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Determination of cinnamic acid and paeoniflorin in traditional chinese medicinal preparations by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic method for the determination of cinnamic acid in *Cinnamomi ramulus* and paeoniflorin in *Paeoniae radix* was established. The samples were separated by a LiChrospher RP-18 column with water–acetonitrile–methanol–acetic acid (61:34:5:0.1 or 80:15:5:0.1, v/v) as the mobile phase at a flow-rate of 1.0 ml/min. Cinnamic acid and paeoniflorin were determined by UV detection at 280 and 250 nm, respectively. The method was applied to determine the optimum conditions for the extraction of the traditional Chinese medicinal preparation Huang Chi Chien Chung Tong, which contains *Cinnamomi ramulus* and *Paeoniae radix*. The results indicate that the best extraction conditions involved the use of an ultrasonic bath at 60°C for 30 min. In this experiment, butyl paraben and methyl paraben were used as the internal standards for cinnamic acid and paeoniflorin, respectively. A good and reproducible separation of cinnamic acid and paeoniflorin was obtained within 15 min. The method was also applicable to other preparations that contain *Cinnamomi ramulus* and *Paeoniae radix* such as Guey Chi Chia Long Ku Muu Li Tong, Kuei Chi Chien Chung Tong and Tang Kuei Chien Chung Tong.

INTRODUCTION

Traditional Chinese medicines, especially the concentrated type, are widely used and suitable assay methods are therefore needed for quality control purposes. In Japan, since 1985, the Ministry of Health and Welfare has required that all concentrated herbal preparations submitted for inspection and registration should include a content analysis with at least two chemical components as markers [1]. It has also regulated that all concentrated herbal preparations produced by pharmaceutical factories should be compared with the standard decoction[a] and the resulting difference in content of their marker components should be within ±30%. However, as our knowledge of the effective components of traditional Chinese medicines is still limited and the chemical compositions are very complicate, to determine accurately the contents of traditional Chinese medicine is very difficult. Research in this area is progressing in order that the process of Chinese medicine manufacture can be established on a scientific basis.

Huang Chi Chen Chung Tong is a prescription often used to treat physical weakness, and was studied in this work. Two components, cinnamic acid (present in *Cinniamomi ramulus*) and paeoniflorin (present in *Phaeoniae radix*) were selected for analysis; their structural formulae are shown in Fig. 1. High-performance liquid chromatography (HPLC) was employed to establish the optimum conditions for determination [2–8], which hopefully would serve as a reference for determining the contents of other prescriptions containing *Cinnamomi ramulus* and *Paeoniae radix*. In this experi-

[a] Standard decoction: to raw herb materials representing 1-day's dosage add a twentyfold weight excess of water and boil for more than 30 min until the liquid has reduced to half of the original volume. Filter the liquid to obtain the preparation.

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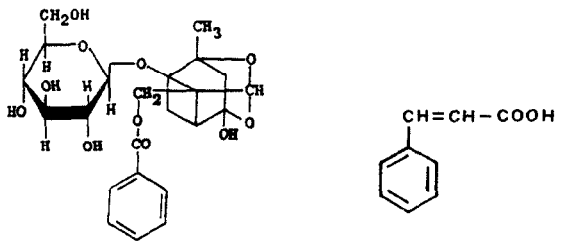

paeoniflorin cinnamic acid

Fig. 1. Structures of marker components.

ment, we also examined the effects of different extraction times and temperatures on the extracted amounts of cinnamic acid and paeoniflorin. The effects of various processess of concentration, drying and the addition of other excipients are also discussed.

EXPERIMENTAL

Materials

According to ref. 9, the following materials are needed to prepare Huang Chi Chen Chung Tong: *Cinniamomi ramulus*, 3 g; *Paeoniae radix*, 6 g; *Zingiberis rhizoma*, 3 g; *Zizyphi fructus*, 3 g; *Glycyrrhizae radix*, 3 g; *Astragali radix*, 1.5 g; and *Saccharum granorum*, 20 g. Other Chinese concentrated herbal preparations containing *Cinniamomi ramulus* and *Paeoniae radix* include Huang Chi Chen Chung Tong, Guey Chi Chia Long Ku Mum Li Tong and Kuei Chi Chen Chung Tong.

