ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
21 (1999) 207–213

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Short communication

Reversed-phase liquid chromatographic determination of cryptotanshinone and its active metabolite in pig plasma and urine

M. Xue [a,*], Y. Cui [a], H.Q. Wang [b], ZH.Y. Hu [a], B. Zhang [a]

[a] *Lanzhou Institute of Animal and Pharmaceuticals, Chinese Academy of Agricultural Sciences, Lanzhou 730050, People's Republic of China*
[b] *Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou 730050, People's Republic of China*

Received 6 August 1998; received in revised form 18 December 1998; accepted 24 January 1999

Keywords: High-performance liquid chromatography; Cryptotanshinone; Metabolite Tanshinone IIA; Plasma; Urine

1. Introduction

Cryptotanshinone is the mainly active component isolated from the rhizome of *Salvia miltiorrhiza* Bunge and *Salvia przewalskii* Maxim (Labiatae) which has been used widely in China to treat coronary heart diseases, particularly angina pectoris and myocardial infarction [1–3]. It reportedly has sedative and tranquilizing effects and is also being used to treat neurasthenic insomnia [4,5]. Cryptotanshinone is a diterpene quinone pigment and its structure has been identified [6–8]. Diterpenoid tanshinones have attracted particular attention of medicinal chemists and clinicians because many of them exhibit significant antibacterial [9,10], antidermatophytic [10], antioxidant [11,12], antiinflammatory [10,13], antineoplastic [14], and antiplatelet aggregation [15] activitis. It is found that tanshinones are effective in the treatment of the mastitis, wound infection and metabolism in order in veterinary practice [16].

In this paper, a simple reversed-phase liquid chromatography (LC) method employing a single-step liquid–liquid extraction and UV detection for the rapid quantitative determination of cryptotanshinone and its active metabolite tanshinone IIA in both porcine plasma and urine is reported. The assay demonstrates excellent specificity, linearity, precision and accuracy for cryptotanshinone and tanshinone IIA.

2. Experimental

2.1. Materials

Cryptotanshinone was obtained from our laboratory, which was isolated and purified from the roots of *S. miltiorrhiza* Bunge and *S. przewalskii*

* Corresponding author.

PII: S0731-7085(99)00098-9

Maxim and identified as pure compound from the melting point, IR, UV, NMR [7,8]. Tanshinone IIA was obtained from the National Institute for Control of Bioproducts and Pharmaceuticals (Beijing, China). The structure of cryptotanshinone and tanshinone IIA are shown in Fig. 1. The internal standard, Dibenyl, was obtained from Shanghai Reagent Company (Shanghai, China). Stock standard solutions of cryptotanshinone, tanshinone IIA and dibenyl (0.5, 0.5, and 1 mg ml^{-1}, respectively) were prepared in methanol. Methanol was of LC grade (Shandong Chemical Reagent Co., Jinan, China).All chemicals and solvents were of the highest grade commercially available. Pooled drug-free porcine plasma and urine were obtained from healthy pigs, stored at -20°C and allowed to thaw at an ambient temperature prior to use.

2.2. Instrumentation

LC analysis was performed using a Waters 510 pump (Waters, Milford, USA), an automatic sample injection system (Waters), and an Model 486 absorbance detector (Applied Biosystems, Millipore Co., MA). The detector wavelength was set at 254 nm. Data acquisition and manipulation were performed on a Model 680 laboratory automation system (AST Research Inco, Irrine, CA). Quantitation was based on linear regression analysis of peak-area ratios of cryptotanshinone or tanshinone IIA to the standard versus cryptotanshinone or tanshinone IIA concentration.

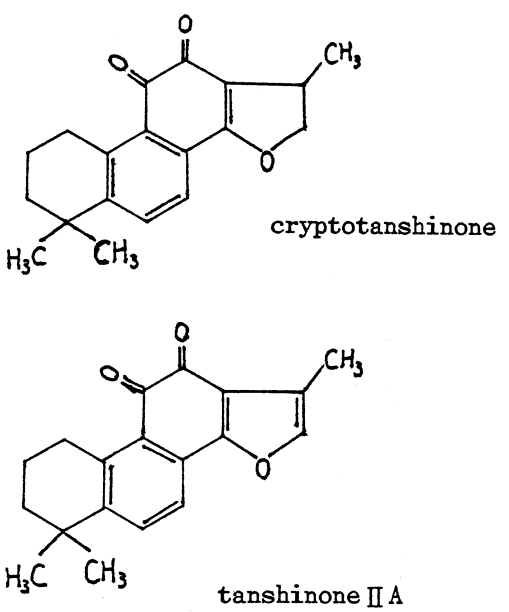

Fig. 1. Structures of cryptotanshinone and tanshinone IIA.

