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Journal of Pharmaceutical and Biomedical Analysis 40 (2006) 788–793
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Sensitive and selective liquid chromatography-electrospray ionisation-mass spectrometry analysis of astragaloside-IV in rat plasma

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Received 23 August 2005; received in revised form 8 October 2005; accepted 10 October 2005
Available online 22 November 2005

Abstract

Astragaloside-IV (3-*O*-beta-D-xylopyranosyl-6-*O*-beta-D-glucopyranosyl-cycloastragenol) is the major active constituent contained in Radix Astragali. This paper describes a rapid, sensitive and specific assay for quantitative determination of astragaloside-IV in rat plasma. After a liquid/liquid extraction (LLE) with *n*-butanol and high-performance liquid chromatography (HPLC) gradient separation with acetonitrile–NH_4Cl solution (0.5 μmol/L) as the mobile phase, the anions adduct $[M + Cl]^-$ at *m*/z 819.4 of astragaloside-IV, and $[M + Cl]^-$ at *m*/*z* 815.35 of internal standard (IS) digoxin were analyzed by electrospray ionisation-mass spectrometry (LC-ESI-MS) in selected ion monitoring (SIM) mode. Chromatographic separation was achieved in less than 9 min and calibration curve was linear over a concentration range of 2–200 ng/ml. The described assay method was successfully applied to the preclinical pharmacokinetic study of astragaloside-IV. After intragastric administration of astragaloside-IV to rats, *C*max and *T*max of astragaloside-IV were 134.73 ± 39.86 ng/ml and 1.5 h, respectively, and the elimination half-life ($t_{1/2}$) was 5.45 ± 0.39 h.

Keywords: Astragaloside-IV; LC-ESI-MS; Pharmacokinetics

1. Introduction

Astragaloside-IV (3-*O*-beta-D-xylopyranosyl-6-*O*-beta-D-glucopyranosyl-cycloastragenol) is the major active constituent of Radix Astragali used widely in traditional Chinese medicine [1]. It possesses well-documented hepatoprotective [2], antiviral [3], anti-inflammatory [4], antinociceptive [5], antihypertensive [6] and immunostimulant activities [7]. Astragaloside-IV, molecular structure shown in Fig. 1, is a purified saponin (molecular weight 784). Studies in vitro have demonstrated that astragaloside-IV is a strong scavenger for superoxide radicals and hydroxyl radicals [8]. It was evidenced that astragaloside-IV has a neuroprotective effect in the murine model of focal cerebral ischemia/reperfusion and its anti-infarction effect might be derived at least in part from its antioxidant properties [9]. Although plenty of works on the pharmacology were reported, pharmacokinetic properties of astragaloside-IV were seldom studied, which is essential for its further research as a novel drug.

Many analysis methods, such as high-performance liquid chromatography (HPLC) [10], HPLC with evaporative light-scattering detection (ELSD) [11] and a precolumn derivatization [12] method have been developed for the determination of astragaloside-IV. However, most of them were developed for the quantitative determination of astragaloside-IV in the plants or medical preparations, in which the content of astragaloside-IV was taken as a parameter for quality control (QC) according to the Chinese Pharmacopoeia. Considering their poor sensitivity, these methods cannot be used for bio-sample analysis. Liquid chromatography–mass spectrometry (LC–MS) [13] and LC–MS/MS with a solid phase extraction (SPE) [14] had been established for the measurement of astragaloside-IV. The same extraction procedure has been established during our previously study in our lab. However, the SPE involved high-cost and

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doi:10.1016/j.jpba.2005.10.015

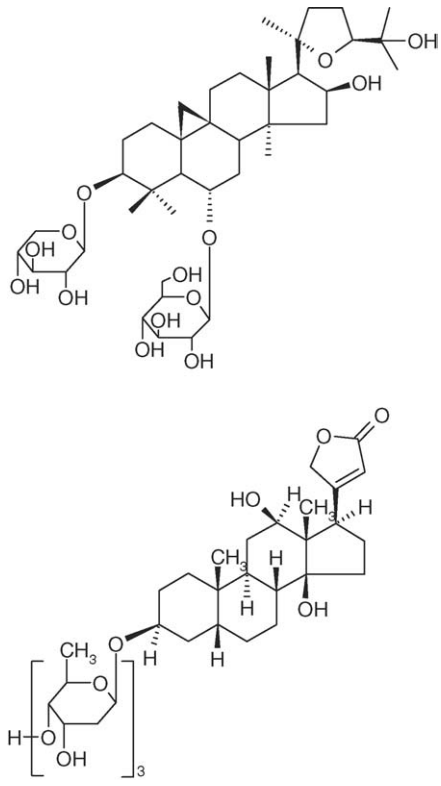

Fig. 1. Structures of astragaloside-IV and digoxin (IS).

