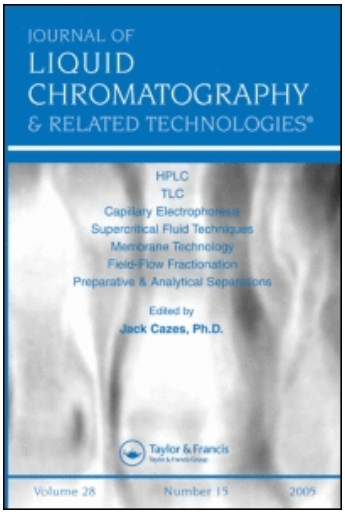

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Seung Hyun Kim[a]; Eun Jung Choi[a]; Ki Yong Lee[b]; Sang Hyun Sung[b]; Heung-Mook Shin[c]
[a] Institute for Life Science, Elcom Science Co. Ltd., Seoul, Korea [b] College of Pharmacy and Research Institute of Pharmaceutical Science, Seoul National University, Seoul, Korea [c] Department of Physiology, College of Oriental Medicine, Dongguk University, Kyongju, Korea

To cite this Article Kim, Seung Hyun , Choi, Eun Jung , Lee, Ki Yong , Sung, Sang Hyun and Shin, Heung-Mook(2008) 'Simultaneous Determination of Alkaloids and Flavonoids in HMC05 Preparation by HPLC-DAD', Journal of Liquid Chromatography & Related Technologies, 31: 19, 2917 — 2926
To link to this Article: DOI: 10.1080/10826070802424501
URL: http://dx.doi.org/10.1080/10826070802424501

Journal of Liquid Chromatography & Related Technologies®, 31: 2917–2926, 2008

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070802424501

Simultaneous Determination of Alkaloids and Flavonoids in HMC05 Preparation by HPLC-DAD

Seung Hyun Kim,[1] Eun Jung Choi,[1] Ki Yong Lee,[2] Sang Hyun Sung,[2] and Heung-Mook Shin[3]

[1]Institute for Life Science, Elcom Science Co. Ltd., Seoul, Korea
[2]College of Pharmacy and Research Institute of Pharmaceutical Science, Seoul National University, Seoul, Korea
[3]Department of Physiology, College of Oriental Medicine, Dongguk University, Kyongju, Korea

Abstract: High performance liquid chromatography with diode array detector (HPLC-DAD) was developed for simultaneous determination of hesperidin, coptisine, palmatine, and berberine in HMC05, a standardized extract of eight different herbs. This method was validated in terms of specificity, linearity ($r^2 > 0.9995$), precision (<5.0% RSD), and recoveries (96.6–110.5%). The LOD of these compounds were ranged from 101.6 to 171.7 ng. In addition, these four compounds in HMC05 were identified or tentatively characterized using HPLC-DAD-electrospray mass spectrometry (HPLC-DAD-ESI-MS).

Keywords: HMC05, HPLC-DAD, Simultaneous determination, Validation

INTRODUCTION

Long practiced outside of conventional medicine, herbal medicines are coming to the attention of many researchers as up to date analysis show their values in the treatment and prevention of diseases.[1] In Traditional Oriental Medicine, there are various composite formulae containing

Correspondence: Sang Hyun Sung, College of Pharmacy and Research Institute of Pharmaceutical Science, Seoul National University, San 56-1, Sillim-Dong, Gwanak-Gu, Seoul 151-742, Korea. E-mail: shsung@snu.ac.kr

several ingredients, based on the notion that multiple constituents are responsible for the pharmacological and biological effects of the herbal medicines. However, it brings definite issues regarding the quality control of herbal medicines that must be addressed to achieve reproducible phytoequivalence without undesirable effects. Recently, the chromatographic fingerprint technique has been internationally accepted as an efficient tool for the integral quality control of herbal medicines.[2] On the other hand, the quality of herbal medicines is highly related to their major constituents in many cases, and thus quantitative analysis of these components is also necessary.

HMC05 is a standardized water extract of eight herbs, *Pinellia ternate*, *Atractylodes macrocephala*, *Gastrodia elata*, *Citrus unshiu*, *Poria cocos*, *Crataegus pinnatifida*, *Siegesbeckia pubescens*, and *Coptidis japonica*. It has been reported that HMC05 has anti-atherosclerotic effects attributable to its anti-inflammatory activity.[3] HMC05 is originated and modified from a traditional herbal medicine, Banhabackchulchunmatang (BCT). BCT has been used for thousands of years as a therapeutic formula in China, Japan, and Korea and is still widely used. BCT itself has been known to be effective against headache, dizziness, and hypertension, and is well tolerated with few side effects.[4] Different methods have been developed for the determination of the active components in the individual crude herb.[5–7] The chemical profiles of the combined herbal extracts are generally different from the mixture of the individual herbal extracts, which causes new bioactivities for new clinical effects. Hence, development of practical and reliable methods for the identification and quantification of multiple constituents in the herbal mixture is required to evaluate the quality of HMC05.

