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Determination of paeoniflorin, ferulic acid and baicalin in the traditional Chinese medicinal preparation Dang-Guei-San by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic method for the determination of paeoniflorin, ferulic acid and baicalin in the traditional Chinese medicinal preparation Dang-Guei-San, which contains Paeoniae Radix, Swertiae Herba, Cnidii Rhizoma and Scutellariae Radix, was established. The samples were separated with a Cosmosil $5C_{18}$-AR column by gradient elution with 0.03% (v/v) phosphoric acid-acetonitrile (0 min 96:4, 5 min 84:16, 7 min 82:18, 14–30 min 78:22) as the mobile phase at a flow-rate of 1.0 ml/min, with detection at 245 nm. Methylparaben was used as the internal standard and three equations were derived showing linear relationships between the peak-area ratios of marker components (paeoniflorin, ferulic acid and baicalin) to methylparaben and concentration. The recoveries of paeoniflorin, ferulic acid and baicalin were 27.86, 33.89 and 49.31%, respectively. The repeatability (relative standard deviation) was generally less than 5% ($n = 5$). The effects of various processes such as concentration by reduced-pressure evaporation, freeze-drying and spray-drying were studied and commercial concentrated herbal preparations containing Paeoniae Radix, Swertiae Herba, Cnidii Rhizoma and Scutellariae Radix were also analysed.

INTRODUCTION

In analysing the constituents of Chinese medicinal preparations, studies on related topics generally started from the examination of a single herb component and then proceeded with the analysis of traditional prescriptions containing that specific component. As a result, most analytical work on Chinese medicines has been confined to single herbs. In order to promote the Good Manufacturing Practices (GMP) of Chinese medicinal preparations, our aim was to develop simple and expedient analytical methods for routine use in quality control.

In this study, we selected the Chinese medicinal preparation Dang-Guei-San and applied HPLC to develop suitable methods for determining the concentrations of its marker components. For determining paeoniflorin in Paeoniae Radix, we used the documented mobile phase [1] and obtained a satisfactory result. For determining baicalin in Scutellariae Radix, we also tried the documented mobile phase [2] with gradient elution. However, a poor peak shape was obtained and the whole process took much longer than expected. Ion-pair chromatography can also separate several flavonoids simultaneously in Scutellariae Radix [3,4], but this method damaged the column and pump. Therefore, we considered that a method using an aqueous acid–acetonitrile eluent would be a more feasible way to perform the analysis.

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EXPERIMENTAL

Materials

According to ref. 5, the materials used to prepare Dang-Guei-San were Atractylodis Rhizoma (1.5 g) and Paeoniae Radix, Swertiae Herba, Cnidii Rhizoma, Scutellariae Radix (3 g

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each). Each material was obtained from the market in Taipei and pulverized (8 mesh). For concentrated herbal preparations containing Paeoniae Radix, Swertiae Herba, Cnidii Rhizoma and Scutellariae Radix, three different commercial brands were obtained from the market.

Chemicals and reagents

Structures of the marker components are shown in Fig. 1. Paeoniflorin and baicalin were purchased from Nacalai Tesque (Kyoto, Japan). Ferulic acid and the internal standard methylparaben were obtained from Sigma (St. Louis, MO, USA). Acetonitrile and methanol (HPLC grade) were purchased from Labscan. Phosphoric acid was of analytical-reagent grade. Ultrapure distilled water with a resistivity greater than 18 MΩ was used.

Instruments

HPLC was conducted with a Waters Model 625 system equipped with a Waters Model 486 UV detector and a Rheodyne Model 9125-080 injector. Peak areas were calculated with a Shiunn Haw computing integrator. A Cosmosil $5C_{18}$-AR reverse-phase column (150 × 4.6 mm I.D.) and used. Concentrations by reduced-pressure evaporation, freeze-drying and spray-drying of a standard decoction were carried out with a Rotavapor (Buchi R110/RE120/EL-13), a freeze-drier (Virtis Freeze Mobile 3) and a mini spray dryer (Buchi Model 190), respectively.

