Quality Evaluation of Cortex Moutan by High Performance Liquid Chromatography Coupled with Diode Array Detector and Electrospary Ionization Tandem Mass Spectrometry

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An high performance liquid chromatography (HPLC) coupled with diode array detector (DAD) and electrospray ionization tandem mass spectrometry (ESI/MS^n) method was developed for quality evaluation of Cortex Moutan through identification of common constituents based on chromatographic fingerprints and determination of key pharmacological compounds. The representative chromatographic fingerprints of Cortex Moutan were obtained by analyzing 10 batches of samples under the optimized HPLC conditions and the results showed that the chromatographic profiles of the analyzed samples were very similar. Total of nineteen common peaks were detected and seventeen of them were identified rapidly by their characteristic UV profile and the information of molecular structure provided by ESI/MS^n experiments. Simultaneously, five key pharmacological compounds, namely gallic acid, oxypaeoniflorin, paeoniflorin, benzoylpaeoniflorin and paeonol, were determined by the validated HPLC-DAD method. The linear calibration curves were acquired with correlation coefficients higher than 0.999. The precisions of intra-day and inter-day were not exceeding 3.1%, and the recoveries of five analytes were from 92.86 to 99.35%. This developed method that combined the chromatographic fingerprints and quantification assay ensured the phytoequivalence and pharmacological effects of Cortex Moutan and was successfully applied to the quality control of Cortex Moutan.

Key words HPLC-DAD-ESI/MS^n; quality evaluation; chromatographic fingerprint; Cortex Moutan

Herbal medicines play an important role owing to its effectiveness in public health. It is widely accepted that the multiple constituents in herbal medicines resulted to their remarkable clinical therapeutics. In order to ensure the stability and efficiency in clinic use, the quality control system of herbal medicines should be reflected the phytoequivalence and pharmacological effects.[1,2] In general, two strategies are applied in the quality control of herbal mediciness: one is based on the chromatographic fingerprints to assess the batch-to-batch consistency of the chemical constituents[3—5] and the other is to determine single or a few key pharmacological compounds for the assessment of quality.[6,7] However, the first strategy is a 'blind analysis' because of the lacking of the definite chemical information of constituents and quantitative information of the major pharmacological constituents, so it is difficult to assess the pharmacological effects of herbal medicines. The other strategy only focuses on a few index compounds and cannot be comprehensively responsible for the quality of multiple constituents in herbal medicine, so it can hardly ensure the phytoequivalence. Thus, it is necessary to develop the quality evaluation method for herbal medicines, which is provided with the comprehensive elucidation of constituents based on chemical fingerprint and quantitative analysis of key pharmacological compounds simultaneously.

Recently, HPLC-DAD-ESI/MS^n has been a powerful analytical tool for the rapid identification of chemical constituents in herbal medicine. It combined the separation of HPLC, the high characteristic of UV spectra, and the structure information provided by ESI/-MS^n used for analysis of complex herbal matrix in contrast with the conventional arduous and time-consuming phytochemistry techniques. In last decades, publications on the use of HPLC-DAD-ESI/MS^n have markedly increased,[8—10] and the analysis of multiple constituents in complex extraction of herbal medicines using this technique can be achieved.[11—14]

Cortex Moutan, the root cortex of *Paeonia suffruticosa* Andr., is one of famous Chinese herbal medicine widely used for treatment of analgesic, anaphylactic, antioxidative, and anti-inflammatory ailments.[15,16] The pharmacological reports were focused on gallic acid, paeonol, monoterpenoid glycosides such as oxypaeoniflorin, paeoniflorin and benzoylpaeoniflorin and these compounds were proved to be the key pharmacological constituents of Cortex Moutan.[17—20] Though only one species *Paeonia suffruticosa* Andr. was documented as the source of Cortex Moutan in China Pharmacopeia (2005 Edition, Vol. I),[21] the crude drug from different local areas had also been traded in markets without authentication and resulted insecurity of clinical use.

The aim of the present study was to develop an HPLC-DAD-ESI/MS^n analytical method for the quality evaluation of Cortex Moutan through simultaneous identification and determination of the chemical constituents in Cortex Moutan.

