Simultaneous determination of 10 components in traditional Chinese medicine Dachaihu Granule by reversed-phase-high-performance liquid chromatographic-diode array detector

Yingfei Hu, Tulin Lu, Chunqin Mao, Hao Wu, Xing Zhang, JV Wang, Juanjuan Gu

College of Pharmacy, Nanjing University of Chinese Medicine, Nanjing, China

Submitted: 25-01-2012 Revised: 22-02-2012 Accepted: 05-03-2013

ABSTRACT

Background: Dachaihu Granule, commonly used for treating cholecystitis, is derived from a famous traditional Chinese formula named Dachaihu Decoction. No analytical method has been reported for simultaneous determination of 10 bioactive compounds for quality control in Dachaihu Granule so far. **Objective:** To develop a high-performance liquid chromatographic (HPLC) method with diode array detector (DAD) for simultaneous determination of 10 bioactive compounds (paeoniflorin, aloe-emodin, rhein, emodin, chrysophanol, physcion, naringin, hesperidin, neohesperidin, and baicalin) in traditional Chinese medicine Dachaihu Granule. **Materials and Methods**: The samples were separated on a Kromasil C_{18} (250 × 4.6 mm,i.d. with 5.0 μm particle size)column with multi-wavelength detection method by a gradient elution using acetonitrile (A) and 0.2% acetic acid (B) as the mobile phase. The column temperature was maintained at 30°C and the detection wavelength was set at 230 nm for paeoniflorin, 254 nm for aloe-emodin, rhein, emodin, chrysophanol, and physcion, 280 nm for naringin, hesperidin, neohesperidin, and baicalin. **Results:** The developed method provided satisfactory precision and the accuracy of this method was in the range from 94.0% to 103.1%, all of the 10 compounds showed good linearity ($r > 0.999$) in a detected concentration range. **Conclusion:** The validated method was successfully applied to the simultaneously of these active components in Dachaihu Granule from different production batches.

Key words: Dachaihu granule, high-performance liquid chromatographic-diode array detector, multi-components, multi-wavelength, quality control

Access this article online

Website: www.phcog.com

DOI: 10.4103/0973-1296.108136

Quick Response Code:

INTRODUCTION

Dachaihu Granule (DCHG) is derived from a well-known traditional Chinese formula named "Dachaihu decoction", which was founded by ZhongJing Zhang, a famous medical scientist during the Eastern Han Dynasty of China. The formula is a combination of eight medicinal materials, including *Bupleuri Radix, Scutellariae Radix, Rhei Radix et Rhizome, Aurantii Fructus Immaturus, Paeoniae Radix Alba, Pinelliae Rhizoma Praeparatum Cum Zingibere et Alumine, Zingiberis Rhizome Recens*, and *Jujubae Fructus*. Recent study showed that DCHG had a significant effect on curing acute or chronic cholecystitis in clinical practice.[1-2] Pharmacological studies and clinical practice also have demonstrated that DCHG has many biological functions, such as antibacterial, antiulcer, preventing atherosclerosis, and protecting liver and gallbladder.[3-7] Moreover, it is also an effective adjunctive therapy for the cure of pediatric high fever, gastritis, and pancreatitis.[8-10] However, it is not yet clear what the bioactive constituents and mechanisms of DCHG are, although it is known that DCHG has numerous and diverse compounds, including flavone, saponin, terpene, coumarin, anthraquinones, etc.

