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Journal of Chromatography B, 822 (2005) 170–177
JOURNAL OF CHROMATOGRAPHY B
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Quantitative determination of Astragaloside IV, a natural product with cardioprotective activity, in plasma, urine and other biological samples by HPLC coupled with tandem mass spectrometry

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Received 31 March 2005; accepted 20 May 2005
Available online 28 June 2005

Abstract

Astragaloside IV is a novel cardioprotective agent extracted from the Chinese medical herb *Astragalus membranaceus* (Fisch) Bge. This agent is being developed for treatment for cardiovascular disease. Further development of Astragaloside IV will require detailed pharmacokinetic studies in preclinical animal models. Therefore, we established a sensitive and accurate high performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (LC/MS/MS) quantitative detection method for measurement of Astragaloside IV levels in plasma, urine as well as other biological samples including bile fluid, feces and various tissues. Extraction of Astragaloside IV from plasma and other biological samples was performed by Waters OASIS™ solid phase extraction column by washing with water and eluting with methanol, respectively. An aliquot of extracted residues was injected into LC/MS/MS system with separation by a Cosmosil C_{18} (5 μm, 150 mm × 2.0 mm) column. Acetonitrile:water containing 5 μM NaAc (40:60, v/v) was used as a mobile phase. The eluted compounds were detected by tandem mass spectrometry. The average extraction recoveries were greater than 89% for Astragaloside IV and digoxin from plasma, while extraction recovery of Astragaloside IV and digoxin from tissues, bile fluid, urine and fece ranged from 61 to 85%, respectively. Good linearity ($R^2 > 0.9999$) was observed throughout the range of 10–5000 ng/ml in 0.5 ml rat plasma and 5–5000 ng/ml in 0.5 ml dog plasma. In addition, good linearity ($R^2 > 0.9999$) was also observed in urine, bile fluid, feces samples and various tissue samples. The overall accuracy of this method was 93–110% for both rat plasma and dog plasma. Intra-assay and inter-assay variabilities were less than 15.03% in plasma. The lowest quantitation limit of Astragaloside IV was 10 ng/ml in 0.5 ml rat plasma and 5 ng/ml in 0.5 ml dog plasma, respectively. Practical utility of this new LC/MS/MS method was confirmed in pilot pharmacokinetic studies in both rats and dogs following intravenous administration.

Keywords: Astragaloside IV; HPLC; LC/MS/MS; Pharmacokinetics

1. Introduction

The saponin Astragaloside IV, a -*O*-beta-D-xylopyranosyl-6-*O*-beta-D-glucopyranosylcycloastragenol (for chemical structure see Fig. 1), was purified from the Chinese medical herb *Astragalus membranaceus* (Fisch) Bge. Pharmacological studies indicated that Astragaloside IV possesses antihypertensive, positive inotropic action, anti-inflammatory activity, treating viral myocarditis, antinociceptive effects, hepato-protective effects, a neuroprotective effect, anti-infarction effect and antiviral activity [1–8]. In addition, Astragaloside IV has been demonstrated to increase T, B lymphocyte proliferation and antibody production in vivo and in vitro; but inhibited productions of IL-1 and TNF-alpha from peritoneal macrophages in vitro. This compound is now being developed as cardioprotective agent for treating cardiovascular diseases. There is merit in

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doi:10.1016/j.jchromb.2005.05.034

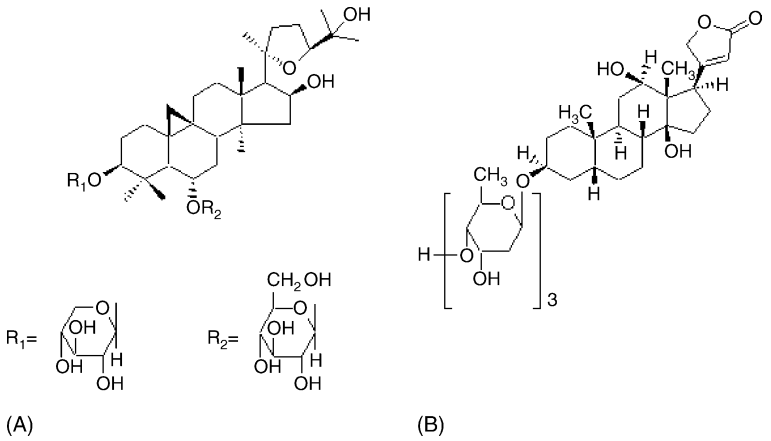

Fig. 1. Chemical structure of Astragaloside IV extracted from the Chinese medical herb *Astragalus membranaceus (Fisch) Bge* (A) and internal standard digoxin (B).

characterizing the pharmacokinetics of Astragaloside IV in animals.