Results and Discussion

Optimization of the HPLC Condition Various eluting conditions for the separations were tried and a gradient of acetonitrile and 0.1% formic acid was employed to achieve well separation. Formic acid was used as modifier of mobile phases owing to its significantly restrained the peak tailing of the constituents in experiments. For comparing the LC-UV chromatogram in different wavelengths, 230 nm was chosen as the monitoring wavelength due to well signal response of most of constituents.

HPLC-UV Fingerprint Analysis of Cortex Moutan All the samples were analyzed under the optimized HPLC condition, and the characteristic HPLC-UV fingerprints were

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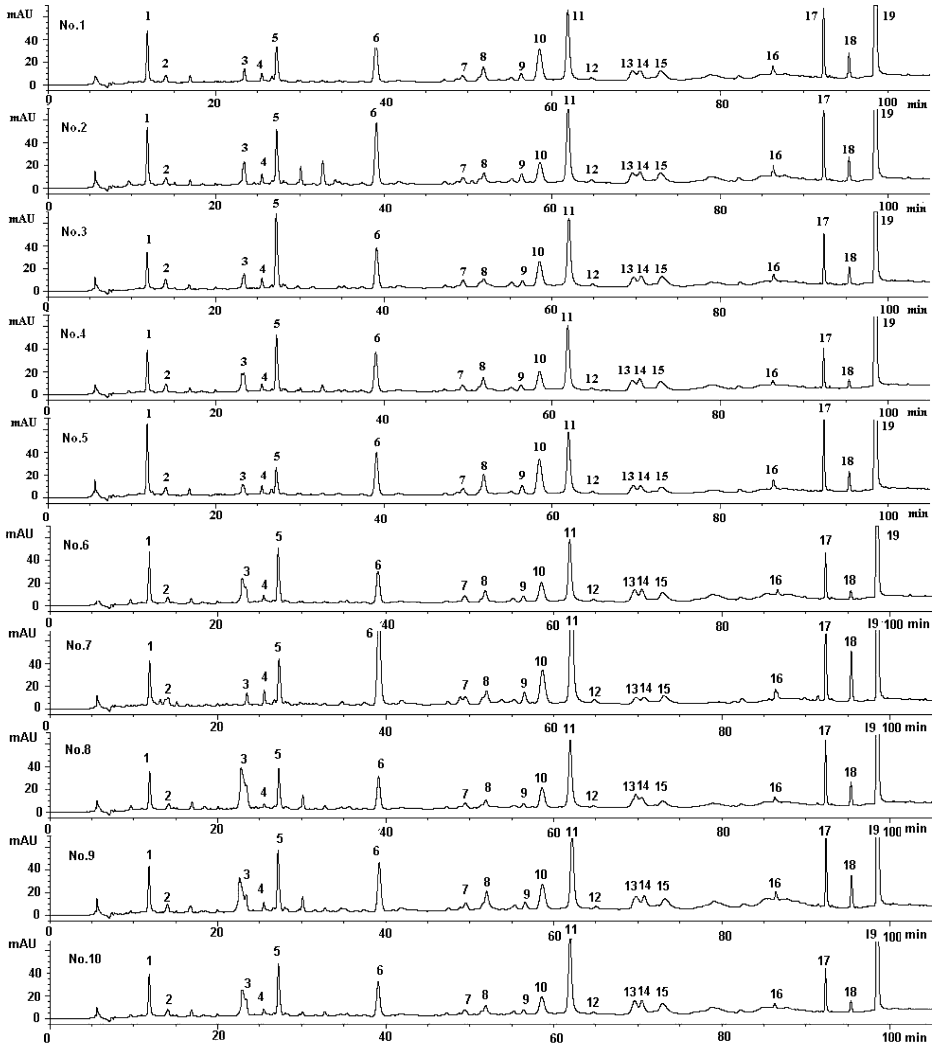

Fig. 1. HPLC Fingerprints of 10 Batches of Cortex Moutan

Marked peaks were common peaks.

presented in Fig. 1. These peaks that appeared in all HPLC profiles were assigned as 'common peaks,' which represented the phytoequivalence of Cortex Moutan.