As we know, paeoniflorin (PA) has a well known effect on treating inflammation.[11] Aloe-emodin (AE), rhein (RH), emodin (EM), chrysophanol (CH), and physcion (PH), which were extracted from Rhei Radix et Rhizome, were proved to be effective components of antiinflammatory, protecting liver and gallbladder.[12-13] Flavone compounds including naringin (NA), hesperidin (HE), neohesperidin (NE), and baicalin (BA)[14-17] also have bioactivity, and

Address for correspondence:
Prof. Tulin Lu, College of Pharmacy, Nanjing University of Chinese Medicine, Nanjing, China. E-mail: lutulin2005@126.com

their chemical structures are shown in Figure 1. Generally, NA, HE, and BA were believed to be the main active constituents and were chosen as marker compounds for the quality evaluation and standardization of DCHG. However, due to multiple compounds that might be associated with the therapeutic functions, a single or a few marker compounds could not be responsible for the overall pharmacological activities of DCHG. Therefore, it is urgently needed to establish a comprehensive quality evaluation method based on analysis of the whole bioactive compounds in order to accurately control the quality of this herbal drug.

Previously, BA from DCHG was analyzed and three marker constituents (NA, HE, and BA) from Dachaihudecocetion were simultaneously analyzed with iso-gradient method for the quality control of the medicine.[18-19] Although HPLC methods have been applied to determine some of the constituents in crude drugs and Chinese patented medicine,[20-22] no analytical method has been reported for simultaneous determination of 10 major constituents in DCHG. Hence, it is very important to establish a method for quality control of these bioactive compounds, which could help to evaluate the quality of the herbal formula. In this study, the method of HPLC-DAD has been developed for the simultaneously qualitative and quantitative analysis of 10 bioactive compounds:PA, AE, RH, EM, CH, PH, NA, HE, NE, and BA.

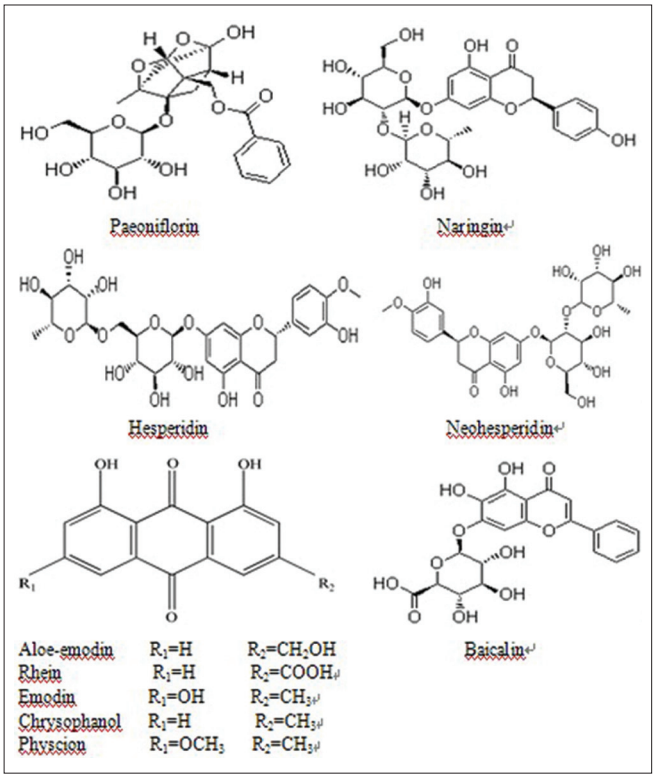

Figure 1: The chemical structures of the investigated components: paeoniflorin (PA), aloe-emodin (AE), rhein (RH), emodin (EM), chrysophanol (CH), physcion (PH), naringin (NA), hesperidin (HE), neohesperidin (NE), and baicalin (BA)

MATERIALS AND METHODS

Chemical and reagents

Sample DCHG was supplied by Nantong Essence Pharmaceutical Co., Ltd. (Jiangsu, China). Standard substances including PA, AE, RH, EM, CH, PH, NA, and BA were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Standard substances including NA and NE were purchased from the Nanjing ZeLang Medicine Photochemistry Technology Co., Ltd. (Jiangsu, China), the purity of these reference compounds were determined to be more than 98% by HPLC analysis. The HPLC grade methanol and acetonitrile were purchased from Tedia (Fairfield, USA). Acetic acid (analytical reagents) was purchased from LingFeng Chemical Reagent Company (Shanghai, China). Other reagents were all of analytical grade. Ultra pure water was obtained in a Milli-Q system (Milford, MA, USA).

