Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Pharmaceutical and Biomedical Analysis 40 (2006) 758–762
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Determination of glycyrrhetic acid in human plasma by LC–ESI–MS

Li Ding [a,*], Xin Huang [a], Jin Yang [a], Xiaojie Bian [a], Zhengxing Zhang [a], Guangyu Liu [b]

[a] *Department of Pharmaceutical Analysis, China Pharmaceutical University, 24 Tongjiaxiang, Nanjing 210009, China*
[b] *Organization for State Drug Clinical Trial, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China*

Received 10 June 2005; received in revised form 26 October 2005; accepted 27 October 2005
Available online 7 December 2005

Abstract

A sensitive liquid chromatography–electrospray ionization–mass spectrometry (LC–ESI–MS) method for determination of glycyrrhetic acid, the active metabolite of glycyrrhizin, in human plasma using ursolic acid as the internal standard (IS) was established. Plasma was extracted with ethyl acetate and separated on a C_{18} column with a mobile phase of 10 mmol/l ammonium acetate–acetonitrile (10:90). Glycyrrhetic acid was determined using electrospray ionization in a single quadrupole mass spectrometer. HPLC–ESI–MS was performed in the selected ion monitoring (SIM) mode using target ions at *m*/*z* 469.5 for glycyrrhetic acid and *m*/*z* 455.5 for IS. Calibration curve was linear over the range of 0.1–400 ng/ml. The limit of quantitation for glycyrrhetic acid in plasma was 0.1 ng/ml. The mean plasma extraction recovery of glycyrrhetic acid was 94.51 ± 3.82%. The method has been successfully applied to study the pharmacokinetics of glycyrrhetic acid in healthy male Chinese volunteers. The assay was proved to be sensitive, accurate and convenient.

Keywords: Glycyrrhetic acid; Glycyrrhizin; LC–MS; Pharmacokinetics

1. Introduction

Glycyrrhizin, (3β, 20β)-20-carboxy-11-oxo-30-norolean-12-en-3-yl-2-*O*-β-D-glucopyranuronosyl-D-glucopyranosiduronic acid (see Fig. 1I), is one of the constituents of *Glycyrrhiza glabra* L. Its marketed preparation, glycyrrhizin capsule, is used clinically for the treatment of chronic hepatitis, allergic disorder and inflammation. When orally ingested, glycyrrhizin is completely transformed to its active metabolite glycyrrhetic acid (see Fig. 1II) in intestine by bacterial hydrolysis, and the parent compound was not detectable in plasma at any time, but glycyrrhetic acid could be detected at a considerable concentration [1]. So, we evaluated the bioavailability of glycyrrhizin capsule mainly by the results of glycyrrhetic acid.

Several HPLC with UV detection methods for determination of the concentration of glycyrrhetic acid in rats or pigs were developed [2–4]. Yoshikazu et al. [5] reported an HPLC method to determine the concentration of glycyrrhetic acid in human plasma, in which the limit of quantitation (LOQ) was 50 ng/ml. Lin et al. [6] developed a more sensitive LC–MS/MS method to determine glycyrrhetic acid and glycyrrhizin in human plasma with a lower LOQ of 10 ng/ml. All these methods were not sensitive enough for pharmacokinetic research of glycyrrhizin preparations for the terminal phase concentrations of glycyrrhetic acid for most volunteers were below 10 ng/ml.

In this paper, we report a more sensitive, accurate and convenient method to determine the concentration of glycyrrhetic acid in human plasma, in which the LOQ was 0.1 ng/ml. The method was successfully applied to study the pharmacokinetics of glycyrrhetic acid in healthy male Chinese volunteers.

2. Experimental

2.1. Materials and reagents

Glycyrrhetic acid and ursolic acid (see Fig. 1III) were supplied by Chia-Tai Tianqing Pharmaceutical Co. Ltd. (Jiangsu, China). The test preparation was an enteric-soluble capsule preparation of diammonium glycyrrhizinate, which containing

* Corresponding author. Tel.: +86 25 8327 1289; fax: +86 25 8327 1289.
E-mail address: dinglidl@hotmail.com (L. Ding).