HPLC-UV, HPLC-ELSD and HPLC-RID have been developed to determine Astragaloside IV in *Radix Astragali* or formulation [9–12]. However, those methods were not sensitive enough for pharmacokinetic studies. HPLC coupled with a single quadrupole mass spectrometer (LC/MS) has also been evaluated for quantitating the Astragaloside IV in plasma and urine [13–16]. To our knowledge, there was no LC/MS/MS using a triple quadrupole mass spectrometer method currently available for analyzing Astragaloside IV in biological samples.

We describe a rapid, selective and sensitive high performance liquid chromatographic technique coupled with tandem mass spectrometry detection for the determination of Astragaloside IV in plasma and other biological samples. Using the described method in the paper, pilot pharmacokinetic studies of Astragaloside IV in rats and dogs were characterized.

2. Materials and methods

2.1. Chemicals and drugs

Methanol and acetonitrile (HPLC grade) were obtained from Merck (Darmstadt, Germany). Heparine sodium was purchased from Huixin Biochemical Reagents Inc (Shanghai, China). All other reagents are analytical pure.

Astragaloside IV (lot no. 030510) was extracted from the Chinese medical herb *Astragalus membranaceus* (Fisch) Bge with purity of 99.2% by HPLC analysis. Internal standard digoxin (lot No. 0015-9706) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Distilled, deionized water was produced by a Milli-Q Reagent Water System (Millipore, MA, USA).

2.2. Standard solutions

Stock solutions of Astragaloside IV (lot no. 030510) were prepared in ethanol-propylene glycol (50:50, v/v) at a concentration of 10 mg/ml and internal standard digoxin (lot No. 0015-9706) was prepared in methanol at a concentration of 0.04 mg/ml. Both stock solutions were stored at −20 °C until use.

2.3. Apparatus and chromatographic conditions

The HPLC system (Palo Alto, CA, USA) consisted of a Hewlett Packard (HP) series 1100 in conjunction with a quaternary pump, an autosampler, an autoelectronic degasser, an automatic thermostatic column compartment and a computer with a Chemstation software program for analysis of the HPLC data. The analytical column used was a Cosmosil C_{18} (5 μm, 150 mm × 2.0 mm), which was provided by Nacalai (Kyoto, Japan). The mobile phase was a mixture of acetonitrile and water containing 5 μM NaAc (40:60, v/v). The mobile phase was degassed automatically using the electronic degasser system. The column was equilibrated and eluted under isocratic conditions utilizing a flow rate of 0.2 ml/min at ambient temperature.

Mass detection was carried out using a triple quadrupole mass spectrometer with TurboIonSpray, which is connected to the liquid chromatography system (Applied Biosystems, Foster City, CA, USA). High-purity nitrogen was provided by a liquid nitrogen tank. The conditions for mass spectrometry were set at positive ion monitoring. The selected ion monitoring (SIM): Astragaloside IV at *m/z*: 807 $[M + Na]^+$, digoxin at *m/z*: 804 $[M + Na]^+$, curtain gas (CUR): 15, ionspray voltage (IS): 5200, temperature (TEM): 400, ion source gas1 (GS1): 20, ion source gas2 (GS2): 50, declustering potential (DP): 120, focusing potential (FP): 250, entrance potential (EP): −8. Analyst™ Software provides data processing and total instrument control of data acquisition.

2.4. Extraction procedures

2.4.1. Plasma samples

The plasma samples (0.5 ml/sample) were spiked with 12.5 μl of known amounts of internal standard digoxin to yield a final concentration of 1 μg/ml. The samples were vortexed and centrifuged at 3500 rpm for 10 min. The supernatant was added to an active Waters OASIS™ solid phase extraction column (Waters (Shanghai) Inc), the column was washed with 4 ml of water and eluted with 1 ml methanol. The elutient was dried under nitrogen blow at 45 °C. The residue was dissolved with 200 μl mobile phase. After centrifugation at 15,000 rpm for 10 min at 4 °C, the supernatant was directly injected into the HPLC system for LC/MS/MS analysis. The ratio of peak area of Astragaloside IV over the internal standard was used for quantitative analysis.

2.4.2. Tissue samples

The rat tissues were accurately weighed, cut into slices and homogenated after adding the appropriate water (1 ml/0.3 g tissue). To tissue homogenate, 12.5 μl of known amounts of internal standard digoxin (0.04 mg/ml) was added to yield a final concentration of 500 ng/tissue. The protein was precipitated by addition of 1.5 ml methanol, centrifuged at 3000 rpm for 5 min after vortexing for 3 min. The supernatant (1.0 ml) was taken, placed into another tube and re-centrifuged at 12000 rpm for 10 min. The supernatant (800 μl) was filtered and 10 μl was injected into HPLC for analysis.