Instrument and chromatographic conditions

The HPLC system 1200 series (Agilent Technologies, USA) equipped with the Chem Station software (Agilent Technologies) and comprised of a double solvent delivery pump, an online vacuum degasser, an auto sampler, a thermostatic compartment and a diode array detector, were used for the chromatographic analysis. All separations were carried out on a Kromasil C_{18} column (250 × 4.6 mm i.d. with 5.0 μm particle size) from Hanbang Science and Technology (Jiangsu, China). Mobile phase A was 0.2% (v/v) acetic acid aqueous solution and phase B was acetonitrile. The elution was performed using a linear gradient of 5–19% B at 0–13 min, 19–26% B at 13–28 min, 26% B at 28–36 min, 26–40% B at 36–50 min, 40–85% B at 50–69 min, 85% B at 69–75 min. The flow rate was 1.0 mL/min and column temperature was maintained at 30°C, detect wavelength was set at 230 nm for PA, 254 nm for AE, RH, EM, CH, and PH, and 280 nm for NA, HE, NE, andBA. The injection volume was 10 μL. The peak identification was based on the retention time and the DAD spectrum against the standard presented in the chromatogram.

Standard solution preparation

The standard stock solutions of PA 0.1254, AE 0.0092, RH 0.0212, EM 0.0097, CH 0.0191, PH 0.0101, NA 0.1938, HE 0.1096, NE 0.1287, and BA 0.4525 mg/mL were prepared in methanol and stored away from light at 4°C. Working solutions of the low concentration were prepared by appropriate dilution of the stock solution.

Sample solution preparation

The powder of DCHG (about 1.0 g) was extracted with 25.00 mL methanol for 30 min in an ultrasonic bath for one time.Adding up the loss of weight by methanol after cooling, then, the solution was filtered through a syringe filter (0.45 μm) before being injected into the HPLC system for analysis.

RESULTS AND DISCUSSION

Chromatographic separation

In order to optimize the extraction conditions for achievement of quantitative extraction, variables involved in the procedure such as solvent, extraction method and extraction time were optimized. Pure and aqueous methanol or ethanol solutions also were tried as the extraction solvent, the best solvent was found to be pure methanol which gave rise to optimum extraction of all the 10 components. Compared with refluxing extraction, the ultrasonic treatment procedure was found to be the better extraction method for all the 10 components. In order to investigate extraction time, powdered DCHG samples were extracted with 25 mL pure methanol 20, 30, 45, and 60 min, respectively. The results suggested that all the 10 components were almost completely extracted by pure methanol at one time for 30 min.

Due to the complex composition of the sample solution, different mobile phases (methanol–water, methanol–water–acetic acid, acetonitrile–water, and acetonitrile–water–acetic acid) were attempted to elute the investigated 10 components. In order to enhance resolution and eliminate tailing of the peaks of the target compounds, formic acid and acetic acid were added in the mobile phase. Considering the total resolution of the chromatographic separation, the running time and solvent consumption, the mobile phase acetonitrile–water–acetic acid was chosen for the separation. The typical chromatographic profiles of the blank, standard solution, and the real sample solution were shown in Figure 2. On the basis of the absorption maxima of the 10 compounds in UV spectra acquired by use of the diode array detector, the monitoring wavelength was set at 230 nm for PA, 254 nm for AE, RH, EM, CH, and PH, and 280 nm for NA, HE, NE, and BA, respectively. The detections at three wavelengths were carried out to improve the sensitivity and selectivity for the quantitative analysis.