Through comparing the HPLC chromatograms, it was generally found that the fingerprint profiles of Cortex Moutan were similar with each other. By carefully studying on the all HPLC profiles of Cortex Moutan collected from different area, total of seventeen common peaks were found out and marked in Fig. 1, which means that their constituents were similar with each other. The common peaks could be used as the marks in distinguishing Cortex Moutan form other crude drug.

HPLC-MS and MS^n Analysis of Common Peaks in Cortex Moutan As lacking of the elucidation data of common peaks, the chemical constituents represented the phytoequivalence of Cortex Moutan were still ambiguous. In order to identify these common peaks found in HPLC profile of Cortex Moutan, the HPLC-ESI/MS and MS^n experiments were carried out. Negative ion mode was employed because of its more sensitive signals of the common constituents than that in positive ion mode and all the common constituents were well detected and exhibited the quasi-molecular ions $[M-H]^-$. Five compounds were positively identified by comparing the retention time and MS data of the analyzed samples with the respective data obtained from the analysis of reference compounds. The other 12 common peaks in Cortex Moutan were identified (Table 1) by carefully studying on these mass spectrums and comparing with standards and reference data, and their structures were exhibited in Fig. 2. When this comparison was not explanatory enough, MS^n experiments were carried out in order to further confirm the identity of the compounds and the characteristic fragment ions were attributed respectively (Table 2).

Validation Results of Quantitative Methods Each calibration curve was performed with five different concentrations in triplicate, and all the correlation coefficients (r) of these calibration curves were higher than 0.999. The limits of detection (LODs) and quantification (LOQs) under the present chromatographic conditions were determined at a signal-to-noise ratio (S/N) of 3 and 10, respectively. The results were demonstrated in Table 3.

The precisions of intra-day and inter-day were evaluated by the relative standard deviations (RSDs) of the peak area (Pa) by injecting the sample solution (No. 1) of Cortex Moutan into HPLC system for six times successively. As exhibited in Table 3, the precisions of Pa were not exceeding

Table 1. HPLC-DAD-MS Data and Constituents Identification in Cortex Moutan

Peak	t_R (min)	Precursor ion $[M-H]^-$ (m/z)	λ_{max} (nm)	Identification
1	11.9	169.3	215, 270	Gallic acid[22),a)]
2	14.1	463.3	215, 275	Mudanoside b[23)]
3	23.4	577.3	203, 280	Unkown
4	25.5	495.2	258	Oxypaeoniflorin[24),a)]
5	27.2	289.2	203, 282	D-Catethin
6	39.1	479.3	195, 235	Paeoniflorin[17),a)]
7	49.4	787.1	218, 280	Tetragalloylglucopyranose isomers[25)]
8	51.9	787.1	218, 280	
9	56.4	121.2	220, 275	Benzoic acid
10	58.5	631.2	230	Galloyl-paeoniflorin[19)]
11	62.0	939.0	212, 280	Pentagalloylglucopyranose[25)]
12	64.8	615.2	215, 270	Mudanpioside h[26)]
13	69.7	1091.1	218, 280	
14	70.7	1091.1	218, 280	Hexagalloylglucopyranose isomers[25)]
15	73.0	1091.1	218, 280	
16	86.3	599.2	230, 260	Benzoyloxypaeoniflorin[24)]
17	92.3	583.1	230	Benzoylpaeoniflorin [17,24),a)]
18	95.3	195.2	230	Paeonilactone b[27)]
19	98.2	165.2	230, 280	Paeonol[28),a)]

a) With standard compounds.