In the present study, the main constituents of HMC05 were screened and identified by the HPLC-ESI-MS technique. In addition, a HPLC-DAD method was developed and validated for simultaneous quantification of one flavonoid and three alkaloids in HMC05.

EXPERIMENTAL

Reagents and Chemicals

The reference compounds hesperidin, coptisine, palmatine, and berberine were purchased from Wako (Osaka, Japan) and Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) (Figure 1). The purity was determined by HPLC-UV with two wavelengths (250 and 280 nm). All the solvents used in this experiment were HPLC grade. Acetonitrile and water were purchased from Mallinckrodt (USA), and methanol from Fischer (USA). Acetic acid (analytical grade) was purchased from Merck (Darmstadt, Germany). Triple deionized water (Millipore, Bedford,

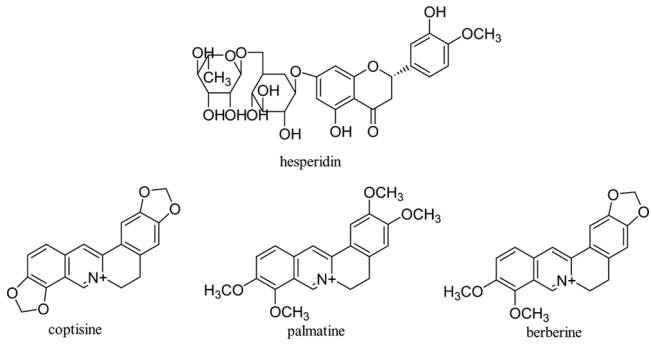

Figure 1. Chemical structures of hesperidin, coptisine, palmatine, and berberine.

MA, USA) was used for all preparations. Phosphoric acid, boric acid, and sodium heptyl sulfate were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

HMC05 Preparation

All herbs used were purchased from Kyungdong traditional herbal market (Seoul, Korea). The samples were authenticated by Dr. Jong Hee Park, a professor of College of Pharmacy, Pusan National University. Voucher specimens have been deposited in Herbarium of the Medicinal Plant Garden, College of Pharmacy, Seoul National University. The herbs had a moisture content of <10% by weight, and were dried. The composition of the mixture was as follows: *Pinelliae Rhizoma* (9 g), *Atractylodis Macrocephalae Rhizoma* (12 g), *Gastrodiae Rhizoma* (6 g), *Citri Pericarpium* (6 g), *Hoelen* (9 g), *Cragaegi Fructus* (9 g), *Siegesbeckiae herba* (9 g), and *Coptidis Rhizoma* (9 g). The herbs (69 g) were mixed, minced with a grinder (Rong Tong Iron Works, Taichung, Taiwan), and extracted with 500 mL of distilled water under reflux for 3 h by boiling the formula. The extract was filtered through a 10 μm cartridge paper. The filtrate was concentrated to about 50 mL with a rotary evaporator at 50°C under vacuum and freeze dried.[3] The freeze dried extract was weighed and dissolved by 50% methanol. This sample solution was filtered through an 0.45 mm membrane filter (Millipore, Nylon, 170 μm) and analyzed with HPLC.

Instrumentation

The HPLC system consisted of a chromatographic pump (P680, Dionex, Germany), and injector (7725i, Rheodyne, USA) equipped with a photodiode array (UVD 340U, Dionex, Germany). The output signal of the

detector was recorded using a Dionex Chromelon^{TM} Chromatography Data System. Chromatographic separation was achieved on a Gemini RP18 (5 μm, 4.6 mm I.D. × 150 mm).

The HPLC-ESI-MS system consisted of Finnigan Surveyor HPLC system with a pump, an autosampler, a PDA plus detector, and Finnigan LCQ advantage MAX with Xcalibur software.