Linearity, range, and limits of detection

Integrated chromatographic peak areas (Y) were plotted against the corresponding concentrations (X μg/mL) of the 10 constituents in the standard solutions to obtain calibration curves based on linear regression analysis. The regression curves were obtained from six concentration

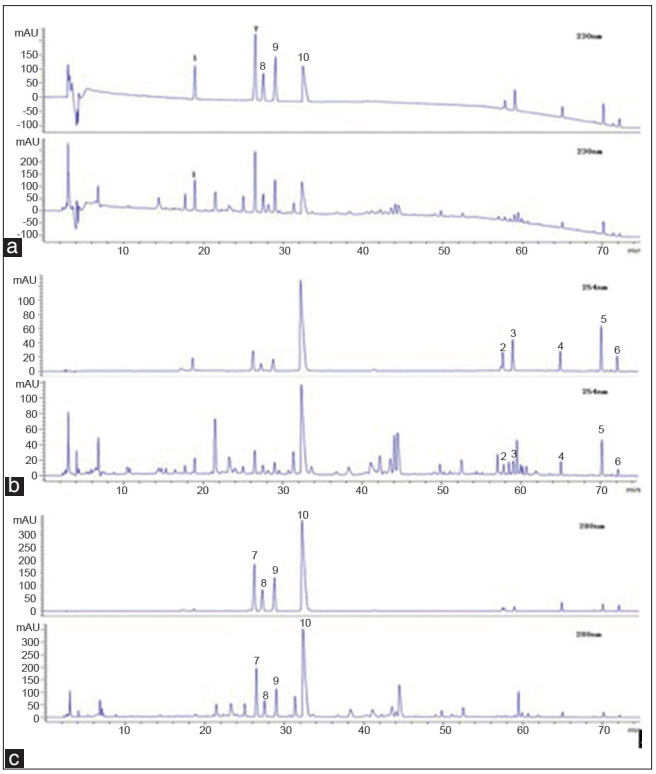

Figure 2: HPLC chromatograms of mixed reference substance and samples at different wavelength;(a) mixed reference substance and sample at 230 nm; (b) mixed reference substance and sample at 254 nm; (c) mixed reference substance and sample at 280 nm; the peak marked was 1-PA, 2-AE, 3-RH, 4-EM, 5-CH, 6-PH 7-NA, 8-HE, 9-NE, 10-BA

levels and all of them had good linearity (r >0.999) in the investigated ranges [Table 1]. The working solutions of the analysis were further diluted with methanol to yield a series of appropriate concentrations. The limits of detection (LOD) and the limits of quantization (LOQ) for each investigated compounds can be seen in Table 1.

Precision, repeatability, and stability

The injection of continuous six times using the same sample was employed for the injection precision, and the injection of six different samples, which were obtained through the same sample preparation procedure, was used for the analysis repeatability. Furthermore, the test of injection precision was determined by the standard solutions, and the test of analysis repeatability was investigated by the real sample solution. The instrument precision was examined by the performance of the intra-day and inter-day assays by six injections of the mixture standard solutions used above. The intra-assay precision was performed with the interval of 2 h in 1 day, and the inter-assay precision was performed over 3 days. The precision result of the solution is presented in Table 2, and it was shown that the RSD(relative standard deviation) values of retention time were lower than 0.25%, while the RSD values of peak area were lower than 2.5% both for the intra-assay and inter-assay precision. Results of

injection repeatability of the solution are shown in Table 3, and the RSD values were lower than 3%. The same real sample was analyzed within 24 h at 30°C for the stability test, and the solution was found to be stable while RSD values of the retention time and peak area were both lower than 2.0%.

Accuracy

Neither a standard method for the determination of these active components nor a standard reference had been established because of the complexity of Chinese medicines. So accuracy of the standard from samples is generally used to evaluate the accuracy of the newly developed analytical method. Three different quantities (low, medium, and high) of the authentic standards were added into the known real sample. The mixtures were extracted as sample preparation described above, and was analyzed using the developed HPLC method mentioned above. Then the quantity of each component was subsequently achieved from the corresponding calibration curves. The recovery of the investigated components ranged from 94.0% to 103.1%, and their RSD values were all less than 3.0% [Table 4]. It was known from the recovery tests that this developed method manifested the reliability and accuracy for the measurement of these components.