time-consuming procedures. Moreover, all of them required a relatively large volume of plasma samples (500 μl) for extraction, which results in the need of considerable rats in pharmacokinetic study. Recently, Yan and Guo [15] established a LC–MS/MS method with precipitation of the plasma protein by acetonitrile. Although this method was simple and sensitive, it might not give the background as clear as the method with SPE or liquid/liquid extraction (LLE) could give which may affect its sensitivity and might cause damage to the column for the long-term usage. In this paper we described a simple, sensitive and reliable LC–MS method using LLE for sample preparation and electrospray ionisation (ESI) in negative mode for the quantitative determination of astragaloside-IV in biological samples.

2. Experimental

2.1. Chemicals and reagents

Astragaloside-IV (>99.0%) and digoxin (>99.5%) (internal standard, IS) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade acetonitrile was obtained from Fisher Scientific (Toronto, Canada). HPLC grade water was produced by a Milli-Q Reagent Water System (Millipore, MA, USA). Methanol, *n*-butanol and others chemicals and solvents used were analytical grade.

Table 1
Gradient elution program for the separation of astragaloside-IV

Time (min)	Solvent A (%)	Solvent B (%)	Gradient curve
0.0	65	35	Linear
2.8	5	95	–
4.8	5	95	Linear
5.0	65	35	–
9.0	65	35	–

A: NH_4Cl solution (0. 5 μmol/L) and B: acetonitrile.

2.2. Animals

Sprague–Dawley rats (190–210 g) were obtained from Sino-British Sippr/BK Lab Animal Ltd. (Shanghai, China) and housed with unlimited access to food and water except for fasting 12 h before the experiment. The animals were maintained on a 12-h light/12-h dark cycle (light on at 8:00) at ambient temperature (22–24 °C) and at 60% relative humidity. Animal experiments were carried out in accordance with the Guidelines for Animal Experimentation of China Pharmaceutical University (Nanjing, China) and protocol was approved by the Animal Ethics Committee of this institution.

2.3. Liquid chromatography–mass spectrometry

The HPLC system consisted of a DGU-14 AM degasser, two LC-10ADvp pumps, a high-pressure mixer, a CTO-10Avp column oven and a Shimadzu 10ATvp Autoinjector (Shimadzu, Kyoto, Japan). A Shimadzu 2010 liquid chromatography–mass spectrometer (Shimadzu, Kyoto, Japan) equipped with electrospray ionization probe, QoQ system (Q-array-Octapole-Quadrupole mass analyzer) was used in the study.

The analysis was carried out on Kromasil C_{18} column (150 mm × 2.1 mm i.d., 3.5 μm, Phenomenex, USA), equipped with an ODS guard column (Security Guard, Phenomenex, USA). A gradient elution mode was adopted using two mobile phases: (A) NH_4Cl solution (0.5 μmol/L) and (B) acetonitrile. The flow rate was 0.2 ml/min. The gradient elution program is indicated in Table 1.

Mass spectrometric conditions were optimized to obtain maximum sensitivity. The final ESI conditions used were as follows: curve dissolution line (CDL) voltage was fixed as that of tuning (15 kV). Probe high voltage (4.5 kV), Q-array voltage of dc −35 V and RF 150 V. Mass spectra were obtained at a dwell time of 0.2 s in SIM mode and 1 s in scan mode. Liquid nitrogen (99.995%, from Gas Supplier Center of Nanjing University, China.) was used as the nebulizing gas at 1.5 and 2.0 L/min as curtain gas. LC Mass Solution Version 2.02 worked on Windows 2000. Vacuum in the mass detector was obtained by Turbo molecular pump (Edwards 28, England).