Separation was accomplished on a Nova-Pak C18 (4 μm; 150 × 3.9 mm i.d.) column (Waters). The column temperature was maintained at 35°C. The mobile phase was methanol–water (85:15,v/v), and was deaerated by sonication prior to use. The flow rate was set at 1.0 ml min^{-1}.

2.3. Sample preparation

Stock standard solution of cryptotanshinone, tanshinone IIA, and dibenzyl were prepared in methanol. Further dilution steps were made in either plasma or urine. Working standard solutions were prepared by adding appropriate volume of cryptotanshinone or tanshinone IIA solution. Quality control samples were also prepared in the same way, using a separately weighed stock solution. The final concentration of cryptotanshinone and tanshinone IIA in plasma standards were 0.025, 0.050, 0.075, 0.10, 0.25, 0.50, 1.00, 2.50 μg ml^{-1}. The final concentrations of cryptotanshinone and tanshinone IIA in urine standards were 0.40, 0.80, 1.60, 3.20, 6.40, and 12.80 μg ml^{-1}. Quality controls of $\sim$0.80, 3.00 and 12.50 μg ml^{-1} were also prepared. After aliquoting, both plasma and urine controls were stored at -20°C until analysis.

2.4. Extraction of samples

To an aliquot of porcine plasma (1.0 ml) or urine (2.0 ml) was added 4 μl of the internal standard stock solution. After the addition of ethyl acetate (2.0 ml), each sample was vortex mixed for 30 s and centrifuged at 3000 × *g* for 10 min. The organic portion was separated. Each sample was extracted three times. After evaporation under nitrogen and reconstitution in 100 l of LC mobile phase, an aliquot (10 μl) was injected into the LC system.

The assay was evaluated in pigs receiving vein dose of cryptotanshinone (500 mg). Blood samples (5 ml) were collected pre-dose and at 2, 5, 10, 15, 30, 45, 60, 90, 120, 240, 360, and 720 min post-dose and were later centrifuged at $3000 \times g$ for 10 min in order to harvest plasma. Urine samples were collected pre-dose and at 0–8, 8–12, 12–24, and 24–48 h periods post-dose. Concentrations of cryptotanshinone were evaluated by determining the ratio of the peak area of cryptotanshinone to that of the internal standard, concentrations of tanshinone IIA were evaluated by determining the ratio of the peak area of tanshinone IIA to that of the internal standard and calculated from the calibration curve obtained after linear regression analysis of the calibration standards.

2.5. Precision and accuracy

Precision and accuracy were assessed by performing replicate analyses of quality control samples against calibration standards. The precision and accuracy of the method were calculated as the relative standard deviation (RSD) and the percentage deviation of observed concentration from theoretical concentration, respectively.

2.6. Recovery

The extraction efficiency (recovery) was determined by calculating the ratio of the amount of extracted compound from drug-free plasma or urine spiked with known amounts of cryptotanshinone (quality control plasma and urine samples) to the amount of compound added at the same concentrations to water just prior to LC injection.

2.7. Stability study

The stability of cryptotanshinone and tanshinone IIA were assessed during all of the storage steps and during steps of the analytical method. During the first days of the study, quality control samples in plasma and urine were prepared from standard solutions of cryptotanshinone and tanshinone IIA. These quality control samples were then placed in freezer storage at -20°C and randomly removed at various times in each analytical sequence during a 12 month period. Additionally, the freeze–thaw stability and the ambient stability in the autosampler after 24 h at room temperature were assessed in quality control samples for both matrices.

2.8. Specificity study

The ability of the assay to quantify cryptotanshinone accurately in the presence of endogenous compounds and major metabolites (tanshinone IIA and hydrotanshinone IIA) was confirmed through the analysis of blanks and spiked quality control samples, respectively.

3. Results and discussion

Typical chromatograms of blank porcine plasma and urine, plasma from a subject containing 0.82 μg ml^{-1} of cryptotanshinone, 1.41 μg ml^{-1} of tanshinone IIA, and urine from a subject containing 8.28 μg ml^{-1} of cryptotanshinone, 3.22 μg ml^{-1} of tanshinone IIA are shown in Fig. 2. The retention times of cryptotanshinone, tanshinone IIA and dibenzyl were 4.06, 5.98, and 5.20 min, respectively.