Sample analysis

For the simultaneous determination of 10 major components in Chinese medicine DCHG from different production batches, the developed HPLC method was used by comparing the retention time with those of standards. The amounts of the 10 compounds in the samples were then calculated. The results shown in Table 5 indicate that the content of anthraquinones was lower than other components between batches. However, the content of some components such as NA, HE, NE, and BA were higher than other compounds. Therefore, the simultaneous determination of all the components contained in DCHG is necessary to improve the quality control of this drug.

CONCLUSION

RP-HPLC with DAD was found to be effective for the simultaneous detection and determination of 10 major

Table 1: Linear relationships between peak area and concentration

Component	Regression equation[a]	r^2	Liner range (μg/ml)	LOD (μg/ml)	LOQ (μg/ml)
PA	$Y = 7.0667\ X + 17.367$	0.9990	25.1–250.9	0.04	0.12
AE	$Y = 18.617\ X + 2.8467$	0.9990	1.83–18.3	0.01	0.03
RH	$Y = 14.698\ X - 0.9508$	0.9999	4.23–42.3	0.23	0.61
EM	$Y = 15.885\ X - 0.0522$	0.9999	1.93–19.3	0.12	0.37
CH	$Y = 19.943\ X + 2.8487$	0.9999	3.83–38.3	0.24	0.65
PH	$Y = 12.398\ X + 1.7793$	0.9999	2.02–20.2	0.14	0.41
NA	$Y = 8.9243\ X - 1.4227$	0.9990	38.7–387.6	0.02	0.07
HE	$Y = 7.1781\ X + 7.7818$	0.9999	21.9–219.2	0.04	0.13
NE	$Y = 9.4416\ X + 11.054$	0.9999	25.7–257.4	0.03	0.10
BA	$Y = 12.324\ X + 50.139$	0.9999	90.5–905.0	0.01	0.03

[a]*Y* is peak area, *X* is the concentration of the compounds (mg/ml) and r^2 is the correlation coefficient of the equation. LOD: Limit of detection. LOQ: Limit of quantification.
Note: All the abbreviations as seen in Figure 1.

Table 2: Intra-assay and inter-assay precision of the method (*n* = 6)

Components	Intra-assay RSD (%)		Inter-assay RSD (%)	
	Retention time	Peak area	Retention time	Peak area
PA	0.22	0.91	0.23	2.40
AE	0.11	1.04	0.22	2.45
RH	0.03	0.79	0.17	1.56
EM	0.02	0.79	0.23	1.78
CH	0.05	1.46	0.14	1.56
PH	0.04	1.29	0.04	2.09
NA	0.03	0.80	0.06	0.97
HE	0.07	0.89	0.12	2.13
NE	0.05	0.79	0.14	1.45
BA	0.08	1.44	0.11	2.31

Note: All the abbreviations as seen in Figure 1.

Table 3: Repeatability of the method (*n* = 6)

Components	RSD of retention time (%)		RSD of peak area (%)	
	Retention time	Peak area	Retention time	Peak area
PA	0.14	0.91	0.23	2.40
AE	0.09	1.04	0.22	2.45
RH	0.03	0.79	0.17	1.56
EM	0.04	0.79	0.23	1.78
CH	0.07	1.46	0.14	1.56
PH	0.04	1.29	0.04	2.09
NA	0.02	0.80	0.06	0.97
HE	0.12	0.89	0.12	2.13
NE	0.06	0.79	0.14	1.45
BA	0.08	1.44	0.11	2.31

Note: All the abbreviations as seen in Figure 1.

