Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 1185–1190
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

HPLC determination of four triterpenoids in rat urine after oral administration of total triterpenoids from *Ganoderma lucidum*

Xiao-Ming Wang [a,b], Shu-Hong Guan [a], Rong-Xia Liu [a], Jiang-Hao Sun [a], Yan Liang [a], Min Yang [a], Wei Wang [a], Kai-Shun Bi [b], De-An Guo [a,*]

[a] *Shanghai Research Center for TCM Modernization, Shanghai Institute of Materia Medica, Shanghai Institute for Biological Sciences, Guo Shoujing Road 199, Zhangjiang, Shanghai 201203, PR China*
[b] *Shenyang Pharmaceutical University, Wenhua Road 103, Shenyang 110016, Liaoning, PR China*

Received 30 June 2006; received in revised form 1 October 2006; accepted 4 October 2006
Available online 20 November 2006

Abstract

A sensitive and simple high-performance liquid chromatography (HPLC) method was applied for the quantitative determination of four major triterpenoids (ganoderic acids C_2, B, K and H) in rat urine after oral administration of total triterpenoids from *Ganoderma lucidum*. The urine sample was extracted with dichloromethane–ethyl acetate (90:10) after acidification by hydrochloric acid (0.2 mol/ml). Chromatographic separation was achieved on a Zorbax SB-C_{18} column (250 mm × 4.6 mm, 5 μm) at 35 °C, with a linear gradient of acetonitrile and 0.03% aqueous phosphoric acid (v/v), at a flow rate of 1.2 ml/min. The four triterpenoids and internal standard (hydrocortisone) were detected at a wavelength 252 nm. The within- and between-day assay coefficients of variation for the four triterpenoids in urine were less than 9% and the extraction recovery of this method was higher than 90%. Using this method, the excretion profile of the triterpenoids in rat urine after oral administration of total triterpenoids of *G. lucidum* was revealed for the first time.

Keywords: *Ganoderma lucidum*; Total triterpenoids; Rat urine; HPLC

1. Introduction

Ganoderma lucidum (Leyss. ex Fr.) Karst, popular medicinal mushroom, has been used as a home remedy in traditional Chinese medicine (TCM) in many Asian countries during the past two millennia [1]. The fruit bodies, spores and cultivated mycelia of *G. lucidum* as well as its extracts have been used to treat a variety of diseases such as hepatitis, neurasthenia, deficiency fatigue and cancer [2–6]. The Shanghai Green Valley Pharmaceutical Co. in China cultivated *G. lucidum* in GAP conditions and developed an anticancer complex prescription comprised with fruit body extract and sporoderm-broken spores of *G. lucidum*, namely SunRecome®. The results of pharmacological research of SunRecome® showed that it not only has the anticancer activity but also has the benefit of reducing chemotherapy-induced side effects [7–10].

A number of bioactive components have been identified from *G. lucidum*, in which the polysaccharide and triterpenoids are the two major bioactive ingredients. The previous studies have reported that the triterpenoids possessed the bioactivities of antioxidation, hepatoprotection, suppresses angiogenesis and anticancer, etc. [11–14]. The potential medicinal values of triterpenoids have attracted intense interest in the search for pharmacological mechanisms. The pharmacokinetic study could help us to understand the biotransformation of triterpenoids in vivo and to predict their toxicities. But the major obstacle for the research of herbal products is their complexity. However, the traditional Chinese medicinal formula is frequently prescribed in combination to obtain the synergistic effects or to diminish the possible adverse reactions [15,16]. Thus, the purpose of the present study was to simultaneously determine the major four triterpenoids (ganoderic acids C_2, B, K and H) in rat urine and to obtain the excretion parameters of these four triterpenoids after oral administration of total ganoderma triterpenoids to rats. In regard to the analytical method, the current study method was mainly based on our previous research on the

* Corresponding author. Tel.: +86 21 5027 1516; fax: +86 21 5027 2789.
E-mail address: gda5958@163.com (D.-A. Guo).

0731-7085/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2006.10.010

determination of triterpenoids in *G. lucidum* by RP-HPLC [17].