2.4.3. Bile fluid

To 0.2 ml of bile fluid, 0.8 ml of water was added, followed by 5 μl of internal standard digoxin (0.04 mg/ml). The mixture was centrifuged at 3500 rpm for 10 min. The supernatant was extracted by an active solid phase extraction column and analyzed as described above.

2.4.4. Urine samples

Twenty-five microlitres of internal standard digoxin (0.04 mg/ml) was added to a 1 ml urine sample. The mixture was centrifuged at 3500 rpm for 10 min and extracted under solid phase extraction and analyzed as described above.

2.4.5. Feces samples

The rat feces was dried and grounded. The mixture was centrifuged at 3500 rpm for 10 min following addition of 2 ml of methanol. To 1 ml of supernatant, 25 μl of internal standard digoxin (0.04 mg/ml) was added. After filtration, 10 μl of filtrated solution was injected into LC/MS/MS for analysis.

2.5. Extraction recovery

Replicate ($N = 3$) plasma samples (0.5 ml/sample) were spiked with known amounts of Astragaloside IV to yield a final concentration of 10, 100 and 2500 ng/ml, and replicate ($N = 3$) plasma samples (0.5 ml/sample) were spiked with 1000 ng/ml digoxin, respectively. To study extraction recovery of Astragaloside IV from various tissues, bile fluid, urine and fece, the Astragaloside IV was spiked into various tissue samples and biological fluids to yield final concentration of 100 and 1000 except 2500 ng/ml in both bile fluid and fece, while digoxin was spiked to yielded the final concentration of 1000 ng/ml except 2500 ng/ml in bile fluid and fece ($N = 2$), respectively. The spiked samples were extracted following the above described extraction procedures. The extraction recovery (ER) was calculated using the formula: $ER\% = \{\text{Peak area [Astragaloside IV or digoxin]}_{\text{extracted}}/\text{peak area [Astragaloside IV or digoxin]}_{\text{unextracted}}\} \times 100\%$.

2.6. Calibration curves

A calibration curve was generated to confirm the linear relationship between the peak area ratio and the concentration of Astragaloside IV in the test samples. Astragaloside IV was added to plasma to yield final concentrations of 10, 50, 100, 500, 1000, 2500 and 5000 ng/ml in 0.5 ml rat plasma, and 5, 10, 50, 100, 500, 1000, 2500 and 5000 ng/ml in 0.5 ml dog plasma. The plasma with known amounts of Astragaloside IV and internal standard were extracted in a solid phase column and analyzed as described above. The standard curves were generated by plotting the peak area ratio against the drug concentrations tested.

2.7. Intra-assay and inter-assay accuracy and precision

Intra-assay and inter-assay accuracy and precision were determined followed method described [17–20]. To evaluate the accuracy, Astragaloside IV was added to drug-free plasma at concentrations of 10, 100 and 2500 ng/ml in 0.5 ml rat plasma and 10, 500 and 2500 ng/ml in 0.5 ml dog plasma. The spiked plasmas were treated as described above. These standard samples were prepared and analyzed within 24 h. The concentrations were calculated using a standard curve. The ratio of the calculated concentration over the known concentration of Astragaloside IV was used as the accuracy of the analytical method.

To evaluate the intra-assay and inter-assay precision, Astragaloside IV was added to drug-free plasma at concentrations of 10, 500 and 2500 ng/ml in 0.5 ml rat and dog plasma. The spiked plasmas were treated as described above and the concentrations were calculated using a standard curve. The coefficient of variance was used as an index of precision. These standard samples were prepared and analyzed within 24 h for intra-assay precision. The inter-assay precision was determined using five independent experiments.

2.8. Stability testing

Astragaloside IV was added to plasma to yield final concentrations of 10, 500 and 2500 ng/ml. The spiked plasma was stored at −70 °C. After a week of storage at this tem-

perature, the sample was removed and thawed at 37 °C and extracted as described above.

2.9. Quality control

In order to determine the reliability of LC/MS/MS method, three control samples containing 10, 100 or 2500 ng/ml were prepared by the Lab Manager, and single blind method was adopted for analytical scientists to measure those samples. The testing was repeated for five times.