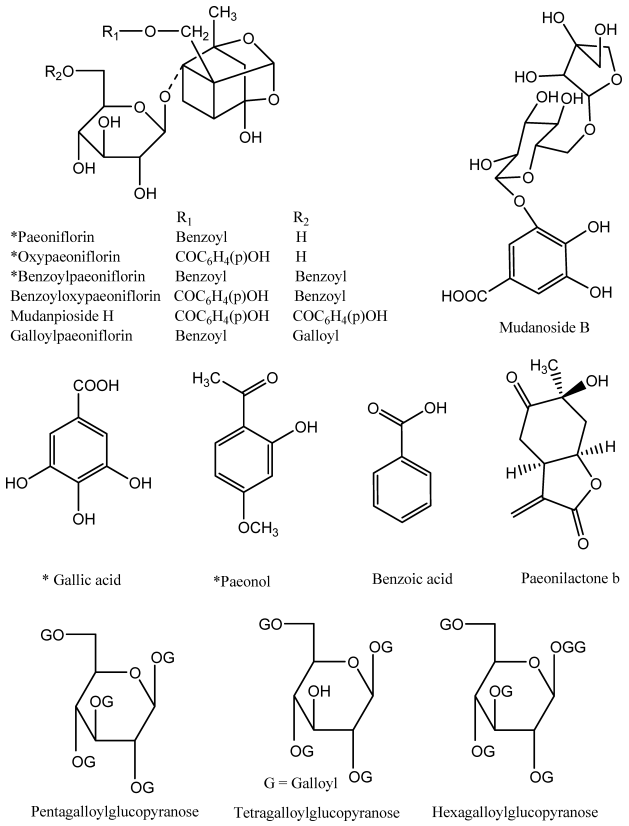

Fig. 2. Structures of Compounds Identified in Cortex Moutan

∗ With standard compounds.

3.1%. The RSDs of Pa were less than 2.7% by injecting the sample continuously in 24 h that indicated the sample solution was stable.

The recovery test were conducted by spiking the accurate amounts for three times at three levels (80%, 100%, 120% amount in the sample) of five reference compounds which were added to approximate 1.0 g of sample (No. 1), and the results (Table 3) shown the analytical recoveries of five analytes were between 92.86% to 99.35%.

Quantification Analysis of Five Key Pharmacological Compounds in Cortex Moutan This method was applied to analyze five compounds in Cortex Moutan which were collected from different areas and the results were listed in Table 4. Thought the chromatographic fingerprints of samples were similar with each other and all the common peaks could be detected, the content of the pharmacological compounds was different remarkably. It was obvious that the content of three monoterpenoids in samples was correlative. In samples 2, 7, 9, the contents of monoterpenoids was higher than average content, whereas they were lower than average content in samples 6, 8, 10. As to the content of gallic acid and paeonol, it was found that the contents of these two compounds in samples 1, 2, 5 were obviously higher than that in samples 7, 8, 9 and 10.

In conclusion, the newly developed an HPLC-DAD-ESI/MS^n method described herein represents a significant improvement in the quality evaluation of Cortex Moutan. In contrast to conventional quality evaluation method for herbal medicine, it allows not only roundly and directly identifies the chemical constituents, but also could be use as quantitative analysis of the major biological active ingredients. Thus, it seems to be the powerful method of choice for routine analysis for herbal medicine.

Experimental

Standards and Samples Gallic acid (**1**), paeoniflorin (**3**) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Oxypaeoniflorin (**2**), benzoylpaeoniflorin (**4**) and paeonol (**5**) were isolated from Cortex Moutan, and their structures were elucidated by UV, MS and NMR spectrums. The purity of these compounds were determined to be more than 98% by normalization of the peak areas detected by HPLC with UV, and showed very stable in methanol solution. Ten batches samples of Cortex Moutan were collected from the markets of different areas including Beipei city, Sichuan Province (No. 1), Chongqing city, Sichuan Province (No. 2), Heze city, Shangdong Province (No. 3), Jinan city, Shangdong Province (No. 4), Luoyang city, Henan Province (No. 5), Bozhou city, Anhui Province (No. 6), Bozhou city, Anhui Province (No. 7), Linan city, Zhejiang Province (No. 8), Hangzhou city, Zhejiang Province (No. 9) and Nanning city, Guangxi Province (No. 10). All the samples were authenticated by one of us (Dr. He) and the voucher speci-

Table 2. The Precursor Ion and Main Fragment Ions of 17 Common Constituents in MS and MS^n Analysis