0731-7085/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2005.10.047

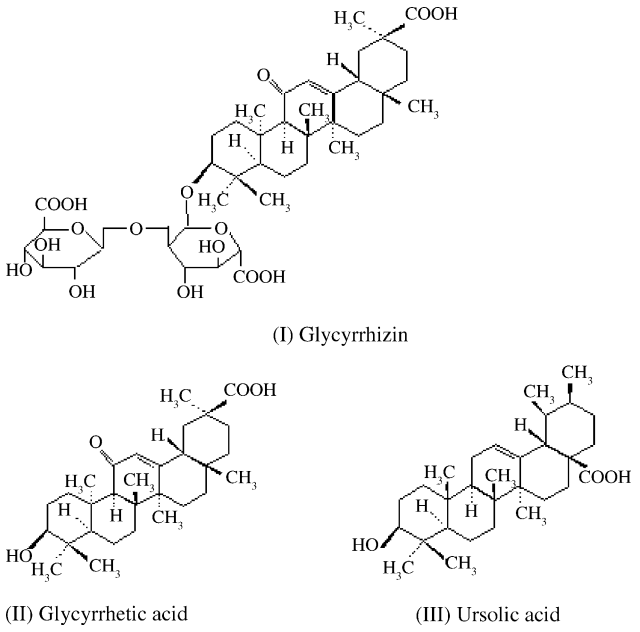

Fig. 1. Chemical structures of glycurrhizin (I), glycyrrhetic acid (II) and ursolic acid (III).

50 mg of glycyrrhizin per capsule (Chia-Tai Tianqing Pharmaceutical Co. Ltd., Jiangsu, China). The reference preparation was diammonium glycyrrhizinate capsule, which containing 50 mg of glycyrrhizin per capsule (Chia-Tai Tianqing Pharmaceutical Co. Ltd., Jiangsu, China). Ethyl acetate was analytical grade; ammonium acetate was HPLC grade, both of those were purchased from Nanjing Chemical Reagent Co. (Nanjing, China). Acetonitril was HPLC grade and was purchased from Merck Company (Dermstadt, Germany).

2.2. Instrument and conditions

HPLC analyses were performed using an Agilent 1100 LC–ESI–MS system (Agilent Technologies, Palo Alto, CA) with a Lichrospher ODS 5 μm 100 mm × 4.6 mm i.d. (Jiangsu Hanbon Science & Technology Co. Ltd., China). The mobile phase was 10 mmol/l ammonium acetate–acetonitril (10:90 (v/v)), the column temperature was maintained at 25 °C. A constant flow-rate of 1.0 ml/min was used throughout the analyses. LC–ESI–MS was carried out using nitrogen to assist nebulization. A quadrupole mass spectrometer equipped with an electrospray ionization source was set with a drying gas (N_2) flow of 10 l/min, nebulizer pressure of 40 psi, drying gas temperature of 350 °C and capillary voltage of 4 kV. The fragmentor voltage was 200 V. LC–ESI–MS was performed in negative ion selected-ion monitoring (SIM) mode using target ions at *m*/*z* 469.5 for glycyrrhetic acid and *m*/*z* 455.5 for the internal standard (IS).

2.3. Preparation of standard solutions, calibration standards and quality control samples [7,8]

Stock solutions of glycyrrhetic acid and IS were prepared at 1 mg/ml in the methanol, respectively, and stored at −20 °C. These solutions were stable for 2 months at least. Standard solutions containing 20, 10, 1 μg/ml, 100, 10 and 1 ng/ml glycyrrhetic acid were prepared by diluting the stock solution with methanol. These standard solutions were used to prepare the calibration standards of glycyrrhetic acid. A solution containing 5 μg/ml IS was also prepared using methanol.

Calibration standards of glycyrrhetic acid at concentrations of 0.1, 0.3, 1, 3, 10, 30, 100, 200, 400 ng/ml were prepared by spiking appropriate amount of the standard solutions in blank plasma obtained from healthy volunteers. Quality control (QC) samples were prepared in blank plasma at concentrations of 0.1, 1, 10, 100 and 400 ng/ml for glycyrrhetic acid and stored at −20 °C.

2.4. Sample preparation

One-milliliter plasma samples were extracted with 5 ml ethyl acetate after addition of 30 μl IS solution (5 μg/ml). Following centrifugation and separation, the organic phase was evaporated to dryness under a stream of nitrogen in a 37 °C water bath. The residue was reconstituted in 100 μl of the mobile phase, and a 40 μl aliquot was injected into the LC–ESI–MS system. Glycyrrhetic acid in human plasma at −20 °C was stable for 1 month at least. Glycyrrhetic acid and IS in extraction solution at assay temperature were stable for 12 h at least.