Chromatographic Conditions

The HPLC-DAD chromatographic separation was carried out using a linear gradient elution of A (acetonitrile) and B (1350 μL of phosphoric acid, 1150 μL of acetic acid, 1.236 g of boric acid in 1000 mL of 5 mM sodium heptyl sulfate in water) at a flow rate of 1.0 mL/min (0 min, 10% A; 10 min, 35% A; 20 min 55% A; 30 min, 10% A; v/v). The diode-array UV/Vis detector (DAD) was used for the detection and the wavelength for quantification was set at 280 nm.

Separation in HPLC/ESI/MS was achieved at 25°C on a Waters XTerra^{TM} RP18 (5 μm, 4.6 mm I.D. × 150 mm). A linear gradient elution of A (0.03% formic acid) and B (100% acetonitrile) was used (0 min, 10% B; 30 min, 40% B; 55 min, 55% B; 56 min, 10% B; 60 min, 10% B; v/v) at a flow rate of 0.3 mL/min. Ion polarity was positive.

Preparation of Standard Solution

Stock standard solutions of hesperidin, coptisine, palmatine, and berberine were prepared in methanol at a concentration of 1 mg/mL, respectively. The appropriate amount of every standard solution was mixed and diluted with methanol as indicated.

RESULTS AND DISCUSSION

Optimization of HPLC-DAD Conditions

The chromatographic conditions were optimized to obtain chromatograms with a good resolution of adjacent peaks. Reversed phase columns have been usefully applied to analyze the components of natural resources. The preferred chromatographic condition was obtained using Gemini RP18 (5 μm, 4.6 mm I.D. × 150 mm) column. Various mixtures of water and acetonitrile in combination with several acids including phosphoric acid, acetic acid, and boric acid were tested as a mobile phase to enhance the resolution and eliminate the peak tailing of the target

compounds.[8] Since *C. japonica*, one of the ingredients of HMC05, is known to contain protoberberine alkaloids such as berberine, palmatine, coptisine, and jatrorrhizine, tris(hydroxymethyl)aminomethane, triethylamine and sodium heptyl sulfate were also tested as the ion pairing

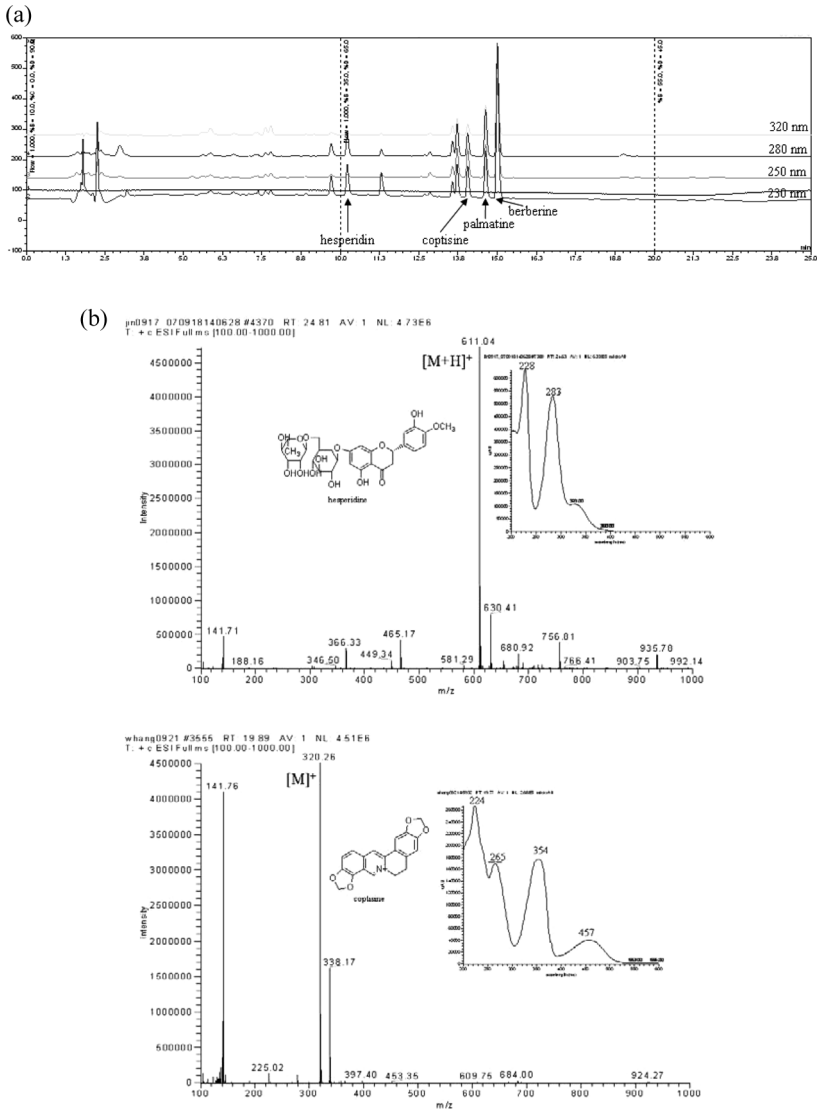

Figure 2. HPLC chromatogram of HMC05 at four different wavelengths (a) and HPLC-DAD-ESI-MS spectrum of hesperidin, coptisine, palmatine, and berberine in HMC05 (b).

