for each standard, were calculated. Intra- and inter-day precision was determined using a standard addition method to prepare spiked samples, employing both standards and controls.

Recovery tests were performed by adding known amounts of reference standards to ORS samples before extraction. An average recovery was calculated using the formula: Recovery (%) = $(Amount_{determined} - Amount_{original})/Amount_{spiked} \times 100$.

III. Results and Discussion

1. Optimization of chromatographic conditions

We obtained satisfactory separation using mobile phases consisting of (A) H_2O and (B) acetonitrile, with a gradient flow of (A)/(B)=75/25 (0 min) → (A)/(B)=0/100 (30 min) → (A)/(B)= 75/25 (35 min; hold for 15 min). Quantitation was achieved by PDA detection at 280 nm, based on peak area. The selectivity of the HPLC protocol is illustrated in Fig. 2, where good separation of marker compounds from other components of the extract can be noted. Using optimized chromatography conditions, all analytes were eluted before 20 min, showing a resolution better than 6.0, and good specificity in sample analysis. Representative HPLC chromatogram of standards and extract was shown in Fig. 2.

2. Recovery

A recovery test was performed by addition of known amounts of cinnamaldehyde and coumarin. Standard compounds, at each of three different levels, were mixed with sample powder, and extracted. The recovery of each standard ranged from 93.01-104.16%, and the RSD range was 0.41-1.61%(Table 2).

Table 2. Recovery of two marker compounds (n=3)

Compound	Original mean (ug/mL)	Spiked (ug/mL)	Detected mean (ug/mL)	Recovery mean (%)	SD	RSD(%)
Coumarin	11.07	2.00	12.93	93.01	1.51	1.62
		5.00	16.23	103.19	0.41	0.41
		10.00	21.26	101.98	0.57	0.57
Cinnamaldehyde	37.27	8.40	45.54	98.37	1.19	1.21
		21.00	57.67	97.14	0.49	0.50
		42.00	81.02	104.16	0.71	0.68

3. Linearity, range, LOD, and LOQ

Calibration curves were obtained using standard solutions containing 1.64-105.00 μg/mL of cinnamaldehyde and 0.78-50.00 μg/mL of coumarin as marker compounds. Line equations representing calibration curves and correlation coefficients

thereof are summarized in Table 3.

The LOD value of cinnamaldehyde and coumarin was 0.04 ug/mL and 0.11 μg/mL, respectively. The LOQ value of cinnamaldehyde and coumarin was 0.15 ug/mL and 0.37 μg/mL, respectively. These data are shown in Table 3.

Table 3. Regression data, linear ranges, LODs and LOQs for marker compounds

Compound	Linear range (ug/mL)	Slope	Intercept	Correlation coefficient (R^2)	LOD (ug/mL)	LOQ (ug/mL)
Coumarin	0.78-50.00	42536.98	-4343.49	1.0000	0.11	0.37
Cinnamaldehyde	1.64-105.00	107074.16	-46100.12	0.9999	0.04	0.15

4. Precision and accuracy

Repeatability or intra-assay precision was assessed by repeatedly measuring retention times and peak areas for three independently prepared samples of analytes. Measurement precision was less than RSD 1.0% for peak responses and less than RSD 0.05% for retention times, with all analytes. Thus, the HPLC assay offered good reproducibility under optimized conditions.

To test the accuracy and precision of our analytical method, intra- and inter-day variations for measurement of two marker compounds were determined, and are summarized in Table 4. The precision of the method in simultaneous determination of the two marker compounds was acceptable because the RSD did not exceed 1.0% at any concentration. The intra-day accuracy ranged from 93.34-101.88%, and the inter-day accuracy from 93.58-101.64%.

5. Determination of the main constituents of *Oryeong-san*

To simultaneous determination, we analyzed two compounds, coumarin and cinnamaldehyde, in ORS. Fig. 2 show chromatogram of reference compounds and water extract of ORS, with detection of eluent at 280 nm. The analytical results for each compound identified are summarized in Table 5.

Table 4. Precision and accuracy of analytical results (n=3)

Compound	Spiked Conc. (ug/mL)	Intra-day			Inter-day		
		Observed Conc. (ug/mL)	Precision (RSD %)	Accuracy (%)	Observed Conc. (ug/mL)	Precision (RSD %)	Accuracy (%)
Coumarin	2.00	1.87	0.14	93.34	1.87	0.77	93.58
	5.00	5.09	0.46	101.88	5.08	0.31	101.64
	10.00	9.98	0.11	99.80	9.98	0.08	99.85
Cinnamaldehyde	8.40	8.40	0.07	100.00	8.40	0.51	100.04
	21.00	20.05	0.21	95.45	20.06	0.16	95.50
	42.00	42.48	0.05	101.14	42.47	0.04	101.12

Table 5. Analytical results for marker compounds in *Oryeong-san*

Batch (#)	Compound					
	Coumarin			Cinnamaldehyde		
	Mean (mg/g)	SD	RSD (%)	Mean (mg/g)	SD	RSD (%)
1	1.10	0.0003	0.025	3.66	0.0004	0.010
2	1.11	0.0001	0.009	3.72	0.0038	0.103
3	1.10	0.0010	0.087	3.67	0.0014	0.039

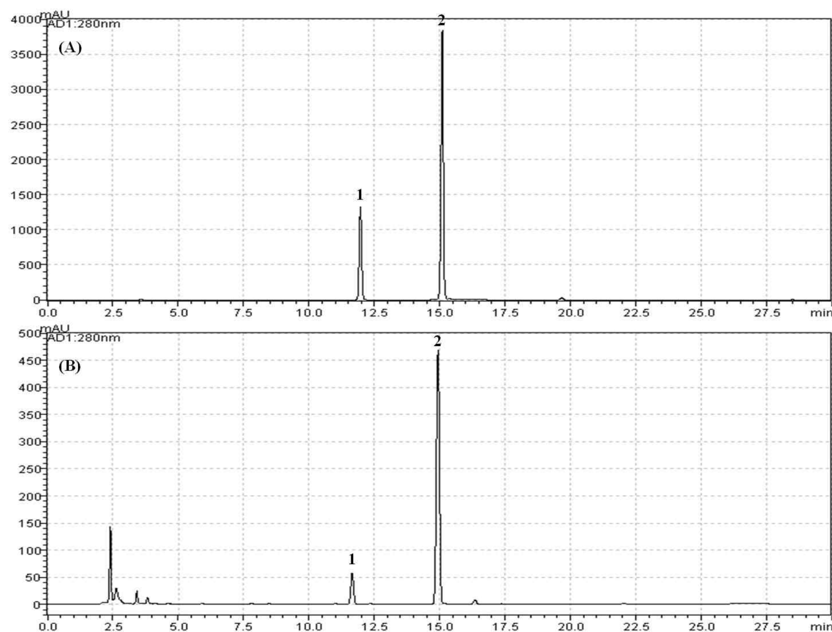

Fig. 2. Representative HPLC chromatogram of the standard mixture (A) and *Oryeong-san* (B).
Number 1 and 2 in the chromatogram indicate coumarin and cinnamaldehyde, respectively.

Ⅳ. Conclusion

To evaluate the quality of ORS, a HPLC method was developed for simultaneous determination of cinnamaldehyde and coumarin. In the present work, simultaneous determination of the two marker compounds in ORS was validated with respect to linearity, precision, and accuracy. The HPLC method will help to improve quality control of ORS.

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