2.4. Preparation of stock solution, standards and QC samples

A stock solution of astragaloside-IV was prepared by dissolving the accurately weighed reference compound in methanol to

give a final concentration of 1 mg/ml. The solution was then serially diluted with methanol to achieve standard working solutions at concentrations of 20, 50, 100, 200, 500, 1000 and 2000 ng/ml for astragaloside-IV. A 500 ng/ml internal standard working solution was prepared by diluting the stock solution of digoxin of 20 μg/ml with methanol. The quality control samples were prepared in a similar way at concentrations of 2 ng/ml (lower limit of quantitation, LLOQ), 5 ng/ml (low), 20 ng/ml (medium) and 100 ng/ml (high).

2.5. Sample preparation

Each collected blood sample was immediately centrifuged at $1500 \times g$ for 5 min and plasma was transferred into a clean Eppendorf tube. The plasma samples were stored at −20 °C until analysis. Aliquots (100 μl) of the plasma, spiked with internal standard working solution (10 μl), were vortex-mixed for 30 s and then extracted with *n*-butanol (1 ml) for 2 min using a vortex mixer (Scientific industries, Inc., USA). After centrifugation at $12000 \times g$ for 5 min, the upper organic phase (800 μl) was transferred into clean tubes and evaporated to dryness in the Thermo Savant SPD 2010 SpeedVac System (Thermo Electron Corporation, USA). The residue was then reconstituted in 100 μl methanol immediately before LC–MS analysis. After centrifugation at $21000 \times g$ for 10 min at 4 °C, the supernatant was transferred to 1.5 ml autosampler vial. A 10 μl of the supernatant was used for analysis.

2.6. Calibration curves and assay validation

The calibration curve consisted of seven concentration levels (2, 5, 10, 20, 50, 100, 200 ng/ml of astragaloside-IV in rat plasma). The calibration curve was constructed by adding 10 μl of IS and varying the concentrations of astragaloside-IV to blank rat plasma. Plasma samples were quantified using the ratio of the peak area of astragaloside-IV to that of IS. Peak area ratios were plotted against concentrations and astragaloside-IV concentrations were calculated using a least squares linear regression. Linear regression analysis was performed using Microsoft Excel 2002.

The precision and accuracy of the assay were obtained by comparing the predicted concentration (obtained from the calibration curve) to the actual concentration of astragaloside-IV spiked in blank plasma. The lower limit of quantitation was considered as the concentration that produced a signal-to-noise (S/N) ratio of 10. The recovery was calculated from the ratio of the peak area of astragaloside-IV after extraction from plasma to the peak area of an equivalent amount of the standard solution. The precision and accuracy were determined by back calculation of spiked plasma samples at four concentrations with respect to a calibration graph prepared each day. The precision was express as the inter-batch and intra-batch coefficient of variation (%). The accuracy was calculated as the mean deviation of each concentration from the theoretical value.

2.7. Stability

The stability was assessed at three concentration levels (5, 20 and 100 ng/ml). The freeze and thaw stability study samples at three concentrations were frozen at −20 °C for 7 days, then thawed at 37 °C. After complete thawing, the samples were refrozen for 24 h under the same conditions. This freeze-thaw cycle was repeated for three times before these samples were analyzed using the method described above. The stability of astragaloside-IV in processed plasma at 4 °C, during storage in