2. Experimental

2.1. Chemicals, reagents and animals

The reference substances of triterpenoids **1**–**4** (structure shown in Fig. 1), namely ganoderic acids C_2, B, K and H, were isolated by the author from the fruit body of *G. lucidum*, and their structures were fully identified by direct comparison of their spectral data (UV, IR, NMR and MS) with those reported in literatures [18–21]. The purity analysis suggested their purity to be above 98%. Hydrocortisone (internal standard, I.S.) was purchased from National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile and phosphoric acid were of HPLC grade (Burdick & Jackson, Honeywell International Inc., USA). HPLC grade water was prepared using a Milli-Q Water purification system (Millipore, MA, USA). Dichloromethane, ethyl acetate and hydrochloric acid were analytical reagent grade (Sinopharm Chemical Reagent Co., Ltd., China). The total triterpenoids were extracted from the fruit body of *G. lucidum* that was provided by Shanghai Green Valley Pharmaceutical Co., China. To calculate the administered doses of ganoderic acids C_2, B, K and H, their contents in total triterpenoids were quantitatively analyzed by HPLC/DAD, which were 46.1, 215.0, 63.2 and 129.1 mg/g, respectively. Male Sprague–Dawley rats weighting 200–220 g were obtained from the Shanghai SLAC Laboratory Animal Co., Ltd., China, and kept in an normally controlled breeding room (temperature: $20 \pm 2\,^{\circ}C$; humidity: $60 \pm 5\%$; 12 h dark:12 h light cycle) with standard laboratory food and water for 1 week prior to the experiments.

2.2. Chromatographic system and conditions

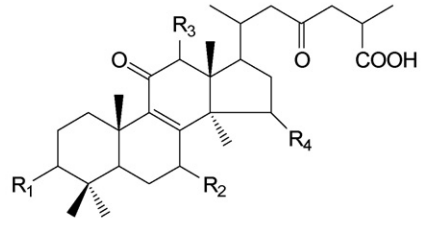
Chromatography was performed using an Agilent 1100 Series HPLC system that comprises a quaternary solvent delivery system, autosampler, degasser and DAD detector. Separation was carried out on a Zorbax SB-C_{18} column (250 mm × 4.6 mm, 5 μm) connected with a Zorbax SB-C_{18} guard column (20 mm × 4 mm, 5 μm) maintained at 35 °C. The mobile phase consisted of (A) acetonitrile and (B) 0.03% aqueous phosphoric acid (v/v) using a gradient elution of 28–33% A at 0–10 min and 33–35% A at 10–40 min. The flow rate was 1.2 ml/min and detection wavelength was set at 252 nm.

2.3. Preparation of standard solutions

The mixture stock solution containing 460 μg/ml for ganoderic acids C_2, 788 μg/ml for ganoderic acid B, 492 μg/ml for ganoderic acid K and 318 μg/ml for ganoderic acid H was prepared by dissolving the reference substances in 50% aqueous acetonitrile. And then the stock solution was subsequently diluted in the same diluent to obtain working standard solution in the range of 460–11.5 μg/ml for ganoderic acid C_2, 788–19.7 μg/ml for ganoderic acid B, 492–12.3 μg/ml for ganoderic acid K and 318–7.95 μg/ml for ganoderic acid H, respectively. The internal standard solution was prepared by dissolving hydrocortisone in same diluent at a concentration of 74.4 μg/ml. All the stock solutions were stored at 4 °C.

2.4. Preparation of calibration standards and quality control (QC) samples

Working standard solution of triterpenoids (20 μl) was added to blank urine (1.5 ml) to yield spiked calibration standards at seven different concentrations ranging from 6.133 to 0.154 μg/ml for ganoderic acid C_2, from 10.507 to 0.263 μg/ml for ganoderic acid B, from 6.560 to 0.164 μg/ml for ganoderic acid K and from 4.240 to 0.106 μg/ml for ganoderic acid H, respectively. Quality control (QC) samples were prepared using the same procedure at high, middle, low and LLOQ concentration levels for the four triterpenoids. These QC samples were stored at −70 °C until analyzed.

