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Determination of gentiopicroside, mangiferin, palmatine, berberine, baicalin, wogonin and glycyrrhizin in the traditional Chinese medicinal preparation Sann-Joong-Kuey-Jian-Tang by high-performance liquid chromatography

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Abstract

High-performance liquid chromatography was employed to determine the contents of several marker substances such as gentiopicroside, mangiferin, palmatine, berberine, baicalin, wogonin and glycyrrhizin in Sann-Joong-Kuey-Jian-Tang. The separation was performed on a Cosmosil $5C_{18}$-AR column by gradient elution with 0.03% (v/v) phosphoric acid–acetonitrile (0 min, 90:10; 10 min, 87:13; 17–27 min, 77:23; 40 min, 62:38; 50 min, 55:45) as the mobile phase at a flow-rate of 1.0 ml/min, with detection at 254 nm. *n*-Propylparaben was used as the internal standard and seven regression equations revealed linear relationships between the peak-area ratios (marker substances/internal standard) and concentrations. The repeatability and reproducibility (relative standard deviation) of the method were in the ranges 0.02–1.78% and 1.44–4.95%, respectively.

Keywords: Sann-Joong-Kuey-Jian-Tang; Pharmaceutical analysis; Gentiopicroside; Mangiferin; Palmatine; Berberine; Baicalin; Wogonin; Glycyrrhizin; Propylparaben

1. Introduction

Traditional Chinese medicine prescriptions have been used for over 1000 years, and in recent years concentrated dosage form have been widely adopted for clinical use. Most of them are composed of many herbs which contain complicate chemical constituents. Whether the effectiveness and safety are comparable to those of traditional decoctions for clinical therapy still remains to be established, hence proper methods for quality control are needed.

Although many high-performance liquid chromatographic (HPLC) methods have been developed for the determination of one or two constituents in crude drugs [1] or preparations [2,3], there have been few reports on the simultaneous determination of multiple constituents in preparations. In order to establish rapid and simple HPLC methods for routine quantitative analysis, we have tried to develop a method to assay multiple constituents in preparation simultaneously. In recent years, we have developed

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and reported [4,5] two HPLC methods for the determination of Dang-Guei-San and Wuu-Ji-San. In this study, seven constituents in the formula of Sann-Joong-Kuey-Jian-Tang was determined as markers. Sann-Joong-Kuey-Jian-Tang, which was recorded in Wan ping hui chun of the Ming dynasty, consists of eighteen kinds of crude drugs. This formula is used for treating swollen lymph glands, goiter, carbuncles, acute inflammation, suppurative ulcers, obstruction and distention beneath the heart [6–8].

For convenience of use, the dosage form of decoction is gradually being replaced by the concentrated dosage form in Taiwan. In the manufacturing process of the concentrated preparation, first the crude drugs are decocted with boiling water and filtered while hot. The filtrate is concentrated under vacuum, then the concentrated filtrate is granulated with starch by a flow coater or concentrated by spray-drying or freeze-drying. Therefore, in this experiment, polar-like alcohol–water mixtures were used as extraction solvents. Seven marker substances of Sann-Joong-Kuey-Jian-Tang, gentiopicroside (present in *Gentianae Scabrae* Radix), mangiferin (in *Anemarrhenae* Rhizoma), berberine and palmatine (in *Phellodendri* Cortex and *Coptidis* Rhizoma), baicalin and wogonin (in *Scutellariae* Radix), glycyrrhizin (in *Glycyrrhizae* Radix) were selected for analysis. HPLC method was developed for the simultaneous determination of the contents of the seven markers by using aqueous acid–acetonitrile as the eluent, and the method was validated. In this study, the influence of the different processing procedures for the concentration and recoveries of marker substances was also investigated.