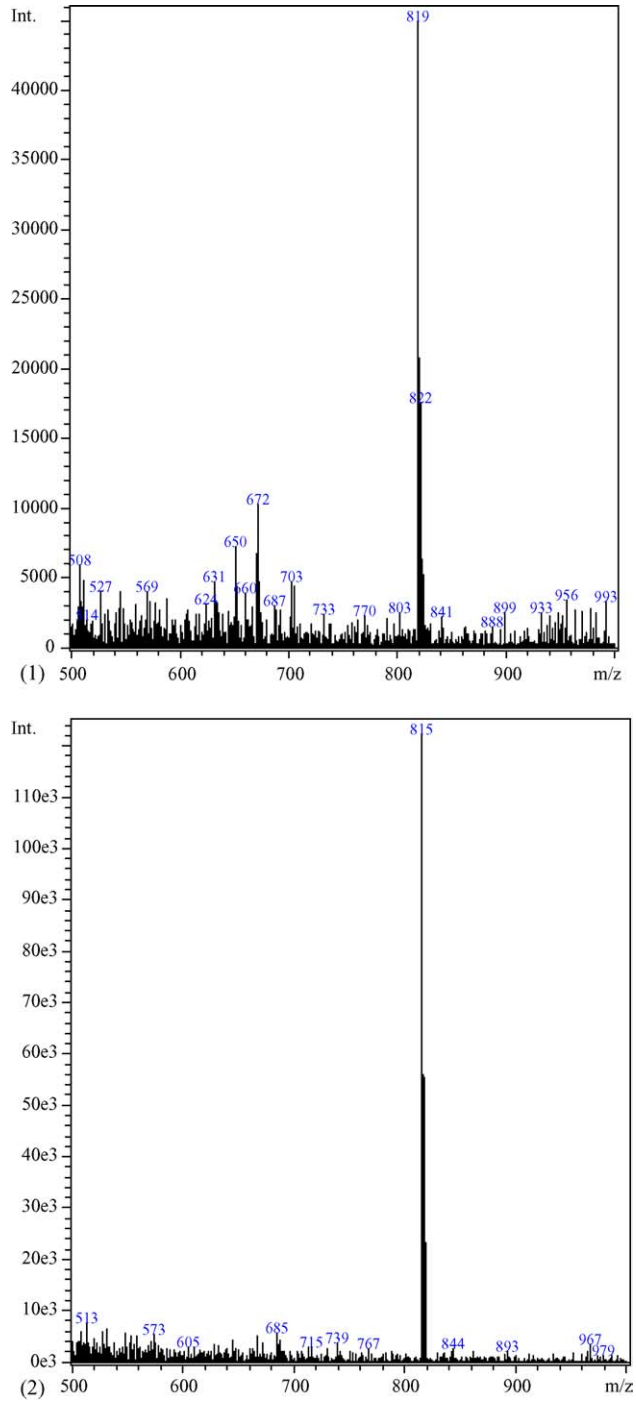

Fig. 2. Mass spectra of astragaloside-IV and digoxin (IS).