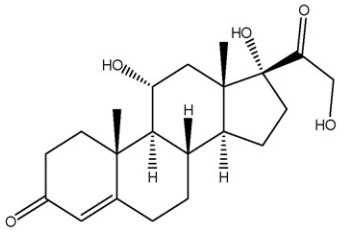


Ganoderic acid C_2 (**1**): R_1=R_2=β-OH, R_3=H, R_4=α-OH

Ganoderic acid B (**2**): R_1=R_2=β-OH, R_3=H, R_4=O

Ganoderic acid K (**3**): R_1=R_2=β-OH, R_3= β-OAC, R_4=O

Ganoderic acid H (**4**): R_1=β-OH, R_2=R_4=O, R_3= β-OAC



Hydrocortisone (I.S.)

Fig. 1. Structures of four triterpenoids in *G. lucidum* and internal standard.

2.5. Sample preparation

Twenty microlitres of I.S. solution and 50 μl of hydrochloric acid (0.2 mol/l) were added into a tube containing calibration standards, QC samples or unknown urine samples. Then, the acidified samples were extracted with 3 ml dichloromethane–ethyl acetate (90:10) by vortexing for about 1 min and centrifuged at 4000 rpm for 5 min at 5 °C, this extraction procedure was repeated twice. The organic layer was combined and transferred into a glass evaporation tube, and then it was dried at 35 °C under a stream of nitrogen. The dry residue was reconstituted in 100 μl of 50% aqueous acetonitrile solution and filtrated through a 0.45 μm membrane filter unit. Then, 20 μl of each sample solution was analyzed by HPLC.

2.6. Method validation

The method was validated for parameters such as linearity, precision, accuracy, extraction recovery and stability following the U.S. Food and Drug Administration guideline [22]. Six different blank urine samples were processed according to Section 2.5 and injected into the HPLC system to investigate whether the endogenous urine components interfere with analytes and internal standard. Four calibration curves were constructed according to Section 2.4 and analyzed on 4 consecutive days. The internal ratios (analyte peak area/I.S. peak area) were calculated for each point, and calibration curves were fit using a weighted least squares linear regression analysis (weighted by $1/y^2$) of internal ratio versus concentrations.

The accuracy and precision were evaluated using the QC samples with high, middle, low and LLOQ concentration levels. Six replicate QC samples at each concentration were analyzed in a sequence to evaluate within-day variation. For evaluation, the between-day variation six replicate QC samples at each concentration were analyzed on 4 different days. The accuracy was expressed by [(mean observed concentration)/(spiked concentration)] × 100% and the precision by relative standard deviation (R.S.D.). The extraction recoveries of four triterpenoids and I.S. were evaluated by comparing the mean peak area of QC samples (high, middle and low) to the mean peak area of pure standards of equivalent concentrations.

To investigate the short-term stability of triterpenoids in the matrices at room temperature, four replicate QC samples (high, middle and low) were analyzed after being thawed at room temperature (20–30 °C) and kept for 24 h. Freeze–thaw stability was determined after three freeze–thaw cycles. Each cycle consisted of freezing at −70 °C for 24 h and then thawing completely at room temperature. To investigate the long-term stability, four replicate QC samples were kept at −70 °C for 30 days.

2.7. Urinary excretion of triterpenoids in rat

Five Sprague–Dawley rats were fasted 18 h with water ad libitum before experiment. Each rat was administered an oral dose of 1.2 g/kg of total triterpenoids (suspended in 2% aqueous tragacanth solution) and held, respectively, in metabolic cages for the collation of urine samples. The urine samples were collected periodically, including 0–4, 4–8, 8–12, 12–20, 20–28, 28–36, 36–48, 48–60 and 60–72 h, and stored at −70 °C until analyzed. The overall urinary volume of each interval was measured and noted and the urine concentrations of the four analytes