2. Experimental

2.1. Materials

According to Ref. [7], the materials used to prepare Sann-Joong-Kuey-Jian-Tang were *Gentianae Scabrae* Radix, *Anemarrhenae* Rhizoma, *Phellodendri* Cortex, *Coptidis* Rhizoma, *Scutellariae* Radix, *Glycyrrhizae* Radix, *Angelicae* Radix, *Platycodi* Radix, *Sparganii* Rhizoma, *Paeoniae* Radix, *Puerariae* Radix, *Zedoariae* Rhizoma, *Cimicifugae* Rhizoma, *Bupleuri* Radix, *Forsythiae* Fructus, *Trichosanthis* Radix, *Laminariae* Thallus and *Zingiberis* Rhizoma (1.5 g each). Each material was purchased from the market and pulverized. For concentrated herbal preparations containing *Gentianae Scabrae* Radix, *Anemarrhenae* Rhizoma, *Phellodendri* Cortex, *Coptidis* Rhizoma, *Scutellariae* Radix and *Glycyrrhizae* Radix, three different commercial brands were purchased from the market.

2.2. Chemicals and reagents

Structures of the marker substances are shown in Fig. 1. Gentiopicroside, palmatine, berberine, baicalin, wogonin and glycyrrhizin were purchased from Nacalai Tesque (Kyoto, Japan) and mangiferin and the *n*-propylparaben from Sigma (St. Louis, MO, USA). Acetonitrile and methanol (HPLC grade) were purchased from Labscan (Dublin, Ireland). Phosphoric acid was of analytical-reagent grade. Ultra-pure distilled water with a resistivity greater than 18 MΩ was used.

2.3. Instruments

The HPLC system consisted of a Waters Model 600E multi-solvent delivery system equipped with a U6K injector and a Model 990 photodiode-array detector (Millipore, Boston, MA, USA). Peak areas were calculated with a Shiunn Haw computing integrator (Scientific Information Service). Concentration by reduced pressure evaporation and freeze-drying of a standard decoction was carried out with a Rotavapor (R110/RE120/EL-13; Büchi, Flawil, Switzerland) and a freeze-drier (Freeze Mobile 3; Virtis, Gardiner, NY, USA), respectively.

2.4. Liquid chromatography

The mobile phase was composed of 0.03% (v/v) phosphoric acid–acetonitrile with gradient elution (0 min, 90:10; 10 min, 87:13; 17–27 min, 77:23; 40 min, 62:38; 50 min, 55:45). The solvents were filtered through a 0.45-μm Millipore filter