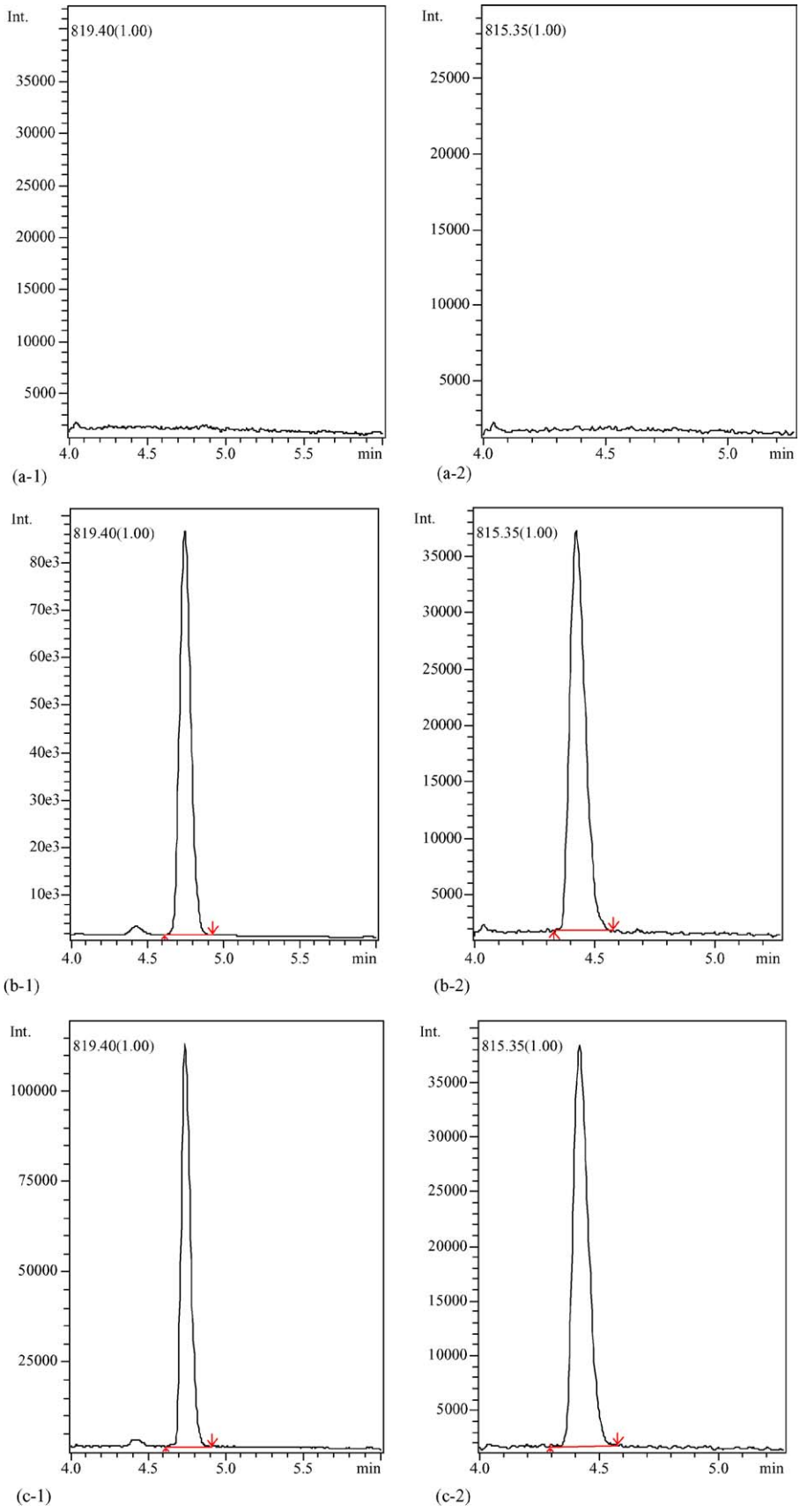

Fig. 3. Representative chromatograms of blank plasma (a); blank plasma spiked with astragaloside-IV(b-1) and IS(b-2); and plasma sample from rat treated with astragaloside-IV (c). The *m/z* 819.4 for astragaloside-IV and *m/z* 815.35 for IS.

Table 2
Intra-batch precision and accuracy of measurement of astragaloside-IV when using negative ion detection with selective ion monitoring ($n = 5$)

Astragaloside-IV nominal concentration (ng/ml)	Astragaloside-IV (mean ± S.D.) determined concentration (ng/ml)	Accuracy (%)	Precision (% R.S.D.)
2	2.25 ± 0.11	112.7	4.68
5	5.19 ± 0.11	103.7	2.07
20	21.03 ± 0.50	105.2	2.37
100	99.59 ± 1.06	99.6	1.06

the autosampler, was performed by repeated injection every 4 h for a period of 24 h. The stability of astragaloside-IV in plasma at ambient temperature was assessed by processing and analysing plasma samples in triplicate after storage for 24 h on the laboratory bench.

2.8. Pharmacokinetic study

After oral administration of astragaloside-IV (10.0 mg/kg), which was suspended in 0.5% sodium carboxymethyl cellulose (CMC-Na), 0.2 ml of blood samples were collected from rats at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 h. Blood samples were centrifuged immediately to separate 100 μl plasma, which were stored at −20 °C until analysis.

3. Results and discussion

3.1. Specificity, linearity and sensitivity

Resolution, sensitivity and mass number calibration were optimized by auto tuning program using polyethylene glycol mixture, before analysis. Fig. 2 shows mass spectra of astragaloside-IV. Mass spectra for astragaloside-IV and digoxin were dominated by the $[M + Cl]^-$ ions. Results of LC–MS analysis of the blank plasma samples showed no interfering peaks present in six different randomly selected samples of drug free mice plasma used for analysis at the retention times of either analyte or internal standard (Fig. 3). Therefore, m/z 819.40 and 815.35 for astragaloside-IV and digoxin, respectively, were chosen for quantification. Sensitivity of the method was further improved by addition of ammonium chloride to the mobile phase. Digoxin was selected as an internal standard because of its similar ionization condition, appropriate m/z value, and good recovery efficiency (95%) as compared to astragaloside-IV.

Visual inspection of the plotted five calibration curves and correlation coefficients (>0.999) confirmed that the calibration curves were linear over the concentration ranges 2–200 ng/ml. The following equation was obtained:

$$y = 0.0235x - 0.0027$$

The LLOQ was 2 ng/ml for determination of astragaloside-IV in 100 μl plasma and is sufficient to support its pharmacokinetic studies. A gradient elution mode was used to obtain baseline separation of the target analytes in the column. HPLC elute was directed to a waste container in the first 4 min of the chromatographic run and then was switched to MS for detection by a six-port switching valve, which assured the target analytes eluted in a more clean way, and thus, with less matrix effects. Therefore, the sensitivity with this method was comparable to the method with SPE.