Chemicals and reagents

Reference standards of *trans*-cinnamic acid and paeoniflorin were purchased from Nacalai Tesque (Kyoto, Japan). The internal standards butyl paraben and methyl paraben were obtained from Sigma (St. Louis, MO, USA). Methanol and acetonitrile (HPLC grade) were purchased from ALPS (Taiwan) and all other reagents were of analytical-reagent grade.

Liquid chromatography

A Waters–Millipore LC system with a U6K injector and a Model 990 photodiode-array detector was used. For reversed-phase HPLC, a LiChrospher RP-18 (5-μm) column (125 × 4 mm I.D.) (Merck) with water–acetonitrile–methanol–acetic acid (61:34:5:0.1, v/v, for cinnamic acid and 80:15:5:0.1, v/v, for paeoniflorin) as the mobile phase at a flow-rate of 1.0 ml/min was adopted, with UV absorbance detection at 280 nm for cinnamic acid and 250 nm for paeoniflorin. Pretreatment of the solvents with a vacuum filter for degassing was applied.

Sample preparation for HPLC

Calibration graph. Cinnamic acid and paeoniflorin were accurately weighed and dissolved in methanol to give various concentrations within the range 0.001–0.01 and 0.025–0.3 mg/ml, respectively. An appropriate amount of internal standard was added to each solution to give concentrations of 0.1 mg/ml of butyl paraben or 0.02 mg/ml of methyl paraben. Calibration graphs were plotted based on linear regression analysis of the peak-area ratios.

Standard decoction. Amounts of crude drug equivalent to a daily dose of Huang Chi Chen Chung Tong were weighed and pulverized. A twentyfold weight excess 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 concentrations of 0.1 mg/ml of butyl paraben or 0.02 mg/ml of methyl paraben.

Concentrated herbal preparations. An amount equivalent to 1 g of *Cinniamomi ramulus* (or *Paeoniae radix*) was weighed and pulverized if necessary. Extraction was carried out by vibrating with an ultrasonic bath in 90 ml of 50% methanol for 30 min. After extraction, the sample was filtered and diluted to 100 ml with the addition of internal standard to give concentrations of 0.1 mg/ml of butyl paraben or 0.02 mg/ml of methyl paraben.

Recovery. Amounts of crude drugs equivalent to 50 doses of Huang Chi Chen Chung Tong without *Cinnamomi ramulus* were weighed and pulverized together. Then four doses of these powders were weighed precisely and separately, each 36.5 g. To these four doses were added 1, 2, 3 and 4 g of *Cinnamomi ramulus* with known cinnamic acid contents of 7.21, 14.42, 21.63 and 28.84 mg, respectively. A twentyfold weight excess 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 0.1 mg/ml of butyl paraben.

Amounts of crude drugs equivalent to 50 doses of Huang Chi Chen Chung Tong without *Paeoniae radix* were weighed and pulverized together. Then four doses of these powder were weighed precisely and separately, each 33.5 g. To these four doses were added 2, 4, 6 and 8 g of *Paeoniae radix* with known paeoniflorin contents of 25.26, 50.52, 75.78 and 101.04 mg, respectively. A twentyfold weight excess 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 0.02 mg/ml of methyl paraben.

All samples were filtered through a Millipore filter and 10 μl of filtrate were injected for HPLC analysis to calculate the concentration of cinnamic acid or paeoniflorin from their calibration graphs.

RESULTS AND DISCUSSION

The calibration graph for cinnamic acid and methyl paraben was obtained over the range 0.001–0.01 mg/ml. The results, through linear regression analysis, showed a good linear relationship between the peak-area ratio and concentration. Table I gives the results and the regression equation. A similar good linear relationship was obtained from the calibration graph for paeoniflorin and butyl paraben (Table II).

Cinnamic acid present in *Cinnamomi ramulus* and paeoniflorin in *Paeoniae radix* were determined by HPLC under the established conditions. The retention times for cinnamic acid and butyl paraben were 4.2 and 14.5 min, respectively (Fig. 2); retention

TABLE I

RELATIONSHIP BETWEEN CONCENTRATION OF CINNAMIC ACID AND THE PEAK-AREA RATIO

Regression equation: $y = -0.001 + 0.042x$ ($r = 0.9996$).