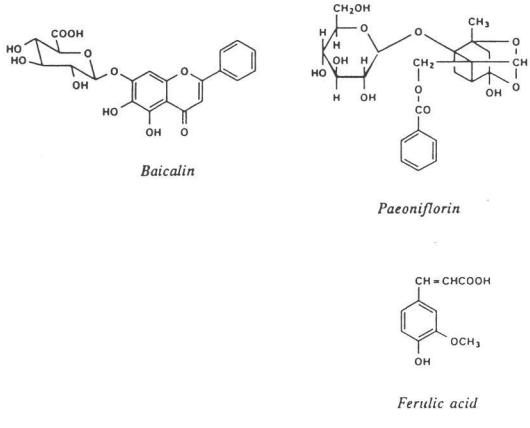

Fig. 1. Structures of marker components.

Liquid chromatography

The mobile phase was 0.03% (v/v) phosphoric acid–acetonitrile (0 min 96:4, 5 min 84:16, 7 min 82:18, 14–30 min 78:22), filtered through a 0.45-μm Millipore filter and degassed prior to used. The flow-rate was 1 ml/min. A constant operating temperature (room temperature) was maintained. The internal standard, methylparaben (3.4 mg), was dissolved in 35 ml of methanol and diluted to 50 ml with water to give the internal standard solution.

Preparation of standard solution

To prepare a standard solution containing paeoniforin, ferulic acid and baicalin, an appropriate amount of internal standard solution was added to an accurately weighed amount of paeoniflorin, ferulic acid and baicalin standards dissolved in 50% methanol to give various concentrations within the ranges 0.0107–0.0535, 0.00124–0.011088 and 0.0115–0.1495 mg/ml, respectively. Calibration graphs were plotted after linear regression analysis of the peak-area ratios with concentration.

Preparation of sample solutions

Standard decocation. Amounts of crude drugs equivalent to a daily dose of Dang-Guei-San were weighed and pulverized, a twentyfold weight of water was added and the mixture was boiled for more than 30 min to halve 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 solution to give a concentration of 0.017 mg/ml of methylparaben.

Interference test. Amounts of crude drug equivalent to a daily dose of Dang-Guei-San without Paeoniae Radix, Swertiae Herba and Cnidii Rhizoma or Scutellariae Radix were weighed and pulverized, a twentyfold weight of water was added and the mixture was boiled for more than 30 min to halve the original volume. After filtration while hot, the filtrate was diluted with methanol to give a 70% methanol solution.

Content of paeoniflorin, ferulic acid and baicalin in raw herbs. Amounts of individual crude drug equivalent to a daily dose of Dang-Guei-San was weighed and pulverized, a twentyfold weight of water was added and the mixture was boiled for more than 30 min to halve 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 solution to give a concentration of 0.017 mg/ml of methylparaben.

Various concentrated products of standard decoction. Concentrations by reduced-pressure evaporation, freeze-drying and spray-drying of a standard decoction were applied. After concentration, each residue was dissolved in a suitable amount of 70% methanol and internal standard was then added to give a concentration of 0.017 mg/ml of methylparaben.

Concentrated herbal preparations from market. An amount of the concentrated herbal preparation equivalent to a daily dose was weighed accurately and extracted with a tenfold weight of water for 30 min in an ultrasonic bath. After extraction, the samples were filtered and diluted with methanol to give a 70% methanol solution and internal standard was then added to give a concentration of 0.017 mg/ml of methylparaben.

Solutions for recovery study

Amounts of crude drugs equivalent to 50 daily doses of Dang-Guei-San without Paeoniae Radix were weighed and pulverized together. Then four doses of this powder, each of 10.5 g, were weighed precisely and separately. To these four doses were added 2, 3, 3.5 and 4 g of Paeoniae Radix with known paeoniflorin contents of 19.94, 29.91, 34.89 and 39.88 mg, respectively. A twentyfold weight of water was added and the mixture was boiled for more than 30 min to halve the original volume. A suitable amount of internal standard was added to the solution to give a concentration of 0.017 mg/ml of methylparaben.