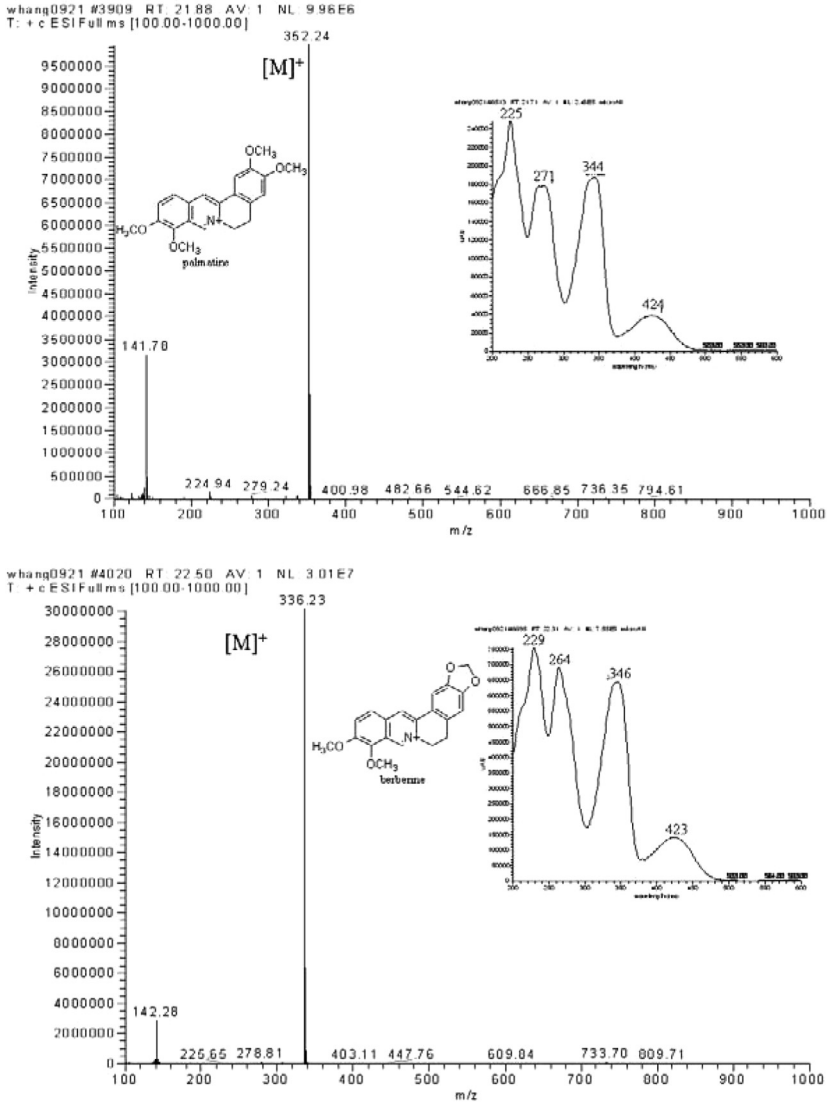

Figure 2b. Continued

agents.[9] Among them, only sodium heptyl sulfate effectively improved the peak separation. As a result, gradient elution of the mobile phases consisting of acetonitrile and a buffer solution (sodium heptyl sulfate, phosphoric acid, acetic acid, and boric acid) were chosen for the HPLC conditions. The wavelength for detection was tested at 230, 250, 280,

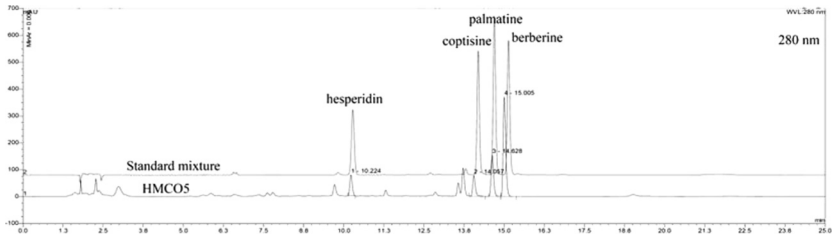

Figure 3. HPLC chromatogram of HMC05 and standard mixtures.

and 320 nm and set at 280 nm, where the four major peaks showed the maximum intensity as measured by a DAD detector as shown in Figure 2. The presence of hesperidin, coptisine, palmatine, and berberine was verified by comparing each retention time (10.2 min, 14.1 min, 14.6 min, 15.0 min) and UV spectrum with those of each standard compound and spiking with authentic standards (Figure 3).