2.5. Assay validation

2.5.1. Linearity

Calibration standards of nine concentrations of glycyrrhetic acid (0.1, 0.3, 1, 3, 10, 30, 100, 200, 400 ng/ml) were extracted and assayed. Peak-area ratios of glycyrrhetic acid to the IS obtained from selected-ion chromatograms were utilized for construction of calibration curves, using weighted linear least-square regression (weighting factor was 1/*C*) [9–11] of the plasma concentrations and the measured ratios. The linearity of the calibration curve was confirmed by plotting the peak-area ratios versus the concentrations of glycyrrhetic acid. The calibration curve was prepared and assayed along with each batch of clinical plasma samples.

2.5.2. Precision, accuracy and specificity

The precision of the assay was determined at low, medium and high concentrations of glycyrrhetic acid by replicate analyses of the QC samples. Intra-day precision was determined by repeated analysis of each QC sample on 1 day ($n = 5$). Inter-day precision was determined by repeated analysis on five consecutive days ($n = 1$ series per day). The concentration of each sample was determined using calibration standards prepared on the same day. Accuracy is defined as the relative deviation in the calculated value (E) of a standard from that of its true value (T) expressed as a percentage (RE%). It was calculated using the formula:

$$\mathrm{RE\%} = (E - T)/T \times 100 \quad (1)$$

Assay precision was defined as the relative standard deviation (S.D.) from the mean (M), calculated using the equation:

$$\text{R.S.D.\%} = \text{S.D.}/M \times 100 \quad (2)$$

The specificity of the assay was checked by analyzing blank plasma samples of 20 volunteers. Each blank plasma sample was tested using the proposed extraction procedure and HPLC–ESI–MS conditions to ensure no interference of glycyrrhetic acid and IS from plasma. The accuracy of the assays was checked by preparation of QC samples at the start of the clinical study. These QC samples were assayed along with clinical samples in each run to monitor the performance of the assay and to assess the integrity and validity of the results of the unknown clinical samples analyzed.

2.5.3. Extraction recovery

The extraction recovery of glycyrrhetic acid was determined at low, medium and high concentrations, respectively. Recovery was calculated by comparison of the peak areas of glycyrrhetic acid extracted from plasma samples with those of injected standards.