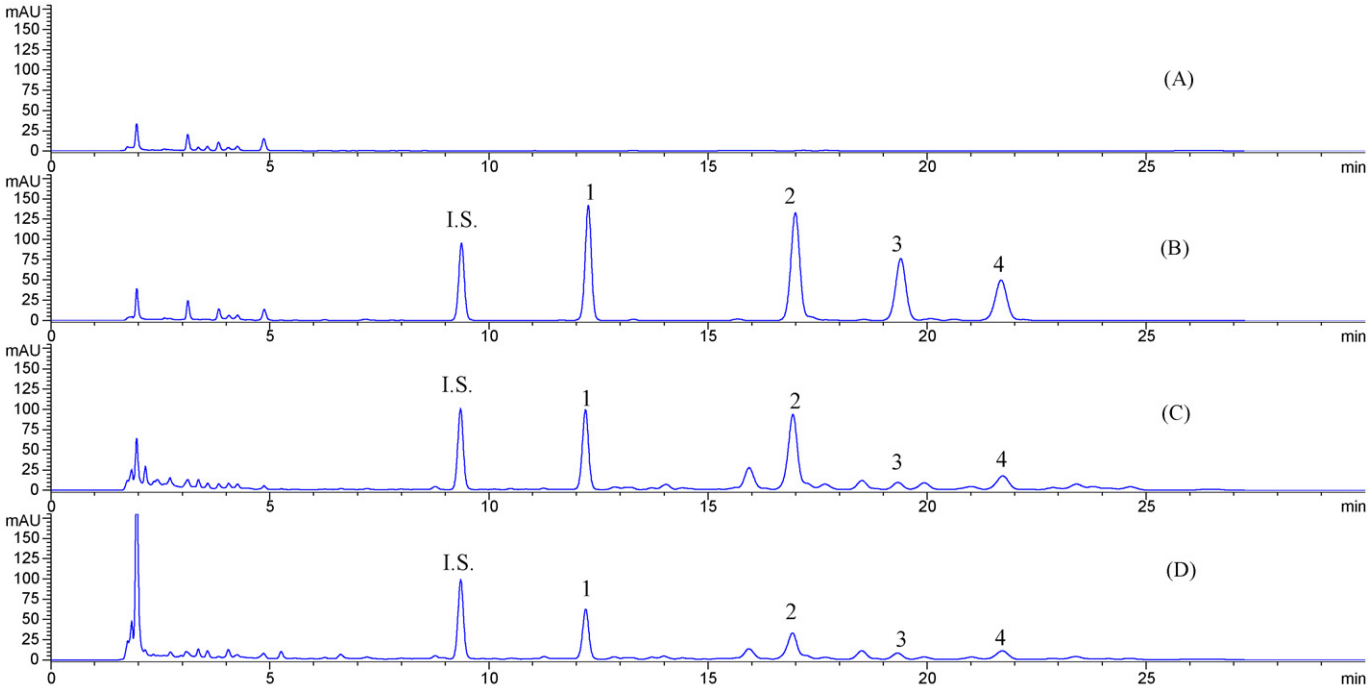


Fig. 2. Representative chromatograms of the four triterpenoids in urine sample. (A) Blank urine. (B) Blank urine spiked with (**1**) ganoderic acid C_2 (4.600 μg/ml), (**2**) ganoderic acid B (7.880 μg/ml), (**3**) ganoderic acid K (4.920 μg/ml), (**4**) ganoderic acid H (3.180 μg/ml) and I.S. (hydrocortisone, 0.992 μg/ml). (C) Urine sample obtained 4 h after oral administration of total triterpenoids of *G. lucidum*. (D) Urine sample obtained 28 h after oral administration of total triterpenoids of *G. lucidum*.

Table 1
Calibration curves of the four triterpenoids in rat urine ($n = 4$)

Compound	Regression equation[a]	r^2	F-test[b]		Linear range (μg/ml)
			F	p-value	
Ganoderic acid C_2	$Y = 0.3348X + 0.0099$	0.995	0.42	0.832	0.133–6.154
Ganoderic acid B	$Y = 0.2702X + 0.0190$	0.993	0.57	0.723	0.263–10.507
Ganoderic acid K	$Y = 0.2585X + 0.0049$	0.996	1.04	0.420	0.164–6.560
Ganoderic acid H	$Y = 0.3322X + 0.0042$	0.998	0.84	0.536	0.106–3.180

[a] Y, peak area ratio (analyte/internal standard); X, concentration of compound in rat urine (μg/ml).
[b] F-test for lack of fit.

at different interval were evaluated by means of linear regression analysis.