HPLC/ESI/MS Identity Confirmation

A HPLC/ESI/MS experiment was also performed to confirm the identity of four marker constituents. A different solvent condition was used for this analysis to reduce the flow rate for the MS detector. The molecular weight of each peak was obtained in positive mode. Figure 2 shows the positive ion ESI-MS spectra for the compounds detected in selected ion monitoring (SIM) mode. Hesperidin showed a molecular ion $[M+H]^+$ at m/z 611.0. Coptisine, palmatine, and berberine showed their molecular ions $[M]^+$ at 320.3, 352.2, and 336.2, respectively.[10] Then using UV absorption, HPLC retention time, and standard chromatogram, the structures of the four marker compounds were verified.

Validation of the HPLC-DAD Methods

Specificity was determined by the calculation of peak purity facilitated by DAD. The peak purity was evaluated using DAD and its corresponding computer software, which confirms the singularity of the peak component. The absorption spectrum of a single component remained invariable at each time point in one peak, which supported specificity of each peak.

Calibration curves were linear in a relatively wide range of concentrations (12.5–250.0 μg/mL for hesperidin, coptisine, palmatine, and berberine) and all showed good linear regressions ($y = 19.4073x + 1.4064$ for

Table 1. Limit of detection (LOD), limit of quantification (LOQ) and characteristic parameters of calibration curves

Compound	Retention time (min)	Regression equation[a]	Correlation coefficient (r^2)	LOD (ng)	LOQ (ng)
Hesperidin	10.2	$y = 19.4073x + 1.4064$	0.9997	160.7	487.0
Coptisine	14.1	$y = 35.2073x + 1.3059$	0.9999	171.7	520.4
Palmatine	14.6	$y = 39.9347x + 4.1876$	0.9996	101.6	307.9
Berberine	15.0	$y = 37.3514x + 3.1636$	0.9999	168.1	509.5

[a]y = peak area, x = amount (μg).

hesperidin, $y = 35.2073x + 1.3059$ for coptisine, $y = 39.9347x + 4.1876$ for palmatine, and $y = 37.3514x + 3.1636$ for berberine) with high correlation coefficient values ($r^2 > 0.9995$) between peak area (y) and amounts of each compound (x, μg) (Table 1).

The limit of detection (LOD) was measured based on the method recommended by ICH ($LOD = 3.3\ \delta/S$, δ = standard deviation of the response, S = slope of the calibration curve). The LOD of hesperidin, coptisine, palmatine, and berberine were 160.7, 171.7, 101.6, and 168.1 ng, respectively, which showed a high sensitivity at this chromatographic condition (Table 2).

Table 2. Analytical results of precision and accuracy

	Precision				Accuracy		
	Inter-day		Intra-day				
Compound	Amount (μg)	RSD[a] (%)	Amount (μg)	RSD[a] (%)	Spiked amount (μg)	Accuracy (%)	RSD[a] (%)
Hesperidin	5.0	1.16	5.0	0.72	2.54	110.5	2.33
	2.5	3.64	1.0	0.64	2.55	103.6	1.82
	1.0	4.10	0.25	0.44	2.57	103.2	3.04
Coptisine	5.0	0.57	5.0	0.89	2.02	107.4	2.48
	2.5	2.12	1.0	0.70	1.77	104.4	1.12
	1.0	3.32	0.25	4.94	1.53	104.3	2.52
Palmatine	5.0	0.49	5.0	0.60	2.19	107.9	1.38
	2.5	1.88	1.0	2.44	2.03	105.8	1.74
	1.0	2.45	0.25	3.42	1.87	106.2	2.27
Berberine	5.0	0.34	5.0	0.07	3.18	106.3	2.59
	2.5	1.18	1.0	1.24	3.52	98.5	1.27
	1.0	2.37	0.25	4.35	3.86	96.6	1.59

[a]RSD (%) = (SD of amount detected/mean of amount detected) × 100 (n = 3).