For baicalin, as mentioned above, 2, 3, 3.5 and 4 g of Scutellariae Radix with known baicalin contents of 170.76, 256.14, 298.83 and 341.52 mg, respectively, were added for a recovery study. For ferulic acid in Swertiae Herba and Cnidii Rhizoma, 50 doses of Dang-Guei-San excluding both herbs were weighed and pulverized. Additionally, Swertiae Herba and Cnidii Rhizoma (50 g of each) were weighed and pulverized together. To these four doses of Dang-Guei-San were added 4, 6, 7 and 8 g of a mixture of Swertiae Herba and Cnidii Rhizoma with known ferulic acid contents of 1.44, 2.16, 2.52 and 2.88 mg, respectively. All samples were filtered through a Millipore filter and were injected for HPLC analysis to calculate the concentration of paeoniflorin, ferulic acid and baicalin from their calibration graphs.

RESULTS AND DISCUSSION

In order to elute the marker components within reasonable retention times, we adopted a method in which the pH was changed by gradient elution. In general, acidic compounds tend to be more hydrophilic when the mobile phase is adjusted to higher pH and more hydrophobic when the pH is lower. In other words, the retention time is lengthened for acidic compounds when the pH is lower than the pK_a value. The relationship between the retention time of a compound and pH was found to be sigmoid [6]. Methylparaben was used as the internal standard because its structure is similar to that of ferulic acid and there was no interference at the same retention time. The detection wavelength was chosen as 245 nm because the contents of paeoniflorin and ferulic acid in the prescription were low, whereas that of baicalin was high, and at 245 nm, baicalin absorbs weakly whereas paeoniflorin and ferulic acid absorb strongly.

A standard decoction was prepared by boiling raw herbs in water for more than 30 min. On one hand, this mimicked the traditional way of making a decoction. On the other hand, studies showed that paeoniflorin is best extractable with water at 60°C for 30 min [7]. The decoctions were eluted with 70% methanol solution in the final stage because the columns tended to become blocked when water was injected. In addition, the solubilities of the marker com-

TABLE I

INTER-DAY AND INTRA-DAY RELATIVE STANDARD DEVIATIONS ($n = 6$) FOR PAEONIFLORIN, FERULIC ACID AND BAICALIN

Marker component	Concentration (mg/ml)	Peak area of marker component/ peak area of methylparaben		R.S.D. (%)	
		Inter-day	Intra-day	Inter-day	Intra-day
Paeoniflorin	0.0321	0.188	0.185	1.01	1.13
Ferulic acid	0.0086	0.312	0.297	0.51	0.26
Baicalin	0.0575	0.739	0.714	1.23	0.42

ponents were satisfactory in 70% methanol solution. For ferulic acid assay, the standard solutions were prepared in 50% methanol solution in the final stage because ferulic acid split into two peaks in the HPLC profile when 70% methanol solution was used. The reason for this difference is that the solvent power of sample solutions should be lower than that of the mobile phase. Increasing the proportion of water can solve this problem.

To check the precision of this method, we injected standard solutions of paeoniflorin, ferulic acid and baicalin at the concentrations of 0.0321, 0.0086 and 0.0575 mg/ml, respectively, six times on the same day. The resulting relative standard deviations (R.S.D.s) were 1.13%, 0.26% and 0.42%, respectively. The R.S.D.s obtained from a 6-day period were 1.01%, 0.51% and 1.23%, respectively (Table I). All these values indicate satisfactory precision. When we measured the contents of marker components in a standard decoction and the preparations sold in the market, the R.S.D.s were about 5%. The reason for the higher R.S.D. values than for a standard solution is that they contain less ferulic acid. The contents of marker components in commercial preparations vary greatly from those in standard decoctions, as shown in Table II and Fig. 2. This is probably due to differences in the manufacturing process. The effects of different concentration devices on the manufacturing process were also explored using reduced-pressure evaporation, spray-drying and freeze-drying, and the results are given in Table III and Fig. 3.