3. Results

3.1. Method validation

Fig. 2 showed the typical chromatograms of blank rat urine (A), urine spiked with four triterpenoids and internal standard (B), and a urine sample obtained 4 h after oral administration of total triterpenoids, which indicated that no significant interfering peak was observed at the retention times of either analytes or internal standard. As shown in Table 1, all calibration curves were found to be good linear regressions ($r^2 > 0.99$) within the test range. Lack of fit test on regression between concentration and four detection values of the peak area ratio exhibited non-significance via F-test at alpha = 0.05 levels. The lowest limit of quantification (LLOQ) of four analytes was chosen at the lowest concentration of calibration curve, for which the R.S.D. was less than 9% and the recovery was ranged from 93 to 104%.

The precision values varied from 8.6 to 6.6% for within- and between-day analyses. Accuracy varied from 110.6 to 96.6%. The results indicate that there is good reproducibility and accuracy for the determination of four triterpenoids in urine samples. As shown in Table 2, the mean extraction recovery of the four triterpenoids at three concentrations was 98–90%, while R.S.D. values were less than 8%. The mean extraction recovery of internal standard was 92.64%, while R.S.D. values were less than 3%. The results showed that the extraction procedure was precise and reproducible.

The stability study showed that the four triterpenoids were stable in urine at room temperature (20–30 °C) for 24 h and −70 °C for at least 30 days, for which the recovery of four analytes ranged from 110.9 to 95.3% with R.S.D. less than 9.4%. The recovery after three freeze–thaw cycles ranged from 106.9 to 97.9% with R.S.D. less than 7%, indicating the four triterpenoids to be stable under these conditions.

3.2. Urinary excretion of triterpenoids in rat

The analytical method has been applied to the determination of four triterpenoids in rat urine samples after oral administration of total triterpenoids extracted from *G. lucidum*. The time variations of urinary excretions of ganoderic acids C_2, B, K and H were shown in Fig. 3. The cumulative urinary excretion of ganoderic acids C_2, B, K and H within 72 h was 1.54, 0.23, 0.13 and 0.09%, respectively. The maximum urinary excretions (the greatest percentage dose of recovered in the urine collected in different time intervals) of ganoderic acids C_2, B, K and H were observed at 4–8 h and the results were presented in Fig. 4.

Table 2
Extraction recovery of the four triterpenoids in rat urine ($n = 3$)

Compound	Concentration added (μg/ml)	Recovery[a] (%mean ± S.D.)	R.S.D. (%)
Ganoderic acid C_2	4.600	94.68 ± 4.82	5.12
	1.535	98.05 ± 4.28	4.37
	0.307	96.43 ± 6.06	6.29
Ganoderic acid B	7.880	95.00 ± 6.97	7.37
	2.627	95.70 ± 3.51	3.67
	0.525	94.82 ± 5.44	5.74
Ganoderic acid K	4.920	94.64 ± 6.98	7.35
	1.640	93.96 ± 4.03	4.30
	0.328	90.32 ± 2.35	2.61
Ganoderic acid H	3.180	95.14 ± 6.68	7.02
	1.060	94.15 ± 4.82	5.12
	0.212	90.37 ± 1.68	1.86
I.S.	0.992	92.64 ± 2.28	2.46

[a] Recovery = (mean peak area of QC samples/mean peak area of pure standards) × 100.