The precision test was carried out by the intra-day and inter-day variability for hesperidin, coptisine, palmatine, and berberine. The intra-day variability was assayed at three concentrations on the same day and inter-day variability at three concentrations on three sequential days (1, 3, 5 days). As listed in Table 2, the RSD of intra-day and inter-day variability was less than 5.0%, which demonstrated good precision of this method.

The accuracy of the method set up in this study was determined by the method of standard addition. The dilute sample solution (30.0 mg/mL) was spiked with the mixture standard samples (0.125 mg/mL for each standard, respectively) at the ratio of 2:1, 1:1, and 1:2, respectively. The resultant samples were analyzed by using the proposed method. For comparison, an unspiked sample was concurrently prepared and analyzed simultaneously. The mean recovery of each compound was 96.6–110.5% (Table 2).

CONCLUSIONS

In this paper, a rapid and reliable HPLC method for simultaneous determination of four active constituents of HMC05, hesperidin, coptisine, palmatine, and berberine, has been developed and validated. The method fulfilled all the requirements to be identified as a reliable and feasible method, showing good specificity, precision, linearity, and accuracy data. Therefore, this established method is useful for the quality control of HMC05 preparations by simultaneous quantitative analysis of these constituents.

ACKNOWLEDGMENTS

This study was supported by grants of the Korea Health 21 R & D Project, Ministry of Health & Welfare, Republic of Korea (B050042A M081505N100020B).

REFERENCES

1. Balunas, M.J.; Kinghorn, A.D. Drug discovery from medicinal plants. Life Sci. **2005**, *78*, 431–441.
2. Liang, Y.Z.; Xie, P.; Chan, K. Quality control of herbal medicines. J. Chromatogr. B **2004**, *812*, 53–70.
3. Kim, K.M.; Choi, J.Y.; Yoo, S.E.; Park, M.Y.; Lee, B.S.; Ko, Y.H.; Sung, S.H.; Shin, H.M.; Park, J.E. HMC05, herbal extract, inhibits NF-κB

expression in lipopolysaccharide treated macrophages and reduces atherosclerotic lesions in cholesterol fed mice. J. Ethnopharmacol. **2007**, *114*, 316–324.
4. Shin, H.M.; Morgan, K.G. Vasodilation by banhabackchulchunmatang, a Chinese medicine, is associated with negative modulation of PKCalpa activation and NO production. Life Sci. **2003**, *74*, 723–732.
5. Chen, P.; Li, C.; Liang, S.; Song, G.; Sun, Y.; Shi, Y.; Xu, S.; Zjang, J.; Sheng, S.; Yang, Y.; Li, M. Characterization and quantification of eight water-soluble constituents in tubers of *Pinellia ternate* and in tea granules from the Chinese multi-herb remedy Xiaochaihu-tang. J. Chromatogr. B **2006**, *843*, 183–193.
6. Zhao, Y.; Cao, Q.; Xiang, Y.; Hu, Z. Identification and determination of active components in *Gastrodia elata* Bl. by capillary electrolysis. J. Chromatogr. A **1999**, *849*, 277–283.
7. Nogata, Y.; Ohta, H.; Yoza, K.I.; Berhow, M.; Hasegawa, S. High-performance liquid chromatographic determination of naturally occurring flavonoid in *Citus* with a photodiode array dectector. J. Chromatogr. A **1994**, *667*, 59–66.
8. Cui, H.; He, C.; Zhao, G.W. Determination of polyphenols by high-performance liquid chromatography with inhibited chemiluminescence detection. J. Chromatogr. A **1999**, *855*, 171–179.
9. Su, X.Z.; Kong, L.; Li, X.; Chen, X.; Guo, M.; Zou, H. Screening and analysis of bioactive compounds with biofingerprinting chromatogram analysis of traditional Chinese medicines targeting DNA by microdialysis/HPLC. J. Chromatogr. A **2005**, *1076*, 118–126.
10. Yu, S.; Pang, X.; Deng, Y.; Li, L.; Liang, Y.; Liu, X.; Xie, L.; Wang, G.; Wang, X. A sensitive and specific liquid chromatography mass spectrometry method for simultaneous determination of berberine, palmatine, coptisine, epiberberine and jatrorrhizine from Coptidis Rhizoma in rat plasma. Int. J. Mass Spectrom. **2007**, *268*, 30–37.

Received April 12, 2008
Accepted May 2, 2008
Manuscript 6333