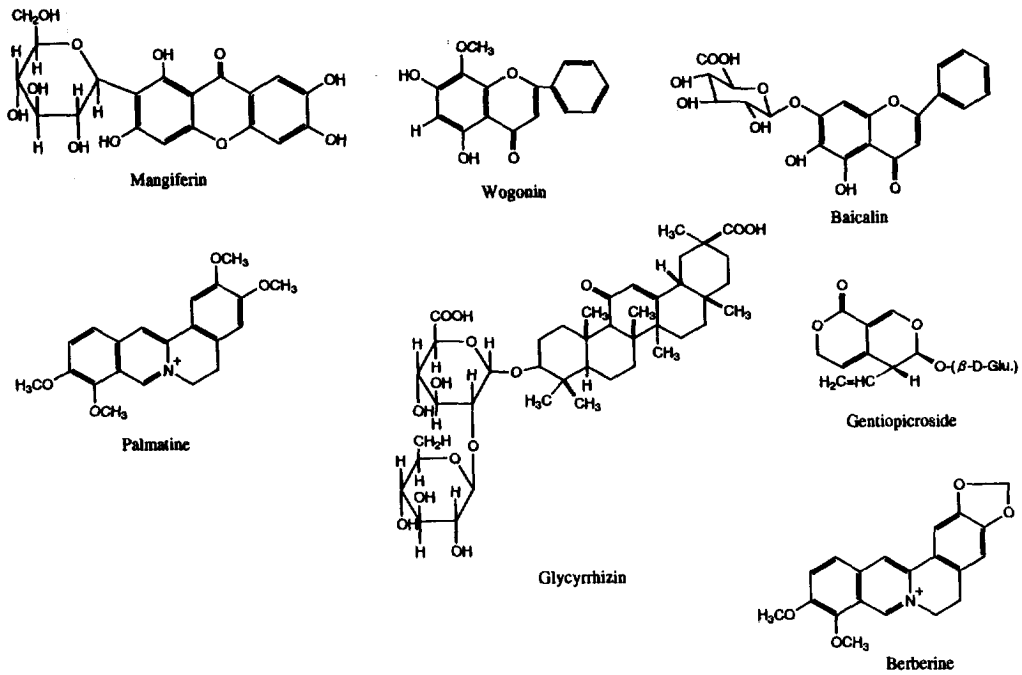

Fig. 1. Structures of marker substances.

and degassed prior to used. A Cosmosil $5C_{18}$-MS reversed-phase column (150 × 4.6 mm I.D.) and a Cosmosil $5C_{18}$-AR guard column (50 × 4.6 mm I.D.) (Nacalai Tesque) were used. The flow-rate was 1 ml/min with UV absorbance detection at 254 nm. The operating temperature was maintained at room temperature.

2.5. Preparation of standard solution

Gentiopicroside, mangiferin, palmatine, berberine, baicalin, wogonin and glycyrrhizin were accurately weighed and dissolved in 70% methanol to give serial concentrations in the ranges 0.010–0.040, 0.121–0.363, 0.012–0.048, 0.0055–0.0220, 0.010–0.040, 0.010–0.040 and 0.0075–0.0300 mg/ml, respectively. An appropriate amount of internal standard was added to each solution to give a concentration of 0.020 mg/ml of *n*-propylparaben. Calibration graphs were plotted by linear regression of the peak-area ratios with concentration.

2.6. Sample preparation for HPLC

Standard decoction and interference test

Amounts of crude drugs equivalent to a daily dose of Sann-Joong-Kuey-Jian-Tang and combinations of the same crude drugs excluding one at a time were mixed, a twentyfold mass of water was added and the mixtures were boiled for more than 30 min to have the original volume. After filtration while hot, the filtrate was diluted with methanol to give a 70% methanol solution and then a suitable amount of internal standard was added to the standard decoction solution to give a concentration of 0.020 mg/ml of *n*-propylparaben.

Contents of gentiopicroside, mangiferin, palmatine, berberine, baicalin, wogonin and glycyrrhizin in crude drugs

Amounts of individual crude drugs of *Gentianae Scabrae* Radix, *Anemarrhenae* Rhizoma, *Phellodendri* Cortex and *Coptidis* Rhizoma, *Scutellariae* Radix and *Glycyrrhizae* Radix equiv-

alent to a daily dose of Sann-Joong-Kuey-Jian-Tang were weighed and pulverized, then processed as above.

Concentrated products of standard decoction

Concentration by reduced-pressure evaporation and freeze-drying of a standard decoction were performed. After concentration, each residue was dissolved in a suitable amount of 70% methanol and internal standard was then added to give a concentration of 0.020 mg/ml of *n*-propylparaben.

Concentrated herbal products from market

An amount of specimen equivalent to 1 g of individual crude drug was weighed accurately and extracted with 70 ml of 70% methanol for 30 min in an ultrasonic bath, then was extracted and filtered as above.

2.7. Recovery tests

An appropriate amount of the concentrated product from the market was weighed accurately and extracted with 70% methanol for 30 min in a ultrasonic bath. The filtrate was divided into five portions (one as a control) and each portion (except the control) was spiked with standard solutions to introduce various concentrations of gentiopicroside (10.0, 20.0, 30.0, 40.0 μg/ml), mangiferin (12.12, 18.18, 24.24, 36.36 μg/ml), palmatine (12.0, 24.0, 36.0, 48.0 μg/ml), berberine (5.5, 11.0, 16.5, 22.0 μg/ml), baicalin (10.0, 20.0, 30.0, 40.0 μg/ml), glycyrrhizin (7.5, 15.0, 22.5, 30.0 μg/ml) or wogonin (10.2, 20.4, 30.6, 40.8 μg/ml), and internal standard was then added to give a concentration of 20.0 μg/ml of *n*-propylparaben. All samples were filtered through a 0.45-μm Millipore filter and injected for HPLC analysis to calculate the recovery.