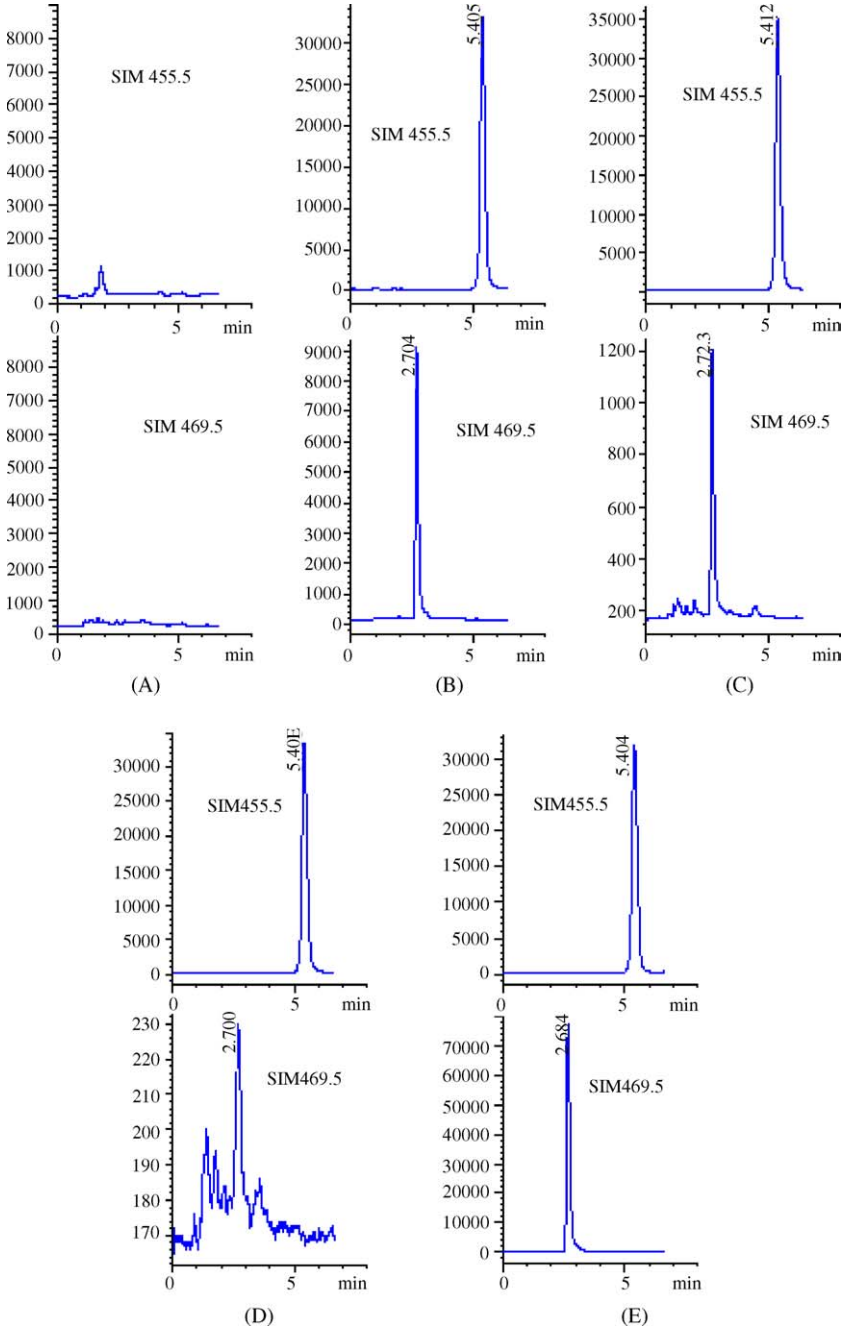

Fig. 2. Typical SIM chromatograms of blank plasma (A), plasma spiked with glycyrrhetic acid (30 ng/ml) and IS (B), plasma obtained from a volunteer after 36 h (C), LOQ for glycyrrhetic acid in plasma (0.1 ng/ml) and IS (D), and the highest concentration of glycyrrhetic acid in plasma (400 ng/ml) and IS in calibration curve (E).

2.6. System suitability test [7,12]

Prior to running each batch of clinical plasma samples, the instrument performance (e.g. sensitivity, reproducibility of chromatographic retention and separation, plate number and tailing factor) was determined by analysis of the reference standard of glycyrrhetic acid, IS, blank plasma and plasma spiked with glycyrrhetic acid and IS.

2.7. Clinical study design and pharmacokinetic analysis

Each of 20 healthy, young, male Chinese volunteers received two capsules of test or reference preparations containing 100 mg diammonium glycyrrhizinate, respectively after overnight fasting. Standard meals were provided at 3 and 9 h post-dose. Blood was sampled predose and at 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0, 16.0, 24.0, 36.0 and 48.0 h post-dose for determination of plasma concentration of glycyrrhetic acid. Model-independent pharmacokinetic parameters were calculated for glycyrrhetic acid. The maximum plasma concentrations (C_{max}) and the time to those (T_{max}) were noted directly. The elimination rate constant (k_{el}) was calculated by linear regression of the terminal point of the semi-log plot of plasma concentration against time. Elimination halflife ($t_{1/2}$) was calculated from the formula:

$$t_{1/2} = 0.693/k_{el} \quad (3)$$

Area under the plasma concentration–time curve (AUC_{0-48}) to the last measurable plasma concentration (C_p) was calculated by the linear trapezoidal rule.

3. Result and discussion

3.1. Conditions of chromatography

Ursolic acid was selected as an internal standard (IS) because its chemical properties and mass spectral fragmentation were similar to those of glycyrrhetic acid. Several tests were performed for optimizing the components of mobile phase in order to achieve good chromatographic peak shape and resolution. The test results showed that the solvent system of ammonium acetate could improve the peak shapes of glycyrrhetic acid. Good separation of target compounds and short run time were obtained using an elution system of 10 mmol/l ammonium acetate–acetonitrile (10:90 (v/v)). Representative chromatograms are shown in Fig. 2 in which the retention times were about 2.7 min for glycyrrhetic acid and 5.4 min for IS.

3.2. Conditions for ESI–MS

The ESI mass spectrum at a fragmentor voltage of 200 V showed that the negative ion $[M-H]^-$ of glycyrrhetic acid was at m/z 469.5. Fig. 3A shows a typical full-scan ESI-negative mass spectrum of glycyrrhetic acid at a 200 V fragmentor voltage. In order to determine the optimal fragmentor voltage, the intensities of selected ion of glycyrrhetic acid at m/z 469.5 was compared at