To ensure the specificity and selectivity of the method, we prepared three blank decoctions for comparison: one with no Paeoniae Radix, one with no Swertiae Herba and no Cnidii Rhizoma and one with no Scutellariae Radix. The chromatograms are shown in Fig. 4. The retention times of the marker components, *i.e.*, paeoni-

TABLE II

CONTENTS OF PAEONIFLORIN, BAICALIN AND FERULIC ACID IN STANDARD DECOCTION AND COMMERCIAL CONCENTRATED HERBAL PREPARATION OF DANG-GUEI-SAN

Sample	Paeoniflorin		Baicalin		Ferulic acid	
	Mean ± S.D. (mg/g)[a]	R.S.D. (%)	Mean ± S.D. (mg/g)[a]	R.S.D. (%)	Mean ± S.D. (mg/g)[a]	R.S.D. (%)
Standard decoction	2.78 ± 0.05	1.97	27.26 ± 1.35	4.98	0.17 ± 0.01	4.05
Commercial preparation A	1.88 ± 0.06	3.06	4.72 ± 0.20	4.24	0.02 ± 0.001	6.25
Commercial preparation B	2.12 ± 0.11	5.64	0.02 ± 0.001	5.02	N.D.	–
Commercial preparation C	N.D.[b]	–	2.44 ± 0.07	2.89	N.D.	–

[a] $n = 5$ with 95% confidence limits.
[b] N.D. = Not determined.

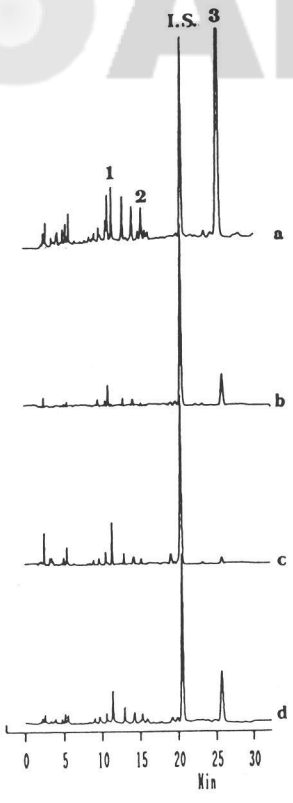

Fig. 2. Chromatograms of paeoniflorin, ferulic acid and baicalin in Dang-Guei-San: (a) standard decoction; (b) commercial preparation A; (c) commercial preparation B; (d) commercial preparation C. Peaks: 1 = paeoniflorin; 2 = ferulic acid; 3 = baicalin; I.S. = internal standard (methylparaben).

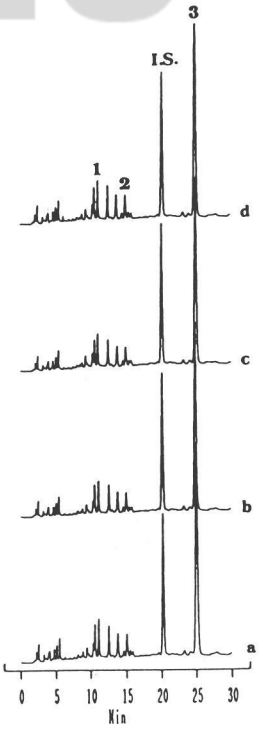

Fig. 3. Chromatograms of paeoniflorin, ferulic acid and baicalin in Dang-Guei-San: (a) standard decoction; (b) after concentration under reduced pressure; (c) after freeze-drying; (d) after spray-drying. Peaks as in Fig. 2.