3. Results and discussion

The selected marker substances in this study belong to glycosides, alkaloids, flavones or triterpene saponins. As mentioned in the Introduction, many HPLC methods have been reported for determining one or two constituents in crude drugs of preparations. Among the HPLC conditions mentioned in previous reports, an ODS column and isocratic eluent were mostly adopted. In those reports, mobile solvents and UV wavelengths used for determinations of marker substances were as follows: 0.1 *M* phosphoric acid–acetonitrile (72:28), $\lambda = 280$ nm for baicalin and wogonin (in *Scutellariae* radix) [1]; *M*/15 potassium dihydrogenphosphate–acetonitrile–sodium lauryl sulfate (300 ml:270 ml:3.3 g), $\lambda = 254$ nm for berberine (in *Coptidis* Rhizoma) [1]; water–acetonitrile–sodium lauryl sulfate (500 ml:450 ml:1.0 g), $\lambda = 254$ nm for berberine (in *Coptidis* Rhizoma) [1]; dilute phosphoric acid (1 → 1000)–acetonitrile–sodium dodecyl sulfate (500 ml:500 ml:3.0 g), $\lambda = 300$ nm for berberine (in *Coptidis* Rhizoma) [1]; *M*/15 potassium dihydrogenphosphate–acetonitrile–sodium dodecyl sulfate (500 ml:50 ml:1.0 g), $\lambda = 340$ nm for berberine (in *Phellodendri* Cortex) [1]; water–acetonitrile–potassium dihydrogenphosphate–sodium lauryl sulfate (500 ml:500 ml:3.4 g:1.7 g), $\lambda = 345$ nm for berberine (in *Phellodendri* Cortex and *Coptidis* Rhizoma) [9]; THF–water–0.8% phosphoric acid (160:825:15), $\lambda = 258$ nm for mangiferin (in *Anemarrhenae* Rhizoma and its preparation) [10]; and dilute acetic acid (1 → 15)–acetonitrile (3:2), $\lambda = 254$ nm for glycyrrhizin (in *Glycyrrhiza* Extract) [9].

In this study, the seven markers of Sann-Joong-Kuey-Jian-Tang could not be separated effectively by using the isocratic mobile solvents mentioned above. In order to find an easy way to analyse the specimen, we employed a gradient solvent system (acetonitrile and phosphoric acid solution), which can effectively separate seven markers simultaneously. Except for gentiopicroside, the UV absorbance maxima of all the markers substances are mostly within the range 250–260 nm, so we selected 254 nm as the detection wavelength. Although the UV absorbance maximum of gentiopicroside is 270 nm, we can find a peak at 254 nm.

We checked the precision of this method by running standard solutions of gentiopicroside, mangiferin, palmatine, berberine, baicalin, glycyrrhizin and wogonin at concentrations of

Table 1
Inter-day and intra-day relative standard deviations for the marker substances in Sann-Joong-Kuey-Jian-Tang ($n = 5$ with 95% confidence limits)

Marker substance	Concentration (μg/ml)	Peak area of marker substance/peak area of *n*-propylparaben		R.S.D. (%)	
		Inter-day	Intra-day	Inter-day	Intra-day
Gentiopicroside	16.00	0.114	0.114	1.40	0.69
Mangiferin	9.09	0.441	0.442	0.43	0.45
Palmatine	9.60	0.157	0.157	1.78	1.33
Berberine	8.80	0.139	0.140	1.15	1.14
Baicalin	16.00	0.143	0.143	1.18	1.53
Glycyrrhizin	40.00	0.064	0.063	0.78	0.02
Wogonin	16.32	0.329	0.328	1.33	0.48