Concentration (mg/ml)	Peak-area ratio[a]
0.001	0.025 (1.48)
0.003	0.071 (1.12)
0.005	0.120 (0.78)
0.010	0.240 (0.90)

[a] Peak area of cinnamic acid/peak area of butyl paraben, with relative standard deviation (%) in parentheses ($n = 6$).

TABLE II

RELATIONSHIP BETWEEN CONCENTRATION OF PAEONIFLORIN AND PEAK-AREA RATIO

Regression equation: $y = -0.00005 + 0.297x$ ($r = 0.9998$).

Concentration (mg/ml)	Peak-area ratio[a]
0.025	0.087 (0.94)
0.050	0.176 (1.27)
0.100	0.339 (1.61)
0.300	1.016 (0.69)

[a] Peak area of paeoniflorin/peak area of methyl paraben, with relative standard deviation (%) in parentheses ($n = 6$).

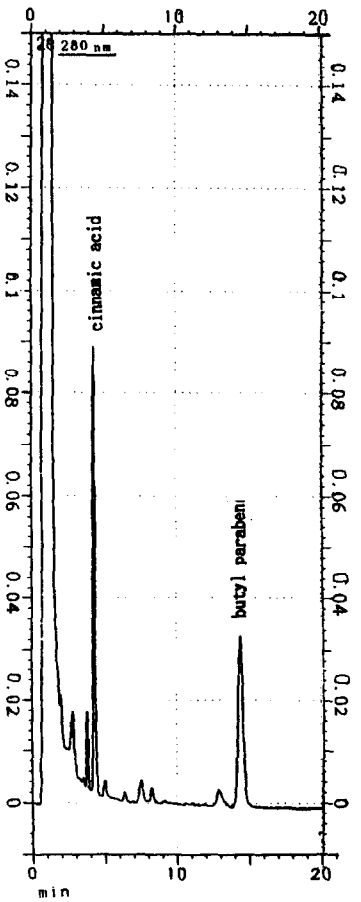

Fig. 2. Chromatogram of ciannamic acid in Huang Chi Chen Chung Tong. Column, LiChrospher RP-18; mobile phase, water–acetonitrile–methanol–acetic acid (61:34:5:0.1, v/v); flow-rate, 1 ml/min.

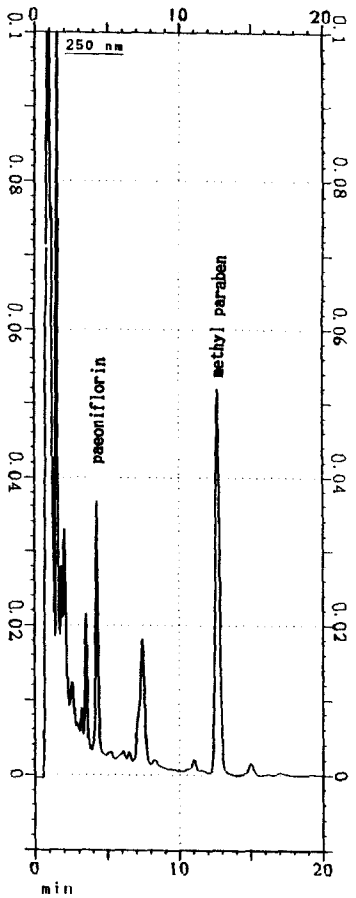

Fig. 3. Chromatogram of paeoniflorin in Huang Chi Chen Chung Tong. Column, LiChrospher RP-18; mobile phase, water–acetonitrile–methanol–acetic acid (80:15:5:0.1, v/v); flow-rate, 1 ml/min.

times for paeoniflorin and methyl paraben are 4.2 and 12.5 min, respectively (Fig. 3). The assay method used has the following advantages that it is easy and convenient to operate, is rapid and the accuracy of the determination is improved by the addition of an internal standard.

Traditional Chinese medicines are usually prepared by boiling with water. However, extraction of components with water from preparations tends to cause problems during the experiment, *e.g.*., the extracted materials may block the column. For this reason, four solvents were tried for extraction: water, methanol, methanol–water (30:70) and methanol–watwr (50:50). Two methods, refluxing and using an ultrasonic bath, were used to extract cinnamic acid from *Cinnamomi ramulus* and paeoniflorin from *Paeoniae radix*. The largest extracted amount was assigned an arbitrary value 100 to compare the efficiencies of the various extraction methods. The results are given in Tables III and IV ($n = 6$) and it indicate that both methods have similar effects on the determination of cinnamic acid and paeoniflorin. They also show that methanol, methanol–water (30:70) and methanol–water (50:50) are the best solvents for cinnamic acid extraction whereas water, methanol–water (30:70) and methanol–water (50:50) are best for paeoniflorin.