3.2. Accuracy and precision

Tables 2 and 3 shows a summary of intra- and inter-batch precision and accuracy at the LLOQ and at low, medium and high concentrations of astragaloside-IV in plasma. The results shown in Table 2 indicate that the assay method is reproducible for replicate analysis within the same day. The results shown in Table 3 suggest that the assay method is reproducible on different 3 days. These results indicated that the present method has a satisfactory accuracy, precision and reproducibility.

3.3. Recovery

The absolute recoveries of astragaloside-IV at concentrations of 2, 5, 20 and 100 ng/ml ($n = 5$) from rat plasma were determined to be 92.03 ± 0.08%, 93.52 ± 0.07%, 97.12 ± 0.05% and 94.69 ± 0.04%, respectively. The mean recoveries of them were more than 90% ($n = 5$).

Table 3
Inter-batch precision and accuracy of measurement of astragaloside-IV when used negative ion detection with selective ion monitoring ($n = 5$)

Astragaloside-IV nominal concentration (ng/ml)	Astragaloside-IV (mean ± S.D.) determined concentration (ng/ml)	Accuracy (%)	Precision (% R.S.D.)
2	2.20 ± 0.19	110.0	8.59
5	5.20 ± 0.25	104.0	4.81
20	21.01 ± 1.10	105.0	5.24
100	102.45 ± 4.11	102.5	4.01

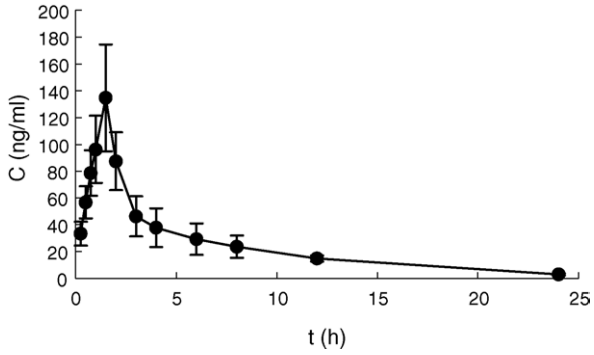

Fig. 4. Plasma concentration–time profile of astragaloside-IV in rat after administration of a single dose of astragaloside-IV.

3.4. Stability

The stability of astragaloside-IV during the sample storing and processing procedures was fully evaluated by analyzing triplicate of QC samples at the concentration of 5, 20 and 100 ng/ml for both analytes. QC samples of astragaloside-IV obtained by extraction showed no significant degradation after at least 24 h at room temperature (7.9% deviation of the spiked values). Standard stock solution of astragaloside-IV was shown to remain stable for at least 2 month at 4 °C. The concentration variations found after three-cycles of freezing and thawing were within ±8.5% of nominal concentrations, indicating no significant substance loss after three repeated freezing and thawing. When processed samples were stored at 4 °C in the autosampler, astragaloside-IV showed a very good stability, and the responses varied no more than ±10% within 24 h of storage at the concentrations studied.

3.5. Pharmacokinetic study

This simple, precise and accurate LC–MS method yields satisfactory results for determination of astragaloside-IV in rat plasma and has been used successfully in a pharmacokinetic study in six rats following a single oral administration. The mean plasma concentration–time profile of astragaloside-IV illustrates in Fig. 4. The *C*max and *T*max of astragaloside-IV were 134.73 ± 39.86 ng/ml and 1.5 h, respectively, and the elimination half-life ($t_{1/2}$) was 5.45 ± 0.39 h.

4. Conclusion

The method described here is simple, rapid, and specific for the determination of astragaloside-IV in rat plasma. The method can be applied successfully to pharmacokinetic study in the rat and may be adequate to measure the drug concentration in other bio-samples, such as in tissues, urine and bile, etc.

Acknowledgements

This study was supported by National “863” Project (no. 2003AA2Z347A), Jiangsu Key Lab of Drug Metabolism and Pharmacokinetics (no. BM2001201) and Jiangsu international cooperation Fund (BZ2004042). The authors would like to thank Professor Fenzhi Sun for the revise of this manuscript.

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