Peak	$[M-H]^-$ (m/z)	MS^2 ion (m/z)	MS^3 ion (m/z)	MS^4 ion (m/z)
1	169.3	125.1 $[M-H-CO_2]^-$	97.0 $[M-H-CO_2-CO]^-$	—
2	463.3	301.1 $[M-H-C_6H_{10}O_5]^-$	—	—
4	495.2	465.1 $[M-H-CH_2O]^-$	326.9 $[M-H-CH_2O-$ (p-hydroxyl-bezoic acid)$]^-$	165.2 $[M-H-CH_2O-$(p-hydroxyl-bezoic acid) $-$glucose$]^-$
6	479.3	448.9 $[M-H-CH_2O]^-$	326.9 $[M-H-CH_2O-$(benzoic acid)$]^-$	164.9 $[M-H-CH_2O-$(benzoic acid)$-$glucose$]^-$
7	787.1	635.2 $[M-H-C_7H_4O_4]^-$	483.1 $[M-H-2\times C_7H_4O_4]^-$	331.2 $[M-H-3\times C_7H_4O_4]^-$
8	787.1	635.2 $[M-H-C_7H_4O_4]^-$	483.1 $[M-H-2\times C_7H_4O_4]^-$	331.2 $[M-H-3\times C_7H_4O_4]^-$
9	121.2	77.1 $[M-H-CO_2]^-$	—	—
10	631.2	613.3 $[M-H-H_2O]^-$	491.3 $[M-H-H_2O-$(benzoic acid)$]^-$	313.2 $[M-H-H_2O-$(benzoic acid)$-C_{10}H_{10}O_3]^-$
11	939.0	769.1 $[M-H-$gallic acid$]^-$	617.2 $[M-H-$gallic acid$-C_7H_4O_4]^-$	465.1 $[M-H-$gallic acid$-2\times C_7H_4O_4]^-$
12	615.2	585.3 $[M-H-CH_2O]^-$	447.2 $[M-H-CH_2O-C_7H_6O_3]^-$	429.1 $[M-H-CH_2O-C_7H_6O_3-H_2O]^-$
13	1091.1	939.4 $[M-H-C_7H_4O_4]^-$	769.2 $[M-H-C_7H_4O_4-$gallic acid$]^-$	599.1 $[M-H-C_7H_4O_4-2\times$gallic acid$]^-$
14	1091.1	939.4 $[M-H-C_7H_4O_4]^-$	769.2 $[M-H-C_7H_4O_4-$gallic acid$]^-$	599.1 $[M-H-C_7H_4O_4-2\times$gallic acid$]^-$
15	1091.1	939.4 $[M-H-C_7H_4O_4]^-$	769.2 $[M-H-C_7H_4O_4-$gallic acid$]^-$	599.1 $[M-H-C_7H_4O_4-2\times$gallic acid$]^-$
16	599.2	569.2 $[M-H-CH_2O]^-$	447.2 $[M-H-CH_2O-$(benzoic acid)$]^-$	429.1 $[M-H-CH_2O-$(benzoic acid)$-H_2O]^-$
17	583.1	553.0 $[M-H-CH_2O]^-$	431.0 $[M-H-CH_2O-$(benzoic acid)$]^-$	413.1 $[M-H-CH_2O-$(benzoic acid)$-H_2O]^-$
18	195.2	176.9 $[M-H-H_2O]^-$	132.9 $[M-H-H_2O-CO_2]^-$	—
19	165.2	147.2 $[M-H-H_2O]^-$	121.1 $[M-H-H_2O-CO]^-$	—

Table 3. Validation Data of Quantitative Method for Five Analytes

Analyte	Calibration curve	r^2	Test range (μg)	LODs (ng)	LOQs (ng)	Precision RSD (%)		Recovery RSD (%)		
						Intra-day variability	Inter-day variability	80%	100%	120%
1	$y=3885x+1.185$	1.0000	0.1549—2.478	4.995	9.910	1.54	1.21	96.99 (2.26)	96.53 (1.29)	96.13 (1.02)
2	$y=632.5x-2.088$	0.9998	0.09515—1.523	45.14	95.15	1.04	1.97	99.33 (0.38)	99.05 (2.87)	96.69 (2.39)
3	$y=2565x-23.91$	0.9997	0.4275—6.837	6.836	13.67	2.36	2.74	99.35 (1.93)	98.64 (2.52)	99.32 (0.94)
4	$y=4369x-3.953$	0.9999	0.07900—2.538	1.011	2.528	2.59	2.59	98.30 (3.01)	98.97 (1.50)	97.98 (2.57)
5	$y=6764x+33.75$	0.9999	0.7975—12.76	1.063	2.126	2.75	3.02	92.86 (0.17)	97.54 (1.63)	93.85 (1.90)