TABLE III

CONTENTS OF PAEONIFLORIN, BAICALIN AND FERULIC ACID AFTER CONCENTRATION BY VARIOUS PROCESSES IN STANDARD DECOCTION AND IN PRODUCTS

Sample	Paeoniflorin		Baicalin		Ferulic acid	
	Mean ± S.D. (mg/g)[a]	R.S.D. (%)	Mean ± S.D. (mg/g)[a]	R.S.D. (%)	Mean ± S.D. (mg/g)[a]	R.S.D. (%)
Standard decoction	2.78 ± 0.05	1.97	27.26 ± 1.35	4.98	0.17 ± 0.01	4.05
Concentration under reduced pressure	2.24 ± 0.10	4.48	23.53 ± 0.31	1.31	0.16 ± 0.01	7.76
Freeze-drying	2.25 ± 0.09	4.04	23.15 ± 0.42	1.81	0.15 ± 0.01	5.88
Spray-drying	2.60 ± 0.12	4.58	26.27 ± 0.93	3.57	0.17 ± 0.01	7.56

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

TABLE IV

RELATIONSHIPS BETWEEN CONCENTRATIONS OF PAEONIFLORIN, FERULIC ACID AND BAICALIN AND THE PEAK AREA RATIO

Paeoniflorin: $y = -3.934 \cdot 10^{-3}x + 0.15$ ($r = 0.9973$); baicalin: $y = -3.689 \cdot 10^{-3}x + 9.11 \cdot 10^{-2}$ ($r = 0.9943$); ferulic acid: $y = -6.645 \cdot 10^{-4}x + 2.47 \cdot 10^{-2}$ ($r = 0.9970$).

Marker component	Concentration (mg/ml)	Peak-area ratio[a]	R.S.D. (%)[b]
Paeoniflorin	0.0107	0.0879	1.25
	0.0214	0.1791	1.31
	0.0321	0.2400	1.13
	0.0535	0.3749	1.00
Baicalin	0.0115	0.1416	0.84
	0.0575	0.6926	0.42
	0.1150	1.3905	0.32
	0.1495	1.6047	0.37
Ferulic acid	0.001232	0.0649	0.52
	0.003696	0.1912	0.40
	0.008624	0.3856	0.26
	0.011088	0.4634	0.31

[a] Peak area of marker components/peak area of methyl paraben.
[b] $n = 5$.

TABLE V

CONTENTS OF MARKER COMPONENTS IN SINGLE HERB AND TURNOVER RATIO IN STANDARD DECOCTION OF DANG-GUEI-SAN

Single herb (daily dose)	Marker component	Content of marker component in single herb		Theoretical content of marker component in standard decoction (mg/g) (daily dose, A)	Content of marker component in standard decoction (mg/g) (daily dose, B)	Turnover ratio (B/A, %)
		Mean ± S.D. (mg/g)[a]	R.S.D. (%)			
Paeoniae Radix (3 g)	Paeoniflorin	9.97 ± 0.12	1.25	29.91	8.44	28.22
Scutellariae Radix (3 g)	Baicalin	85.38 ± 1.80	2.11	256.14	84.05	32.81
Swertiae Herba (3 g) and Cnidii Rhizoma (3 g)	Ferulic acid	0.35 ± 0.01	4.19	2.10	1.05	50.00

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

florin, ferulic acid and baicalin, are 11, 15 and 25.5 min, respectively. On inspection of three-dimensional chromatograms, these three components all showed no interference. There was also no peak at their retention times in blank decoctions. The three commercial preparations also showed satisfactory separations.

The calibration graph for paeoniflorin and methylparaben was obtained over the range 0.0107–0.0535 mg/ml. The results, through linear regression analysis, showed a good linear relationship between peak-area ratio and concentration. Table IV gives the results and regression equation. Good linear relationships were also obtained from the calibration graphs for ferulic acid and baicalin.