Table 4: Recovery of the 10 compounds (*n* = 3)

Compound	Quantity original/mg	Quantity added/mg	Quantity detected/mg	Recovery[a] (%)	RSD (%)
PA	2.016	1.645	3.661	98.26	2.24
	2.016	2.056	4.072	98.65	
	2.016	2.468	4.484	98.74	
AE	0.102	0.081	0.183	99.04	2.79
	0.102	0.101	0.203	99.25	
	0.102	0.120	0.222	97.00	
RH	0.163	0.132	0.295	99.11	1.97
	0.163	0.165	0.328	99.68	
	0.163	0.198	0.361	95.60	
EM	0.115	0.101	0.216	103.1	2.40
	0.115	0.127	0.242	99.84	
	0.115	0.152	0.267	97.63	
CH	0.284	0.210	0.494	95.34	2.29
	0.284	0.262	0.546	100.5	
	0.285	0.315	0.600	97.58	
PH	0.083	0.066	0.149	95.89	2.61
	0.083	0.082	0.165	100.8	
	0.084	0.099	0.183	96.03	
NA	2.856	2.142	4.998	97.63	1.65
	2.856	2.677	5.533	97.87	
	2.861	3.213	6.074	101.0	
HE	1.430	1.093	2.523	101.0	2.08
	1.430	1.366	2.796	96.61	
	1.433	1.639	3.072	100.3	
NE	1.655	1.250	2.905	97.26	2.10
	1.655	1.563	3.218	98.54	
	1.658	1.875	3.533	94.00	
BA	8.194	6.421	14.615	93.68	2.65
	8.194	8.062	16.256	99.25	
	8.194	9.675	17.869	94.88	

[a]Calculated as [(amount detected- quantity original)/(amount added)] × 100. Data are means from three experiments (*n* = 3). Data are means from three experiments (*n* = 3).
Note: All the abbreviations as seen in Figure 1.

Table 5: Content of 10 Compounds in Samples of Dachaihu granule

No. of batches	Content (mg/g)									
	PA	AE	RH	EM	CH	PH	NA	HE	NE	BA
100103	2.97	0.24	0.43	0.24	0.41	0.13	2.82	4.82	1.77	13.07
101201	4.33	0.19	0.36	0.21	0.51	0.15	6.60	2.61	3.94	10.45
101202	4.21	0.17	0.32	0.22	0.53	0.15	5.86	3.21	3.44	16.27
110801	4.02	0.20	0.32	0.23	0.57	0.17	5.69	2.85	3.40	16.34
110802	4.59	0.20	0.39	0.22	0.53	0.15	6.98	2.77	4.17	11.72
110803	3.77	0.16	0.27	0.19	0.47	0.13	5.49	2.97	3.22	16.63
Average	3.98	0.19	0.35	0.22	0.50	0.15	5.57	3.20	3.32	14.08
RSD (%)	14.29	13.84	16.69	7.76	10.58	8.38	26.24	25.42	25.32	19.11

Note: All the abbreviations as seen in Figure 1.

bioactive constituents (PA, AE, RH, EM, CH, PH, NA, HE, NE, and BA) in Chinese traditional medicine DCHG. This is also the first report of an accurate and reliable analytical method for the simultaneous determination. High linearity, repeatability, precision, accuracy and reliability were presented in the method validation procedure. The proposed method is promising to improve the quality control of DCHG.

REFERENCES

1. Zhang ZZ, Li M. Clinical observation of chronic cholecystitis treated in old man with Dachaihu Granule. J Inter Dige Dis 2011;