We also studied the optimum temperature and time for extracting cinnamic acid and paeoniflorin. The samples were vibrated with an ultrasonic bath for 30 and 60 min at 30, 60 and 80°C. The results are shown in Tables V and VI. As can be seen, the

TABLE III

YIELDS OF CINNAMIC ACID EXTRACTED FROM *CINNAMOMI RAMULUS* BY VARIOUS SOLVENTS

Method	Yield (%)			
	Methanol	Methanol–water (30:70)	Methanol–water (50:50)	Water
Reflux (30 min)	100	98.0	98.3	81.8
Agitation with ultrasonic bath	100	98.9	99.7	84.3

TABLE IV

YIELDS OF PAEONIFLORIN EXTRACTED FROM *PAEONIAE RADIX* BY VARIOUS SOLVENTS

Method	Yield (%)			
	Methanol	Methanol–water (30:70)	Methanol–water (50:50)	Water
Reflux (30 min)	46.4	99.0	99.2	100
Agitation with ultrasonic bath	48.9	99.2	99.5	100

TABLE V

YIELDS OF CINNAMIC ACID EXTRACTED WITH METHANOL–WATER (50:50) FROM HUANG CHI CHIEN CHUNG TONG AT VARIOUS TEMPERATURES

Time (min)	Yield (%)		
	30°C	60°C	80°C
30	82.7	100	99.6
60	87.5	99.8	99.5

TABLE VI

YIELDS OF PAEONIFLORIN EXTRACTED WITH METHANOL–WATER (50:50) FROM HUANG CHI CHIEN CHUNG TONG AT VARIOUS TEMPERATURES

Time (min)	Yield (%)		
	30°C	60°C	60°C
30	79.3	100	99.9
60	84.7	99.3	99.1

amounts of the two components extracted did not increase with time or elevated temperature after extraction at 60°C for 30 min.

After comparing the various extraction methods with respect to solvent, temperature and time, and considering the conditions that might be applied during the manufacture of Chinese medicines, we conclude that the best way to extract cinnamic acid and paeoniflorin is to vibrate the sample with an ultrasonic bath for 30 min at 60°C with methanol–water (50:50) as solvent.

The accuracy of the HPLC assay method was determined by recovery tests and the precision was evaluated by measuring the reproducibility [relative standard deviation (R.S.D.)]. The recoveries were 95.3% for cinnamic acid and 95.9% for paeoniflorin

TABLE VII

RECOVERY OF CINNAMIC ACID IN HUANG CHI CHIEN CHUNG TONG

Amount of *Cinnamomi ramulus* taken (g)	Content of cinnmic acid in *Cinnmomi ramulus* (mg)	No. of injections (*n*)	Amount measured (mg)	Recovery (%)	Mean ± S.D. (%)	R.S.D. (%)
1	7.21	6	6.73	93.3	95.3 ± 1.58	1.66
2	14.42	6	13.70	95.0		
3	21.63	6	20.76	96.0		
4	28.84	6	27.97	97.0		