4. Discussion

In order to remove endogenous impurities in urine to an acceptable level, several extraction methods were tried, such as solid-phase extraction and liquid–liquid extraction. The solid-phase extraction using the Extraction-Clean™ cartridge (Alltech Associates, Inc.) was optimized firstly, however, the method was found to have low extraction efficiency and poor reproducibility of the internal standard and ganoderic acid C_2. The liquid–liquid extraction with different organic solvents such as acetone, ethyl acetate, dichloromethane and different ratio of dichloromethane–ethyl acetate was also optimized. Although high extraction efficiency with ethyl acetate, it was abandoned because endogenous impurities were co-eluted with the internal

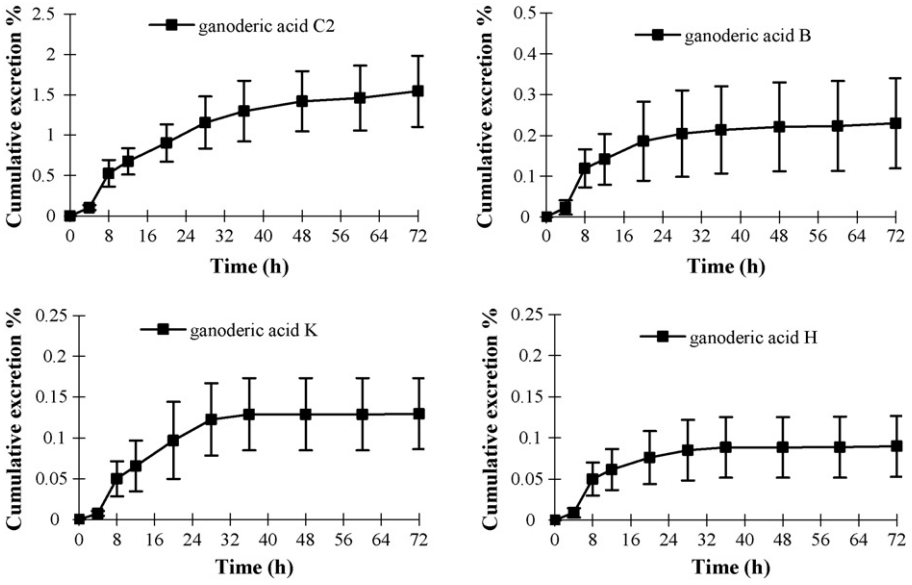


Fig. 3. Cumulative urinary excretion of the four triterpenoids in rat urine after oral administration of total triterpenoids extracted from *G. lucidum*. Each point with bar represents mean ± S.D. ($n = 5$) as the percentage of the administered dose.

standard. The dichloromethane–ethyl acetate (90:10) was finally selected as extracting solvent since it induces less interference from the co-eluted endogenous materials. Due to the acidity of the four triterpenoids, the hydrochloric acid was added to adjust the pH value of urine samples.

The results of our previous studies on determination of triterpenoids in *G. lucidum* by RP-HPLC were used as the basis for mobile phase selection and optimization. Due to the poor resolution of the internal standard, the gradient of mobile phase was optimized and the flow rate was increased to 1.2 ml/min. After the adjustment of the chromatographic conditions, the resolution of the internal standard was improved.

In summary, this method was the first report on the simultaneous determination of the four triterpenoids in rat urine. It was developed and validated with adequate accuracy and precision and successfully applied to study urinary excretion of four triterpenoids in Sprague–Dawley rat after oral administration of total triterpenoid extracted from *G. lucidum*.

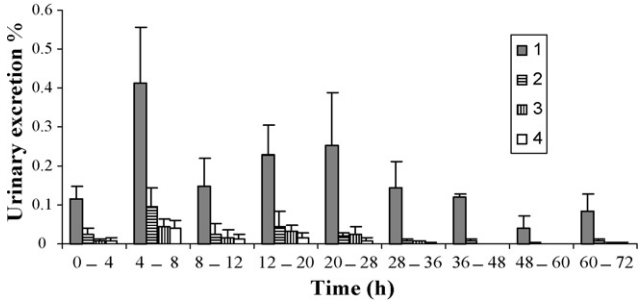


Fig. 4. Urinary excretion–time profile of ganoderic acid C_2 (**1**), ganoderic acid B (**2**), ganoderic acid K (**3**) and ganoderic acid H (**4**) after oral administration of total triterpenoids to rats. Urinary excretion (percentage dose recovered) = amount excreted to urine/amount administered to rats ($n = 5$).