Table 4. Concentration of Five Key Pharmacological Constituents in Cortex Moutan

Sample	Content (mg/g, $n=3$)				
	1	2	3	4	5
1	1.761	1.539	4.361	1.782	18.95
2	1.990	1.728	6.072	2.004	22.11
3	1.229	1.539	4.014	1.100	18.80
4	1.426	1.180	3.903	0.8682	17.56
5	2.418	1.509	4.210	1.887	20.90
6	1.746	0.8843	3.382	1.008	11.75
7	1.533	2.594	9.825	2.655	8.667
8	1.309	0.5837	3.233	1.409	14.61
9	1.580	1.655	4.874	1.8019	15.62
10	1.426	0.9761	3.410	0.9508	10.87

mens were deposited in herbarium of department of Chinese Medicine Science & Engineering, Zhejiang University.

Solvents and Reagents HPLC grade acetonitrile from Merck (E. Merck, Darmstadt, Germany) was used for analysis. Analytical-grade methanol and acetic acid for analysis were from Hangzhou Reagent Company (Hangzhou, PR China). Water was purified with a Mili-Q academic water purification system (Millipore, Bedford, MA, U.S.A.).

Sample Preparation The samples were pulverized and dried in desiccator at 40 °C until constant weight. 1 g of powder was transferred to a 100 ml of flask, extracted under reflux for 1 h with 40 ml of methanol on a water bath and filtrated. The filtration was transferred accurately to a 50 ml of volumetric flask and filled to the mark with methanol. The resultant solution was filtered through a 0.45 μm PTFE film before HPLC analysis.

HPLC System and Conditions HPLC analysis was carried out using an Agilent 1100 series HPLC system (Waldbronn, Germany) equipped with a quaternary pump, an on-line degasser, an autosampler, a column oven and a diode-array detector (DAD). A Zorbax Stable Bond C_{18} column (4.6 mm×250 mm, 5 μm, Agilent) was utilized for separation at the column temperature of 25 °C. The mobile phase consisted of acetonitrile (A) and 0.1% formic acid (B) with a flow-rate of 0.5 ml/min. The gradient programmer was used the according to the following procedure: 0—20 min, linearly increase A from 5% to 15%, 20—30 min, hold on 15% A, 30—75 min linearly increase to 25% A, 75—90 min linearly increase to 45% A; 90—95 linearly increase to 70% A; 95—105 min hold on 70% A. The UV spectra were recorded from 190 to 400 nm, and the monitor wavelength was set at 230 nm.

HPLC System and Conditions A LCQ DECA XP^{plus} Ion Trap mass spectrometer (Thermo Finnigan, San Jose, U.S.A.) equipped with an ESI interface was used to carry out MS and MS^n experiments. Data were acquired and analyzed by Thermo Finnigan $Xcalibur_{1.3}$ workstation. The operating conditions for the ESI interface were as follows: negative ionization mode, temperature of the capillary, 350 °C, spray voltage, 3.0 kV, capillary voltage, 20 V; sheath gas (N_2) flow, 30 A.U.; auxiliary gas (N_2) flow, 10 A.U. Molecular weight data acquisition was performed from m/z 100 to 1500 in full MS scan mode. MS^n experiments were performed by collision of the precursor ions with Helium gas at 2.0 mass isolation widths. The collision energy values were automatic selected.

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References

1) WHO, General Guidelines for Methodologies on Research and Evaluation of Traditional Medicines, 2000, p. 1.