Table 2
Recovery of the marker substances in Sann-Joong-Kuey-Jian-Tang

Marker substance	Added (mg)	Found* (mg)	Relative recovery (%)[a]	Mean ± S.D. (%)	R.S.D. (%)
Gentiopicroside	10.00	8.76	87.6	84.40 ± 3.37	3.99
	20.00	16.04	80.2		
	30.00	25.98	86.6		
	40.00	32.28	83.2		
Mangiferin	12.12	10.93	90.2	90.08 ± 1.70	1.89
	18.18	16.78	92.3		
	24.24	21.72	89.6		
	36.36	32.07	88.2		
Palmatine	12.00	10.60	88.3	89.17 ± 2.65	2.97
	24.00	21.65	90.2		
	36.00	33.19	92.2		
	48.00	41.28	86.0		
Berberine	5.50	4.96	90.2	90.70 ± 1.31	1.44
	11.00	10.00	90.9		
	16.50	15.25	92.4		
	22.00	19.65	89.3		
Baicalin	10.00	9.32	93.2	91.22 ± 1.77	1.94
	20.00	18.08	90.4		
	30.00	26.76	89.2		
	40.00	36.84	92.1		
Glycyrrhizin	7.50	4.90	65.3	69.80 ± 3.46	4.95
	15.00	10.38	69.2		
	22.50	16.04	71.3		
	30.00	22.02	73.4		
Wogonin	10.20	9.19	90.1	89.50 ± 1.98	2.21
	20.40	18.77	92.0		
	30.60	27.05	88.4		
	40.80	35.70	87.5		

[a] $n = 5$ with 95% confidence limits.

16.00, 9.09, 9.60, 8.80, 16.00, 40.00 and 16.32 μg/ml, respectively. The inter-day relative standard deviations (R.S.D.s) obtained for a 5-day period were 1.40, 0.43, 1.78, 1.15, 1.18, 0.78 and 1.33%, respectively, and the intra-day R.S.D.s were 0.69, 0.45, 1.33, 1.14, 1.53, 0.02 and 0.48% (Table 1), respectively. The recoveries of gentiopicroside, mangiferin, palmatine, berberine, baicalin, glycyrrhizin and wogonin were 84.4, 90.1, 89.2, 90.7, 91.2, 69.8 and 89.5% (Table 2), respectively. The results showed that the recoveries of gentiopicroside and glycyrrhizin were lower. The reason may be that the detection wavelength of the former is not the absorbance maximum, and the detectable amount of gentiopicroside in the specimen was much lower than those of the other constituents. On the other hand, the extraction ratio of glycyrrhizin in the specimen may be interfered with by berberine [1].

The linear regressions of gentiopicroside, mangiferin, palmatine, berberine, baicalin, glycyrrhizin, wogonin showed good linear relationships between peak-area ratio and concentration. To ensure the specificity and selectivity of the method, five blank decoctions excluding each crude drug one at a time were prepared for comparison. An example of a chromatogram is shown in Fig. 2. The retention times of the marker substances and internal standard were 12.5, 14.4, 27.4, 27.9, 34.5, 49.2, 51.0 and 55.0 min, for gentiopicroside, mangiferin, palmatine, berberine, baicalin, *n*-propylparaben, glycyrrhizin and wogonin, respectively. On inspection of the three-dimensional chromatograms, these constituents all demonstrated good purity. No peak was found at their retention times in corresponding blank decoctions. Three commercial preparations also showed satisfactory results.