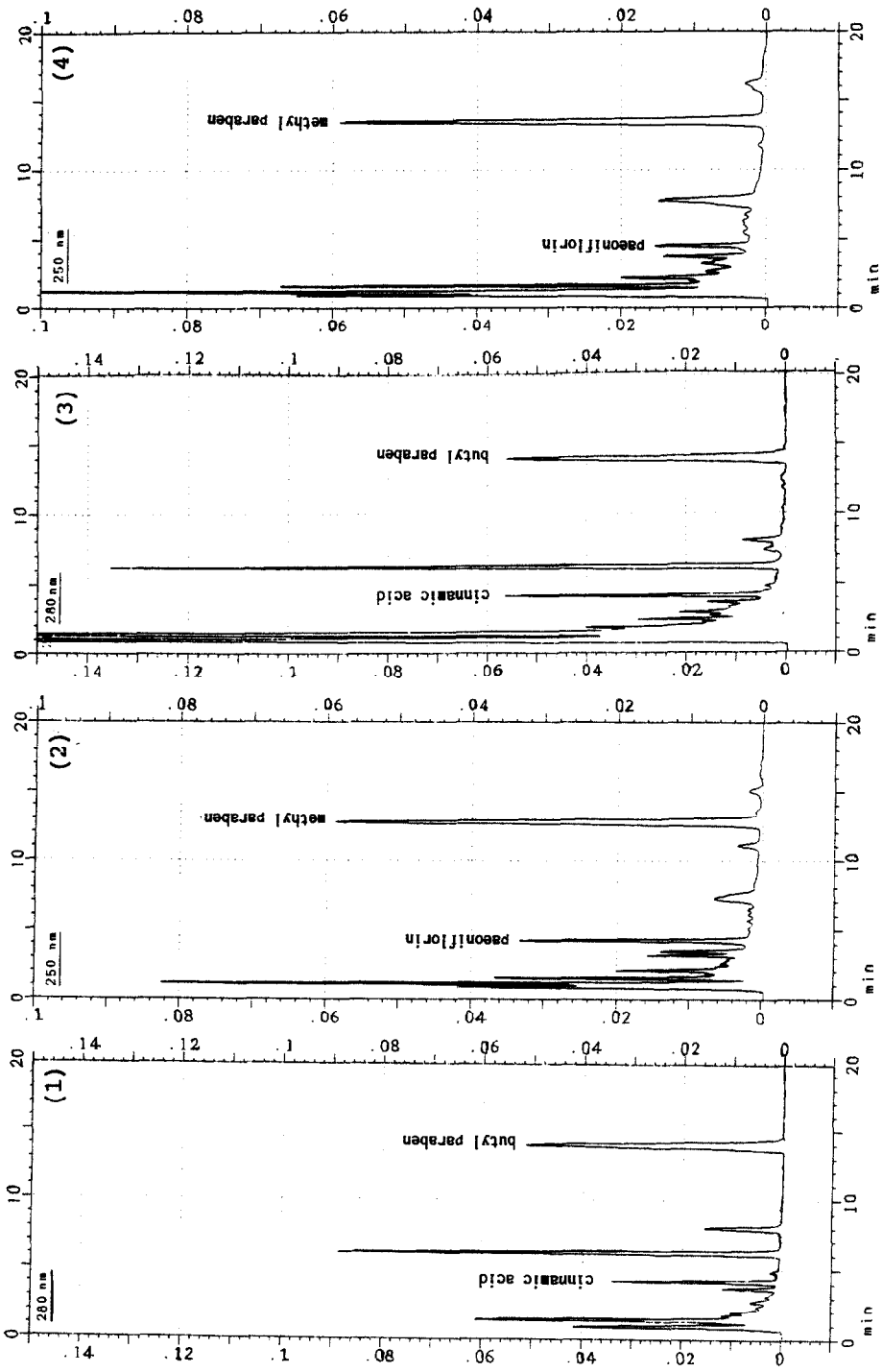

Fig. 4. Chromtograms of different samples: (1) and (2) Guey Chi Chia Long Ku Mum Li Tong; (3) and (4) Kuei Chi Chien Chung Tong. HPLC conditions: (1) and (3) as in Fig. 2; (2) and (4) as in Fig. 3.

TABLE VIII

RECOVERY OF PAEONIFLORIN IN HUANG CHI CHIEN CHUNG TONG

Amount of *Paeoniae radix* taken (g)	Content of paeoniflorin in *Paeoniae radix* (mg)	No. of injections (*n*)	Amount measured (mg)	Recovery (%)	Mean ± S.D. (%)	R.S.D. (%)
2	25.26	6	24.05	95.2	95.9 ± 2.07	2.16
4	50.52	6	48.80	96.6		
6	75.78	6	73.81	97.4		
8	101.04	6	92.27	91.3		

TABLE IX

REPRODUCIBILITY OF THE DETERMINATION OF CINNAMIC ACID IN HUANG CHI CHIEN CHUNG TONG

Injection No.	Amount measured (mg/g)	Mean ± S.D. (%)	R.S.D. (%)
1	7.24	7.212 ± 0.017	0.23
2	7.22		
3	7.19		
4	7.21		
5	7.20		
6	7.21		