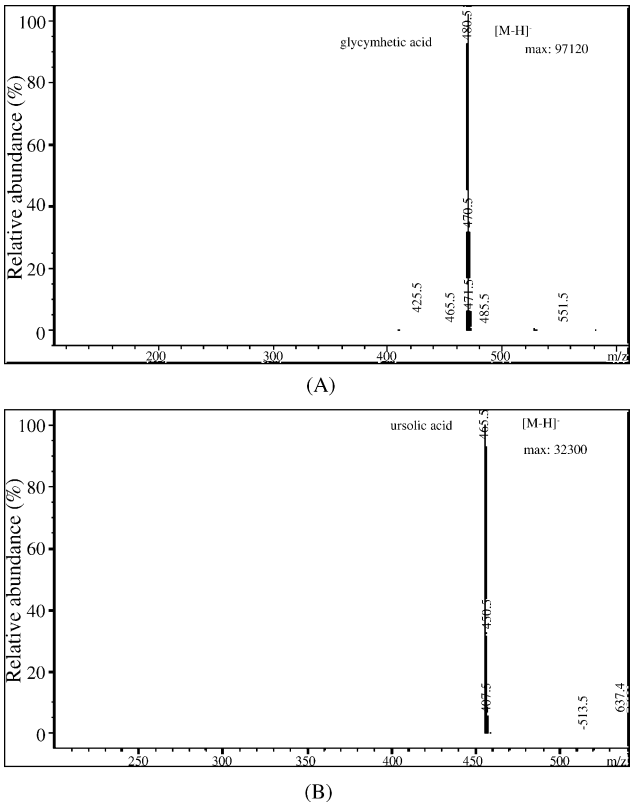

Fig. 3. Mass spectra of the negative ion of glycyrrhetic acid (A) and ursolic acid (B) at 200 V fragmentor voltage.

fragmentor voltages of 50, 70, 90, 100, 120, 150, 200 and 250 V. The result showed that the highest sensitivity was obtained using a fragmentor voltage of 200 V. Therefore, a fragmentor voltage of 200 V was used to carry out LC–ESI–MS in the SIM mode. At this fragmentor voltage, the most intensive ion of IS was at m/z 455.5, see Fig. 3B. Therefore, the negative ion $[M–H]^-$ (m/z 455.5) of IS was selected as the target ion of IS in the SIM.

3.3. Method validation

3.3.1. Calibration curve and sensitivity

The calibration curves, which relate the concentrations of glycyrrhetic acid to the area ratio of glycyrrhetic acid to IS, showed good linearity in the range of 0.1–400 ng/ml. The typical calibration curve for glycyrrhetic acid had a slope of 0.005523 ± 0.000114, and an intercept of 0.000122 ± 0.000075 and $R = 0.9995$. The lower limit of quantification (LOQ) for glycyrrhetic acid in plasma, defined as the lowest concentration at which both precision and accuracy were less than or equal to 20%, was 0.1 ng/ml. Those data show that this assay is sensitive enough for pharmacokinetic study of glycyrrhetic acid. Calibration curves were prepared with each batch of clinical samples.

3.3.2. Precision and accuracy

The intra- and inter-day ($n = 5$) precision and accuracy, shown in Table 1, were satisfactory for our purpose. Those results in Table 1 demonstrate that the precision and accuracy of this assay are acceptable.

Table 1
Precision and accuracy of the assay for determination of glycyrrhetic acid in plasma

Added to plasma (ng/ml)	Intra-assay measured concentration (mean ± S.D.) (ng/ml)	RE%	R.S.D.%	Inter-assay measured concentration (mean ± S.D.) (ng/ml)	RE%	R.S.D.%
0.1	0.101 ± 0.0108	0.97	10.65	0.104 ± 0.0106	3.91	10.17
1	0.990 ± 0.0337	−1.01	3.41	1.00 ± 0.0386	0.26	3.85
10	9.99 ± 0.151	−0.06	1.51	9.83 ± 0.145	−1.68	1.48
100	100.3 ± 1.4	0.28	1.43	100.1 ± 1.5	0.07	1.53
400	402.6 ± 6.2	0.64	1.53	397.5 ± 5.8	−0.63	1.47

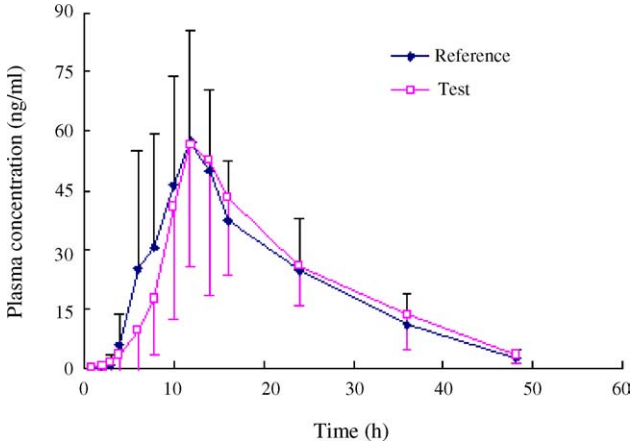

Fig. 4. Mean plasma concentration of glycyrrhetic acid–time profile in 20 healthy volunteers after a 100 mg oral dose.