3.1. Precision and accuracy

Precision and accuracy were assessed in both plasma and urine by performing replicate analyses of spiked samples against calibration standards. The procedure was repeated on the same day and for different days on the same spiked standards at concentrations in range of the standard series. Assay linearity was demonstrated in both plasma and urine, as shown by regression analysis of calibration curves (Table 1). the method was shown to be linear for cryptotanshinone in the range 0.025–2.50 μg ml^{-1} in plasma and 0.80–12.5 μg ml^{-1} in urine, and for tanshinone IIA in the range 0.05–2.50 μg ml^{-1} in plasma and 0.80–12.50 μg ml^{-1} in urine. The between-day preci-

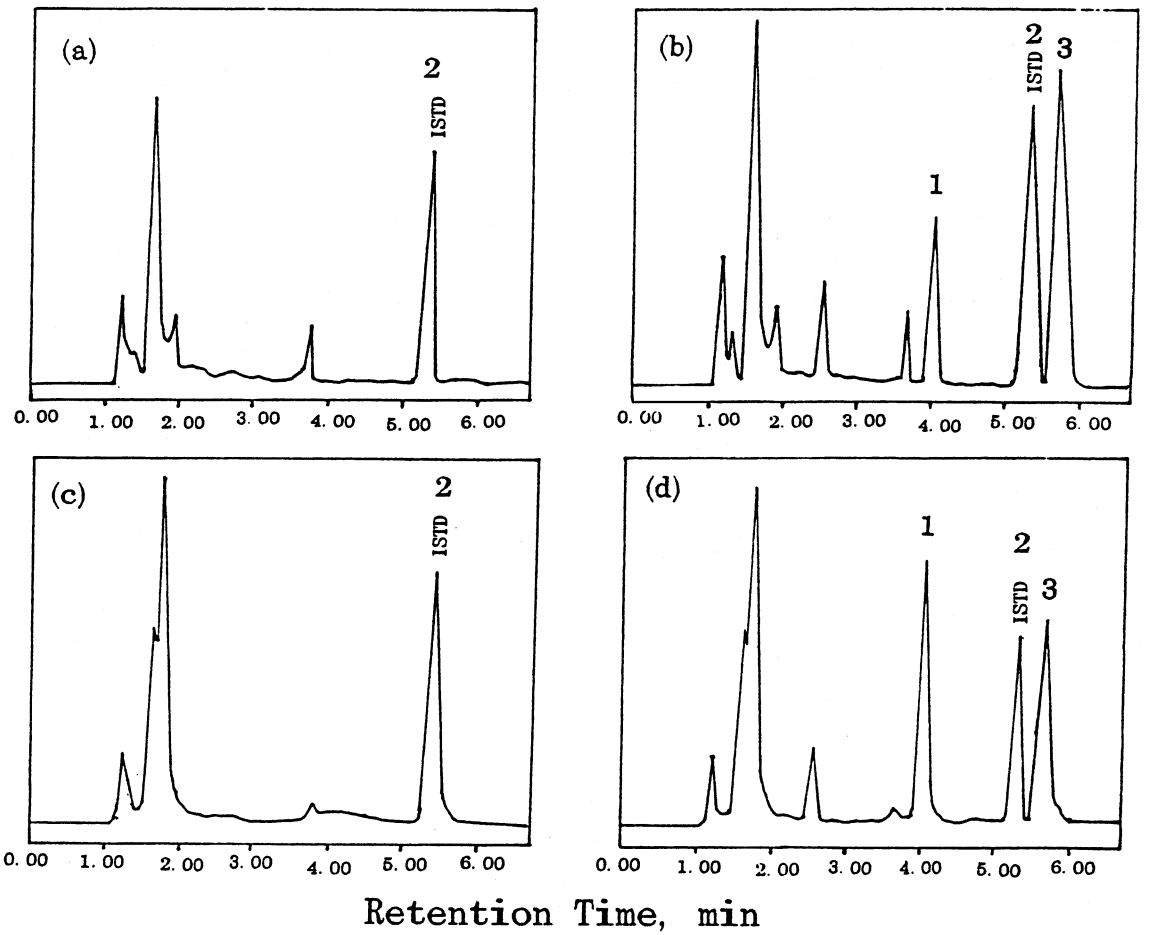

Fig. 2. Representative chromatograms of: (a) blank plasma; (b) a plasma sample from a pig containing 0.82 μg ml^{-1} of cryptotanshinone and 1.41 μg ml^{-1} of tanshinone IIA; (c) blank urine; and (d) a urine sample from a pig containing 8.28 μg ml^{-1} of cryptotanshinone and 3.22 μg ml^{-1} of tanshinone IIA. Peaks: 1, cryptotanshinone; 2, dibenzy (internal standard); 3, tanshinone IIA. For chromatographic conditions, see Section 2.

Table 1
Assay linearity

Matrix/compound	Correlation coefficient ($r \pm$ SD)	Slope $\pm$ SD	Intercept $\pm$ SD
Cryptotanshinone			
Plasma ($n = 6$)	0.9994 ± 0.0004 (RSD = 0.04%)	0.3068 ± 0.0071 (RSD = 2.3%)	$-(0.0337 \pm 0.002)$ (RSD = 3.6%)
Urine ($n = 6$)	0.9998 ± 0.0008 (RSD = 0.08%)	0.7727 ± 0.0317 (RSD = 4.1%)	$-(0.0317 \pm 0.0007)$ (RSD = 2.1%)
Tanshinone IIA			
Plasma ($n = 6$)	0.9998 ± 0.0005 (RSD = 0.5%)	1.1783 ± 0.0224 (RSD = 1.9%)	$-(0.0428 \pm 0.0013)$ (RSD = 3.1%)
Urine ($n = 6$)	0.9998 ± 0.0008 (RSD = 0.08%)	1.1116 ± 0.0289 (RSD = 2.6%)	0.0048 ± 0.0002 (RSD = 4.2%)