TABLE X

REPRODUCIBILITY OF THE DETERMINATION OF PAEONIFLORIN IN HUANG CHI CHIEN CHUNG TONG

Injection No.	Amount measured (mg/g)	Mean ± S.D. (%)	R.S.D. (%)
1	12.73	12.625 ± 0.183	1.45
2	12.28		
3	12.62		
4	12.81		
5	12.67		
6	12.64		

TABLE XI

CONTENTS OF CINNAMIC ACID IN COMMERCIAL PREPARATIONS CONTAINING *CINNAMOMI RAMULUS* AND *PAEONIAE RADIX*

Sample	No.[a]	Loss on drying (%)	Content of cinnamic acid in *Cinnamomi ramulus*-containing preparations (%)
Huang Chi Chen Chung Tong	1g	2.67	0.26
	2p	3.01	0.77
	3p	2.73	0.40
Guey Chi Chia Long Ku Mum Li Tong	1p	3.52	0.32
	2p	2.44	0.66
	3g	3.79	1.87
Kuei Chi Chien Chung Tong	1p	2.83	0.54

[a] p = Powder; g = granules.

TABLE XII

CONTENTS OF PAEONIFLORIN IN COMMERCIAL PREPARATIONS CONTAINING *CINNAMOMI RAMULUS* AND *PAEONIAE RADIX*

Sample	No.[a]	Loss on drying (%)	Content of paeoniflorin in *Paeoniae radix*-containing preparations (%)
Huang Chi Chen Chung Tong	1g	2.67	0.26
	2p	3.01	0.77
	3p	2.73	0.40
Guey Chi Chia Long Ku Mum Li Tong	1p	3.52	0.32
	2p	2.44	0.66
	3g	3.79	1.87
Kuei Chi Chien Chung Tong	1p	2.83	0.54

[a] p = Powder; g = granules.

($n = 6$), and R.S.D.s were 0.23% for cinnamic acid and 1.45% for paeoniflorin. The results of these tests are given in Tables VII–X. We conclude that the method is precise and accurate for the determination of cinnamic acid and paeoniflorin in commercial preparations.

Three different commercial brands of Huang Chi Chen Chung Tong were extracted with the above method and their contents of cinnamic acid and paeoniflorin were determined as described. The results were satisfactory. Other preparations containing *Cinnamomi ramulus* and *Paeoniae radix*, such as Guey Chi Chia Long Ku Mum Li Tong and Kuei Chi Chien Chung Tong, were also analysed with the same method and the results were satisfactory (Fig. 4). The contents of cinnamic acid and paeoniflorin determined in the different preparations are given in Tables XI and XII.

To elucidate the effects of the concentration process and starch excipient on the determination of cinnamic acid and paeoflorin, the process was applied under reduced pressure at 50°C and with lypholization, as adopted in pharmaceutical factories. The results indicated that the process conditions and starch content did not significantly affect the determination of the two components. The results are given in Tables XIII and Table XIV.

The cinnamaldehyde, which is also present in *Cinnamomi ramulus*, was chosen as a marker [10]. However, the content of cinnamaldehyde tends to decrease with increase in boiling time and temperature. Taking into account the possibility than the

TABLE XIII

EFFECTS OF CONCENTRATION BY REDUCED PRESSURE AND LYOPHILIZATION ON CONTENTS OF CINNAMIC ACID AND PAEONIFLORIN IN HUANG CHI CHIEN CHUNG TONG

Method	Content (%)	
	Cinnamic acid	Paeoniflorin
Decoction without concentration	0.72	1.26
Concentrated by reduced pressure at 50°C	0.70	1.26
Lyophilization	0.68	1.20

TABLE XIV

EFFECT OF STARCH ON CONTENTS OF CINNAMIC ACID AND PAEONIFLORIN IN HUANG CHI CHIEN CHUNG TONG

Excipient	Content (%)	
	Cinnamic acid	Paeoniflorin
Without starch	0.72	1.26
With starch	0.69	1.21

method used here might be applied to the analysis of concentrated preparations, cinnamic acid is to be preferred as a marker because it is not affected by time and temperature. The chromatogram of the standard decoction shows that the cinnamaldehyde content decreased with prolonged boiling and because so low that it was difficult to measure. However, the chromatogram of the commercial preparations showed highly concentrated cinnamaldehyde. More research on this aspect needs to be done.

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