31:183-4.
2. Sun JF. Clinical observation of acute cholecystitis treated with Dachaihu decoction. J Sichuan Chin Med 2005;23:53.
3. Cai CX, Chang YZ, Yao HL. Antibacterial effect of Dachaihu Granulation. J Huazhong Univ Sci Tech [Health Sci] 2004;33: 619-21.
4. Zhou YY, Zhou AF, Cai LF. Protection of Dachaihu decoction against gastric stress ulcer. Chin Arch Tradi Chin Med 2006;24:1056-8.
5. Miao HL, Lin MS, Zhang LJ. Influence and mechanism of modified major decoction of bupleurum on bile acid metabolism in rat with obstructive jaundice. Chin J Exp Surg 2006;23:934.
6. Dong XN, He QF, Hu XM. 24 cases of senile obstructive pyogenic cholangitis treated with major Bubleurum decoction and ChenHao decoction. J Zhejiang Tradi Chin Med Coll 2006;30:26.
7. Wang FR, Zheng T, Zheng X. The protective effect of Dachaihu decoction on atherosclerosis induced by fatty foodstuff in rabbit. Chin J Inter Med Cardio Dis 2007;5:36.
8. Sun EH, Sun CX. Study of Dachaihu decoction in the treatment of bile reflux gastritis. J Heilongjiang Med 2007;5:30.
9. Wang WL, Zhang XL, Zhuang XH. Clinical experience in the treatment of pediatric fever of Dachaihu decoction. Chin J Inter Tradi Chin and West Med Fir Aid 2005;12:299.
10. Li JP, Liang GS, Cai YX. Adjuvant treatment of 26 acute pancreatitis patients of Dachaihu decoction. J Zhejiang Chin Med Coll 2007;42:641-2.
11. Wang R, Lu L, Li YW. A comparison on pharmacological actions between Radix PaeoniaeRubra and Radix Paeoniae Alba. Chin J Exp Tradit Med Form 2010;16:112-4.
12. Zhuang JN. The main components of rhubarb and clinical pharmacology study progress. Chin J Mili Surg in Southwest 2009;11:931-3.
13. Zhang SS. Research status of the pharmacological effects of emodin. Chin Med Hera 2006;3:12-4.
14. Chen HF, Zhang WG, Yang WL. Research progress of flavonoids ingredients in dielectric lei citrus isatis. Lishizhen Med Mater Med Res 2008;19:2863-5.
15. Ceng CZ, Liu ZX, Han L. Baicalin extraction technology and its total antibacterial activity. Lishizhen Med Mater Med Res 2009;20:1342-3.
16. Yang L, Cui XY, Zhang H. Antiinflammatory and immunomodulation effects of the extract of Scutellariabaicalensis Georgi. Chin Pharm 2007;18:1856-7.
17. Wu R, Feng LS, Wang JD. Stay on the inhibition effect of baicalin and baical in metallic coordination. Sci Technol Rev 2006;24:212.
18. Liu B, Yang XD, Shi XD. Determination of baicalin in Dachaihu Granule by HPLC. Chin J Hubei Tradi Med 2007;29:51-2.
19. Xu LN, Han X, Chou XH, Yin LH, Wang XN, Peng JY. RP-HPLC Simultaneous determination of three flavonoid glycosides in Dachaihu decoction. Chin J Pharm Anal 2008;28:1686-8.
20. Ouyang EH, Zhang CG, Li XM. Simultaneous determination of geniposide, chlorogenic acid, crocin1, and rutin in crude and processed fructusgardeniae extracts by high performance liquid chromatography. Pharmacogn Mag 2011;7:267-70.
21. Tan XJ, Li Q, Chen XH, Wang ZW, Shi ZY, Bi K, *et al.* Simultaneous determination of 13 bioactive compounds in HerbaArtemisiaeScopariae (YinChen) from different harvest seasons by HPLC-DAD. J Pharm Biomed Anal 2008;47:847-53.
22. Qian ZM, Li HJ, Li P, Ren MT, Tang D. Simultaneous Qualitation and Quantification of Thirteen Bioactive Compounds in FlosLonicerae by High-Performance Liquid Chromatography with Diode Array Detector and Mass Spectrometry. Chem Pharm Bull (Tokyo) 2007;55:1073-6.

Cite this article as: Hu Y, Lu T, Mao C, Wu H, Zhang X, Wang JV, *et al.* Simultaneous determination of 10 components in traditional Chinese medicine Dachaihu Granule by reversed-phase-high-performance liquid chromatographic-diode array detector. Phcog Mag 2013;9:33-8.

Source of support: The Transformation of Scientific and Technological Achievements Special Funds of Jiangsu Province of China (No. BA2010094). **Conflicting Interest:** None declared.