sion and accuracy of the method are presented in Table 2. When the extraction was performed robotically, the r values were at least 0.9994 and the precision and accuracy were excellent with an RSD of less than 6 and 12%.

3.2. Recovery

On analysis of quality control samples (triplicate) at concentrations of 0.05, 0.25 and 0.50 μg ml^{-1} (cryptotanshinone, plasma), 0.16, 0.50, 2.50 μg ml^{-1} (tanshinone IIA, plasma) and 0.80, 3.0 12.50 μg ml^{-1}(cryptotanshinone and tanshinone IIA, urine), the overall recoveries were 88–95% (plasma) and 86–102% (urine), respectively. (see Table 3).

3.3. Stability

The stability of stock and standard solution kept at 15°C and frozen (−20°C) plasma and urine samples as well as frozen plasma and urine

Table 2
Precision and accuracy of the LC analysis of cryptotanshinone and tanshinone IIA

Matrix	Theoretical concentration (μg ml^{-1})	n (mean ± SD)	Experimental concentration (μg ml^{-1})	Precision (RSD%)	Accuracy percent error (%)
Cryptotanshinone					
Plasma	Within-day				
	0.075	5	0.071 ± 0.001	1.10	5.33
	0.20	5	0.176 ± 0.006	3.81	12.0
	0.50	5	0.439 ± 0.009	2.42	12.2
	Between-day				
	0.075	6	0.071 ± 0.001	0.83	5.33
	0.20	6	0.191 ± 0.018	3.87	4.5
	0.50	6	0.471 ± 0.042	5.91	5.80
Urine	Within-day				
	0.80	5	0.752 ± 0.013	1.96	5.0
	3.0	5	3.310 ± 0.027	0.90	9.37
	12.5	5	12.303 ± 0.069	0.63	1.58
	Between-day				
	0.80	6	0.757 ± 0.023	3.44	5.37
	3.0	6	3.420 ± 0.087	2.85	12.28
	12.5	6	12.570 ± 0.190	1.77	0.56
Tanshinone IIA					
Plasma	Within-day				
	0.16	5	0.152 ± 0.002	1.46	5.0
	0.50	5	0.449 ± 0.002	0.42	10.2
	2.50	5	2.315 ± 0.009	0.55	7.4
	Between-day				
	0.16	6	0.158 ± 0.004	3.30	1.25
	0.50	6	0.445 ± 0.003	0.79	11.0
	2.50	6	2.326 ± 0.103	5.70	6.96
Urine	Within-day				
	0.80	5	0.801 ± 0.037	2.11	0.13
	3.0	5	2.928 ± 0.026	1.25	2.41
	12.5	5	11.41 ± 0.045	0.27	8.72
	Between-day				
	0.80	6	0.832 ± 0.026	3.51	3.85
	3.0	6	2.981 ± 0.045	1.69	0.64
	12.5	6	11.73 ± 0.157	1.64	6.16