2) Tyler V. E., *J. Nat. Prod.*, **62**, 1589—1592 (1999).
3) Gong F., Liang Y. Z., Xie P. S., Chau F. T., *J. Chromatogr. A*, **1002**, 25—40 (2003).
4) Liang Y. Z., Xie P. S., Chan K., *J. Chromatogr. B*, **812**, 53—70 (2004).
5) Hu P., Liang Q. L., Luo G. A., Zhao Z. Z., Jiang Z. H., *Chem. Pharm. Bull.*, **53**, 677—683 (2005).
6) Chai X. Y., Li S. L., Li P., *J. Chromatogr. A*, **1070**, 43—48 (2005).
7) Yan S. K., Xin W. F., Luo G. A., Wang Y. M., Cheng Y. Y., *Chem. Pharm. Bull.*, **53**, 1392—1395 (2005).
8) Bringmann G., Messer K., Wohlarth M., Kraus J., Dumbuya K., Ruckert M., *Anal. Chem.*, **71**, 2678—2686 (1999).
9) He X. G., *J. Chromatogr. A*, **880**, 203—232 (2000).
10) Gelpi E., *J. Chromatogr. A*, **1000**, 567—581 (2003).
11) Yi T., Leung K. S., Lu G. H., Zhang H., Chan K., *Chem. Pharm. Bull.*, **53**, 1480—1483 (2005).
12) Zhang H. J., Shen P., Cheng Y. Y., *J. Pharmaceut. Biomed.*, **34**, 705—713 (2004).
13) Hu P., Liang Q. L., Luo G. A., Zhao Z. Z., Jiang Z. H., *Chem. Pharm. Bull.*, **53**, 677—683 (2005).
14) Ueng Y. F., Yu H. J., Lee C. H., Peng C., Jan W. C., Ho L. K., Chen C. F., Don M. J., *J. Chromatogr. A*, **1076**, 103—109 (2005).
15) Shinichi T., Tamaki M., Tetsuya A., Li X., Toshiaki M., Seiji I., *Neurosci. Lett.*, **370**, 130—134 (2004).
16) Kim S. H., Kim S. A., Park M. K., Kim S. H., Park Y. D., Na H. J., Kim H. M., Shin M. K., Ahn K. S., *Int. J. Immunopharmacol.*, **4**, 279—287 (2004).
17) Ishida H., Takamatsu M., Tsuji K., Kosuge T., *Chem. Pharm. Bull.*, **35**, 849—852 (1987).
18) Kosato H., Arichi S., Kubo M., Matsuda H., Kimura Y., Kitagawa I., Yoshikawa M., *Wakanyaku Shinpojium*, **14**, 86 (1984).
19) Yoshikawa M., Uchida E., Kawaguchi A., Kitagawa I., Yamahara J., *Chem. Pharm. Bull.*, **40**, 2248—2250 (1992).
20) Kwon O. G., Kim S. H., Chun B. Y., Park C. K., Son K. H., *J. Pharmacogn.*, **30**, 340—344 (1999).
21) Pharmacopoeia of the People's Republic of China, "Pharmacopoeia Commission of P.R. China," 2005 ed., Vol. I, Chemical Industry Press, Beijing, 2000, p. 119.
22) Wang S., Wang F. P., *Acta Pharm. Sin.*, **27**, 117—120 (1992).
23) Lin H. C., Ding H. Y., Wu. Y. C., *J. Nat. Prod.*, **61**, 343—346 (1998).
24) Lin H. C., Ding H. Y., Wu T. S., Wu P. L., Wu T. S., *Phytochemistry*, **41**, 237—242 (1996).
25) Nishizawa M., Yamagishi T., Nonaka G., Nishioka I., Nagasawa T., Oura H., *Chem. Pharm. Bull.*, **31**, 2593—2600 (1983).
26) Ding H. Y., Wu Y. C., Lin H. C., Chan Y. Y., Wu P. L., Wu T. S., *Chem. Pharm. Bull.*, **47**, 652—655 (1999).
27) Hayashi T., Shinbo T., Shimizu M., Arusawa M., Morita N., Kimura M., Matsuda S., Kikuchi T., *Tetrahedron Lett.*, **26**, 3699—3702 (1985).
28) Yu J., Lang H., Xiao P., *Acta Pharm. Sin.*, **21**, 191—197 (1986).