Traditional Chinese medicines are usually pre-

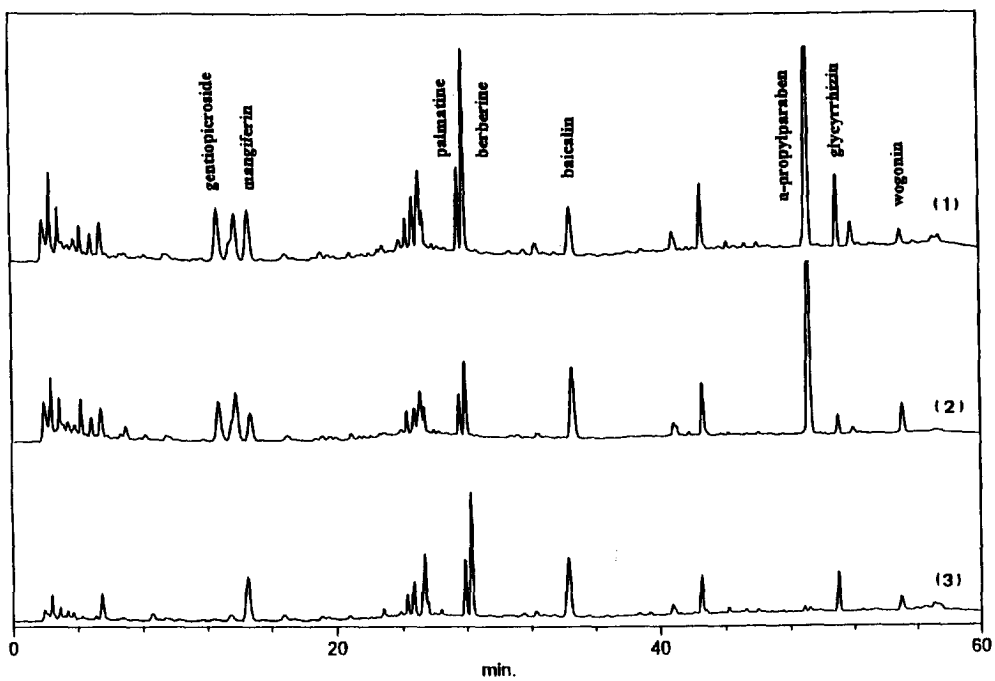

Fig. 2. Chromatogram of the marker substances gentiopicroside, mangiferin, palmatine, berberine, baicalin, glycyrrhizin and wogonin in Sann-Joong-Kuey-Jian-Tang. Column, Cosmosil $5C_{18}$-MS, 150 × 4.6 mm I.D.; guard column, Cosmosil $5C_{18}$-AR, 50 × 4.6 mm I.D., mobile phase, 0.03% phosphoric acid–acetonitrile (0 min, 90:19; 10 min, 87:13; 17–27 min, 77:23; 40 min, 62:38; 50 min, 55:45); flow-rate, 1.0 ml/min. (1) Standard decoction; (2) commercial preparation; (3) standard decoction without *Gentianae Scabrae* Radix.

pared by boiling with water. However, extraction of constituents from commercial product with water tends to be a difficult procedure. For this reason, five solvents, water, methanol, methanol–water (70:30), methanol–water (50:50) and methanol–water (30:70), were tried for extraction. The results indicate that the best extraction was obtained with methanol–water (70:30).

By comparing commercial products with a standard decoction, the contents of marker substances showed great variations. This is probably due to the different sources of crude drugs and manufacturing processes. The results of the effect of both concentration processes showed good stability of all the marker substances after reduced-pressure evaporation and freeze-drying processes. The turnover ratio of these constituents was defined as the percentage yields of these constituents detected in the Chinese medicinal preparation, calculated on the basis of their contents in the individual crude drug. The turnover ratios of these markers varied greatly (68.19, 39.67, 32.64, 23.42, 18.73, 27.65 and 33.51%, respectively). In the present case of Sann-Joong-Kuey-Jian-Tang containing eighteen kinds of crude drugs, each of them was known to contain various chemical constituents. The reason for the poor turnover ratio caused by molecular interactions or insufficient extraction remains to be clarified.

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