Table 3
Recovery

Treatment	Added (μg ml^{-1})	Determined (μg ml^{-1})	Recovery ± SD (%)	RSD (%)
Cryptotanshinone				
Plasma	0.05	0.044 ± 0.001	88.21 ± 2.61	2.96
	0.25	0.227 ± 0.023	90.92 ± 2.93	3.22
	0.50	0.478 ± 0.042	95.52 ± 1.64	1.72
Urine	0.80	0.688 ± 0.024	86.02 ± 1.84	2.14
	3.0	0.307 ± 0.029	102.36 ± 1.01	0.98
	12.5	12.304 ± 0.811	98.43 ± 0.62	0.63
Tanshinone IIA				
Plasma	0.16	0.152 ± 0.019	94.94 ± 1.39	1.46
	0.50	0.449 ± 0.014	89.93 ± 0.37	0.41
	2.50	2.265 ± 0.116	90.59 ± 0.44	0.49
Urine	0.80	0.808 ± 0.038	101.05 ± 5.17	5.12
	3.0	2.928 ± 0.271	97.58 ± 1.22	1.25
	12.5	11.670 ± 0.515	93.36 ± 0.41	0.43

extracts was checked. These samples were: (1) stored frozen at −20°C for at least 12 months (plasma and urine); (2) allowed to stand at ambient temperature in the autosampler for at least 24 h after extraction; and (3) subjected to three freeze–thaw cycles. Analysis of these samples consistently afforded values which were nearly identical with those of freshly prepared quality control samples. thus confirming the overall stability of cryptotanshinone and its metabolite tanshinone IIA in both matrices under long-term frozen storage, assay processing and freeze–thaw conditions.

3.4. Specificity

Endogenous compounds in plasma and urine did not interfere with the cryptotanshinone and tanshinone IIA or the internal standard peaks. Standards to both tanshinone IIA and hydroxytanshinone IIA were added to plasma and quality control samples at concentrations of 2.0 μg ml^{-1}, and their peaks were well resolved from those of cryptotanshinone and the internal standard (by the retention times of tanshinone IIA and hydroxytanshinone IIA were ~5.9 and 2.6 min, respectively). Cryptotanshinone and tanshinone IIA was reliably quantified in these quality control samples with an accuracy within 12% of the target concentration and an RSD 6%. Thus, the presence of high concentration of both metabolites did not interfere with the determination of cryptotanshinone. Fig. 3 represents the plasma and urinary concentration of cryptotanshinone and tanshinone IIA over a 48 h period from a pig which had been administered intravenously (500 mg) of cryptotanshinone.

4. Discussion

The determination of cryptotanshinone and its active metabolite, tanshinone IIA in biological fluids by reversed-phase LC has not been reported, but the determination of tanshinones in plant by LC has been described by several investigators [17,18], and there was difference in the application and results. The biotransformation of cryptotanshinone in pigs has been reported by our laboratory [19]. The method described involves a rapid and specific assay for the determination of cryptotanshinone and its active metabolite, tanshinone IIA in porcine plasma and urine. The normal and heamolysis plasma, and blood were extracted and determined respectively on the same conditions. The results showed the parent drug