Table 2
Pharmacokinetic parameters of glycyrrhetic acid for 20 volunteers after administration of a single dose of 100 mg diammonium glycyrrhizinate (mean ± S.D.)

Parameters	Test capsule	Reference capsule
$t_{1/2}$ (h)	8.8 ± 2.3	8.0 ± 2.2
C_{max} (ng/ml)	68.0 ± 37.2	65.2 ± 26.7
t_{max} (h)	12.9 ± 1.8	11.5 ± 2.3
AUC_{0-48} (h ng/ml)	1018 ± 429	1006 ± 426

3.3.3. Extraction recovery

Ethyl acetate was chosen as the extraction solvent for its higher extraction efficiency to the two target compounds. The mean extraction recovery of glycyrrhetic acid from human plasma with ethyl acetate was 94.51 ± 3.82%.

3.4. Application

The method described above was successfully applied to the pharmacokinetics study in which plasma concentrations of glycyrrhetic acid in 20 healthy, male Chinese volunteers were determined up to 48 h after administration of 100 mg diammonium glycyrrhizinate capsule. The mean plasma concentration of glycyrrhetic acid–time curve is shown in Fig. 4. The mean pharmacokinetic parameter values are calculated and summarized in Table 2. The relative bioavailability of test preparation was 103 ± 13%, based on the test–reference ratios of AUC. Results of variance analysis and two one-side *t*-test showed that there was no statistical significant difference between the two preparations in the AUC and C_{max}. In the case of T_{max}, comparison between the two preparations was carried out by the Wiloxon-ranked sign test for the matched pairs, and the statistical result showed that there was significant difference between the two preparations, and comparing with the reference capsule, the test preparation, an enteric-soluble capsule, showed a one and a half-hour delay to T_{max}.

4. Conclusion

This assay achieved higher sensitivity and better specificity for analysis of glycyrrhetic acid in human plasma. No significant interference caused by endogenous compounds was observed. This simple and rapid assay can be successfully used in pharmacokinetic studies of glycyrrhetic acid in human.

References

[1] S. Takeda, K. Ishthara, Y. Wakui, S. Amagaya, M. Maruno, T. Akao, K. Kobashi, J. Pharm. Pharmacol. 48 (1996) 902–905.
[2] Q.T. Gao, X.H. Chen, K.S. Bi, Biol. Pharm. Bull. 27 (2004) 226–228.
[3] Q. Xiang, G. Chen, J. Chen, J. Shenyang Pharm. Univ. 16 (1999) 107–109.
[4] P. Ren, L. Zhang, L. Wang, X. Huang, Y. Li, J. Nanjing TCM Univ. (Nat. Sci.) 16 (2002) 345–346.
[5] Y. Yamamura, J. Kawakami, T. Santa, H. Kotaki, K. Uchino, Y. Sawada, N. Tanaka, T. Iga, J. Pharm. Sci. 81 (1992) 1042–1045.
[6] Z.J. Lin, S.X. Qin, A. Wufuer, L. Shum, J. Chromatogr. B 814 (2005) 201–207.
[7] Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), May 2001.
[8] B. Boulanger, P. Chiap, W. Dewe, J. Crommen, Ph. Hubert, J. Pharm. Biomed. Anal. 32 (2003) 753–765.
[9] C. Hartmann, J. Smeyers-Verbeke, D.L. Massart, R.D. McDowall, J. Pharm. Biomed. Anal. 17 (1998) 193–218.
[10] G.S. Land, W.J. Leavens, B.C. Weatherley, in: E. Reid, I.D. Wilson (Eds.), Methodological Surveys in Biochemistry and Analysis, vol. 22, Royal Society of Chemistry, 1992, pp. 103–110.
[11] E.K. Kimanani, J. Lavigne, J. Pharm. Biomed. Anal. 16 (1998) 1107–1115.
[12] L.R. Snyder, J.J. Kirkland, J.L. Glajch, Practical HPLC Method Development, Wiley, New York, 1997, pp. 705–706.