Acknowledgements

We thank the Shanghai Commission of Science and Technology (04DZ19848), National Administration of Traditional Chinese Medicine of China (2004ZX01) and Shanghai Green Valley Pharmaceutical Co. for financial support of this work.

References

[1] Z.B. Lin, Modern Research of *Ganoderma lucidum*, second ed., Beijing Medical University Press, Beijing, 2001, pp. 1–7.
[2] Z.B. Lin, C.H. Li, S.S. L, L.S. Kan, Life Sci. 72 (2003) 2381–2390.
[3] X. Liu, J.P. Yuan, C.K. Chung, X.J. Chen, Cancer Lett. 182 (2002) 155–161.
[4] H.N. Zhang, J.H. He, L. Yuan, Z.B. Lin, Life Sci. 73 (2003) 2307–2319.
[5] Y.S. Kim, S.K. Eo, K.W. Oh, C.K. Lee, S.S. Han, J. Ethnopharmacol. 72 (2000) 451–458.
[6] Y.S. Song, S.H. Kim, J.H. Sa, C. Jin, C.J. Lim, E.H. Park, J. Ethnopharmacol. 90 (2004) 17–20.
[7] C.Z. Wang, D. Basila, H.H. Aung, S.R. Mehendale, W.T. Chang, E. McEntee, X.F. Guan, C.S. Yuan, Am. J. Chin. Med. 33 (2005) 807–815.
[8] S.Y. Li, W.Y. Wang, Chin. J. Med. 5 (2005) 37–39.
[9] W.S. Ren, W.Y. Wang, J.S. Chen, S.C. Huang, W.Q. Song, G.S. Dai, Chin. J. Med. 4 (2004) 113–115.
[10] J.S. Chen, W.S. Ren, Chin. J. Med. 3 (2003) 689–693.
[11] M. Zhu, Q. Chang, L.K. Wong, F.S. Chong, R.C. Li, Phytother. Res. 13 (1999) 529–531.
[12] M.Y. Wang, Q. Liu, Q.M. Che, Z.B. Lin, Acta Pharm. Sin. 35 (2002) 326–329.
[13] G. Stanley, K. Harvey, V. Slivova, J.H. Jiang, D. Sliva, Biochem. Biophys. Res. Commun. 330 (2005) 46–52.
[14] K.J. Hong, D.M. Dunn, C.L. Shen, B.C. Pence, Phytother. Res. 18 (2004) 768–770.
[15] E.M. Williamson, Phytomedicine 8 (2001) 401–409.
[16] W. Jia, W.Y. Gao, Y.Q. Tan, J. Wang, Z.H. Xu, W.J. Zheng, P.G. Xiao, Phytother. Res. 18 (2004) 681–686.
[17] X.M. Wang, M. Yang, S.H. Guan, R.X. Liu, J.M. Xia, K.S. Bi, D.A. Guo, J. Pharm. Biomed. Anal. 41 (2006) 838–844.

[18] A. Morigiwa, K. Kitabatake, Y. Fujimoto, N. Ikekawa, Chem. Pharm. Bull. 34 (1986) 3025–3028.
[19] T. Kikuchi, S. Kanomi, S. Kadota, Y. Murai, K. Tsubno, Z.I. Ogita, Chem. Pharm. Bull. 34 (1986) 3695–3721.
[20] T. Kubota, Y. Asaka, I. Miura, H. Mori, Helv. Chim. Acta 65 (1982) 611–619.
[21] T. Kikuchi, S. Kanomi, Y. Murai, S. Kadota, K. Tsubono, Z.I. Ogita, Chem. Pharm. Bull. 34 (1986) 4018–4029.
[22] Food and Drug Administration, Centre for Drug Evaluation and Research (CDER), Guidance for Industry, Bioanalytical Method Validation, May 2001. http://www.fda.gov/cder/guidance/4252fnl.htm.