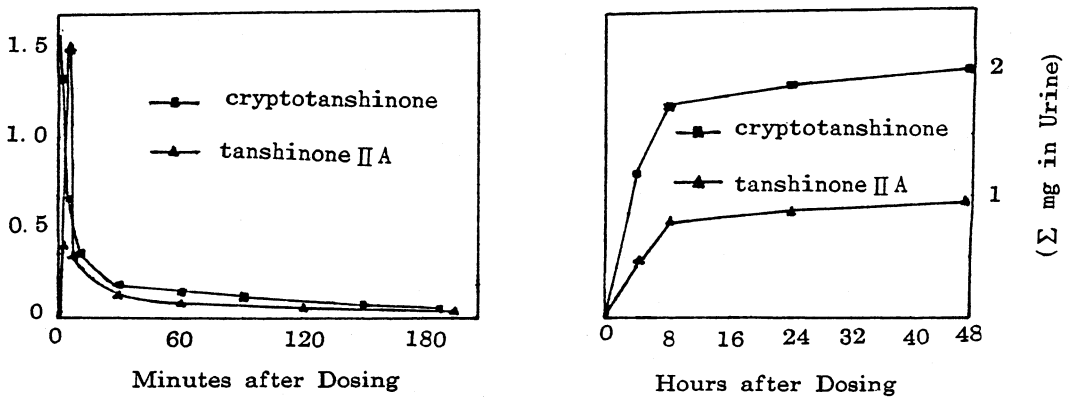

Fig. 3. Pharmacokinetic profile of cryptotanshinone and tanshinone IIA in plasma and in urine following administration of a single intravenous dose of cryptotanshinone (500 mg) to a pig.

and the metabolite were mostly distributed in the plasma and the heamolysis did not affect the extract efficiency. Ethyl acetate and chloroform were used to compare the extract result and the liquid chromatographic separation of the drug and the metabolite on a reversed-phase C_{18} column in different pH (3, 7, and 9) eluents showed less difference for the recovery. The described method was successfully applied to the routine analysis of plasma and urine samples collected during pharmacokinetics and drug metabolism studies. Low detection limits are a prerequisite since the observed half-lives of cryptotanshinone and tanshinone IIA were very short (64.78 and 189 min). cryptotanshinone and tanshinone IIA plasma levels dropped below the detection limit 3 h after an intravenous single dose administration of 10 mg kg^{-1}. The application of the described method to the investigation of pharmacokinetics and metabolism of cryptotanshinone in pigs is presently being studied at the authors' institute.

Acknowledgements

This work was supported by a grant from the National tackle key projects of the People's Republic of China. The authors thank Y.J. Luo, Y.B. Shi and Z.T. Zhou for their technical assistance. They also thank Professor W.J. Xia and Professor R.C. Zhao for a critical review of the manuscript.

References

[1] W.Z. Chen, Y.L. Dong, G. Wang, Acta Pharm. Sin. 14 (1979) 277–282.
[2] W.Z. Chen, Acta Pharm. Sin. 19 (1984) 876–881.
[3] H.M. Chang, Pharmacology and Applications of Chinese Materia Medica, vol. 1, World Scientific Publishing, Singapore, 1986, pp. 255–268.
[4] H.Y. Zhang, O.Y. Rong, Acta Pharm. Sin. 14 (1979) 288–294.
[5] S.F. Fan, L.Q. Sun, Z.H. Wang, Acta Pharm. Sin. 14 (1979) 199–206.
[6] K. Takiura, J. Pharm. Soc. Jpn. 61 (1941) 475–482.
[7] Q.N. Fang, P.L. Zhang, Z.P. Xu, Acta Chem. Sin. 34 (1976) 197–209.
[8] M.K. Qian, B.J. Yang, Acta Chem. Sin. 36 (1978) 199–205.
[9] G. Honds, Y. Keezuka, M. Tabata, Chem. Pharm. Bull. 36 (1988) 408–415.
[10] Y.G. Gao, Y.M. Song, Y.Y. Yang, J.X. Tang, W.F. Liu, Acta Pharm. Sin. 14 (1979) 75–81.
[11] W. Jiang, Y. Zhao, B.L. Zhao, W.J. Xi, Acta Biophys. Sin. 10 (1994) 685–689.
[12] E.H. Cao, X.Q. Liu, J.F. Li, Acta Biophys. Sin. 12 (1996) 339–343.
[13] Y.G. Gao, L.Z. Wang, K.S. Tang, Chin. J. Integr. Trad. West. Med. 3 (1983) 300–306.
[14] W.L. Wu, W.L. Chang, J. Med. Sci. 6 (1985) 159–164.
[15] H.W. Luo, X.J. Hu, N. Wang, J. Jaing, Acta Pharm. Sin. 23 (1988) 830–834.
[16] J.G. Liu, J. Trad. Chin. Vet. Med. 14 (1995) 45–47.
[17] M.Z. Wang, F.Sh. Yang, F.Y. Gao, J. Drug Anal. 5 (1985) 340–350.
[18] P. Song, L.Y. He, Acta Pharm. Sin. 30 (1995) 711–714.
[19] M. Xue, Y. Cui, B. Zhang, Chin. J. Vet. Med. 27 (1997) 28–30.