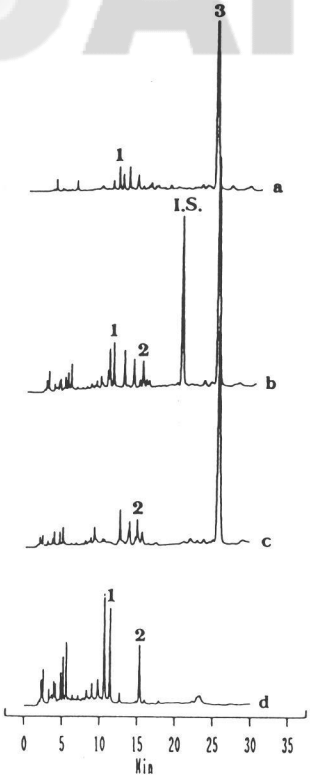

Fig. 4. Chromatograms of paeoniflorin, ferulic acid and baicalin in Dang-Guei-San and their blank solutions: (a) standard decoction without Swertiae Herba and Cnidii Rhizoma; (b) standard decoction; (c) standard decoction without Paeoniae Radix; (d) standard decoction without Scutellariae Radix. Peaks as in Fig. 2.

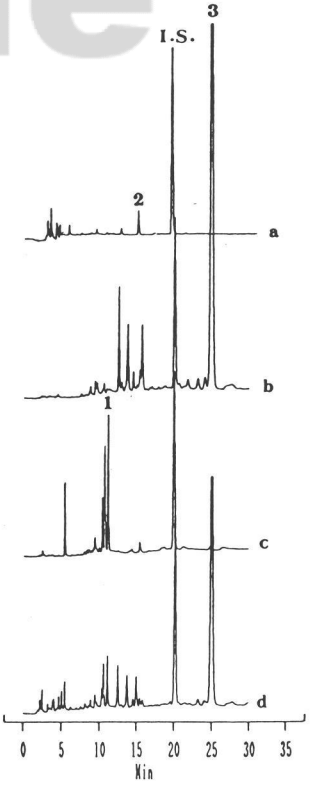

Fig. 5. Chromatograms of paeoniflorin, ferulic acid and baicalin in raw herbs and prescription: (a) Swertiae Herba and Cnidii Rhizoma; (b) Scutellariae Radix; (c) Paeoniae Radix (d) Dang-Guei-San. Peaks as in Fig. 2.

The turnover ratios of these constituents were defined as the percentage yields of these constituents in the Chinese medicinal preparations, calculated on the basis of their contents in the respective crude herbs. The turnover ratios for a certain marker component in different preparations vary greatly because each preparation has its own combination of raw herbs. The turnover ratios of the marker components of Dang-Guei-San prescription are shown in Table V and Fig. 5.

The recoveries of paeoniflorin, ferulic acid and baicalin were 27.86%, 33.89% and 49.31%, respectively, corresponding to turnover ratios of 28.22%, 32.81% and 50.00%, respectively. In fact, the turnover ratio was the same as the recovery when the added amount was a daily dose of Dang-Guei-San. When interaction is part of the procedure, the response measured from the matrix is generally compared with the response from the single raw herb, both subjected to the entire experimental procedure. In this case, the recovery is referred to as the relative recovery. When the amount of raw herb materials added was increased, the more the relative recovery decreased. It seems that a twentyfold weight of water was not enough when the amount of raw herb materials added was too great (Table VI).

TABLE VI

RECOVERY OF PAEONIFLORIN, BAICALIN AND FERULIC ACID FROM DANG-GUEI-SAN

Raw herb	Marker component	Raw herb material added (g)	Equal to marker component added (mg)	Found (mg)	Relative recovery (%)	Mean ± S.D. (%)[a]	R.S.D. (%)
Paeoniae Radix	Paeoniflorin	2.00	19.94	5.76	28.87	27.86 ± 0.86	3.08
		3.00	29.91	8.44	28.22		
		3.50	34.89	9.57	27.43		
		4.00	39.88	10.74	26.92		
Scutellariae Radix	Baicalin	2.00	170.76	60.82	35.62	33.89 ± 1.21	3.56
		3.00	256.14	84.02	32.81		
		3.50	298.83	100.71	33.70		
		4.00	341.52	114.24	33.45		
Swertiae Herba and Cnidii Rhizoma	Ferulic acid	4.00	1.44	0.77	53.74	49.31 ± 3.49	7.06
		6.00	2.16	1.08	50.00		
		7.00	2.52	1.21	48.04		
		8.00	2.88	1.31	45.47		

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

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