2.10. Drug administration and sampling

Thirty six male Sprague–Dawley rats (200 ± 20 g) were provided by Sino-British Sippr/BK Lab Animal Ltd. (Shanghai, China) (Permit No. SCXK Shanghai 2003-0002) and housed six to a cage with unlimited access to food and water except for 12 h before and during the experiment. The animals were maintained on a 12 h light–dark cycle (light on from 8:00 to 20:00 h) at ambient temperature (22–24 °C) and ca. 60% relative humidity. Three male Beagle dogs, 8–10 kg were provided by Experimental Animal Inc of Second Military Medical University (Shanghai, China). Animal studies were approved by Experimental Animal Inc. of Second Military Medical University (Shanghai, China). Animal studies were approved by the Second Military Medical University Animal Ethics Committee. The experimental procedures were carried out in accordance with the Guidelines for Animal Experimentation of Second Military Medical University (Shanghai, China).

In order to determine the pharmacokinetics of Astragaloside IV after intravenous administration, the animals were fasted for 12 h. Astragaloside IV was dissolved in ethanol–propylene glycol (50:50, v/v) immediately before pharmacokinetic studies and the injected volume was adjusted at 0.25 ml/100 g for rats and 0.5 ml/kg for dogs. The intravenous bolus dose was 0.75 mg/kg for rats and 0.5 mg/kg for dogs. The plasma samples (4 ml) were withdrawn at 2, 10, 20, 30, 45 and 60 min and 1.5, 2, 3, 4, 6 and 8 h (1 sample/group $n = 3$) in rats after intravenous injection. The plasma samples (2 ml) were withdrawn at 2, 10, 20, 30, 45 and 60 min and 1.5, 2, 3, 4, 6, 8 and 12 h from femoral vein of dogs. The plasma samples were placed in heparinized tubes and were separated following centrifugation at 3000 rpm for 10 min and stored at −20 °C until analysis.

2.11. Pharmacokinetic analysis

Data fitting and pharmacokinetic parameter calculations were carried out using the 3p87 pharmacokinetic program (Chinese Pharmacological Society). An appropriate pharmacokinetic model was chosen on the lowest Akaike's information criterion (AIC) value under equal weight scheme. The area under the curve (AUC) was calculated by the trapezoidal rule between first (0 h) and last sampling time plus C_n/λ_n, where C_n is the concentration of last sampling, and λ_n is the elimination rate constant. $AUC_{0\rightarrow t} = \Sigma(C_i + C_{i-1})(t_i - t_{i-1})/2$; $AUC_{0\rightarrow\infty} = AUC_{0\rightarrow t} + C_n/\lambda_n$. The elimination half-life was estimated by linear regression analysis of the terminal phase of the plasma concentration-time profile.

3. Results and discussions

3.1. Chromatographic separations

Several combinations of acetonitrile and water (with 5 μM NaAc) were evaluated as possible mobile phases. It was determined that the combination of acetonitrile and water (with 5 μM NaAc) (40:60, v/v) described herein was found to be the most suitable for separating Astragaloside IV and internal standard digoxin. Under the described chromatographic conditions with a mobile phase of acetonitrile/water containing 5 μM NaAc (40:60, v/v), the retention time was about 3.4 min (ranging from 3.2 min to 3.7 min) for Astragaloside IV and 2.4 min for the internal standard. At the retention time, Astragaloside IV and the internal standard were eluted without an interference peak from the blank rat plasma (Fig. 2A–C) and also from the blank dog plasma (Fig. 3A–C). Similarly, there was no interference peaks from urine, bile fluid, fece samples and other tissue samples (Figures not shown). We also tested co-extract and co-elute with other saponins including Bacopaside I, Bacopaside II and Bacopasaponin C, no interference occurred. Therefore, the described method has good specificity.

It was interesting to find that no fragmentation occurred under declustering potential tested from 0 to 200 V. Therefore, selected reaction monitoring is actually selected ion monitoring. In our lab, we have found that some of other saponins such as Bacopaside I, Bacopaside II and Bacopasaponin C are difficulty to be fragmented, even if fragmentation occurred, the abundant of fragmentation was very low (unpublished data).

3.2. Extraction recovery

Extraction of Astragaloside IV and digoxin from both rat and dog plasma using solid phase extraction described above was demonstrated to be satisfactory, extraction recoveries for Astragaloside IV was 93.2 ± 4.1, 90.3 ± 1.97 and 92.7 ± 2.26% from rat plasma, 94.5 ± 4.57, 91.4 ± 4.11 and 93.1 ± 5.75% from dog plasma at final concentration of 10, 100 and 2500 ng/ml, respectively. The extraction recovery of digoxin was 90.7 ± 4.52% from rat plasma and 89.6 ± 5.20% from dog plasma, respectively. Extraction recovery of Astragaloside IV and digoxin from tissues, bile fluid, urine and fece ranged from 61 to 85%, respectively (Table 1).

3.3. Calibration curve

The standard curve obtained from solid phase extraction of plasma containing known amounts of Astragaloside IV

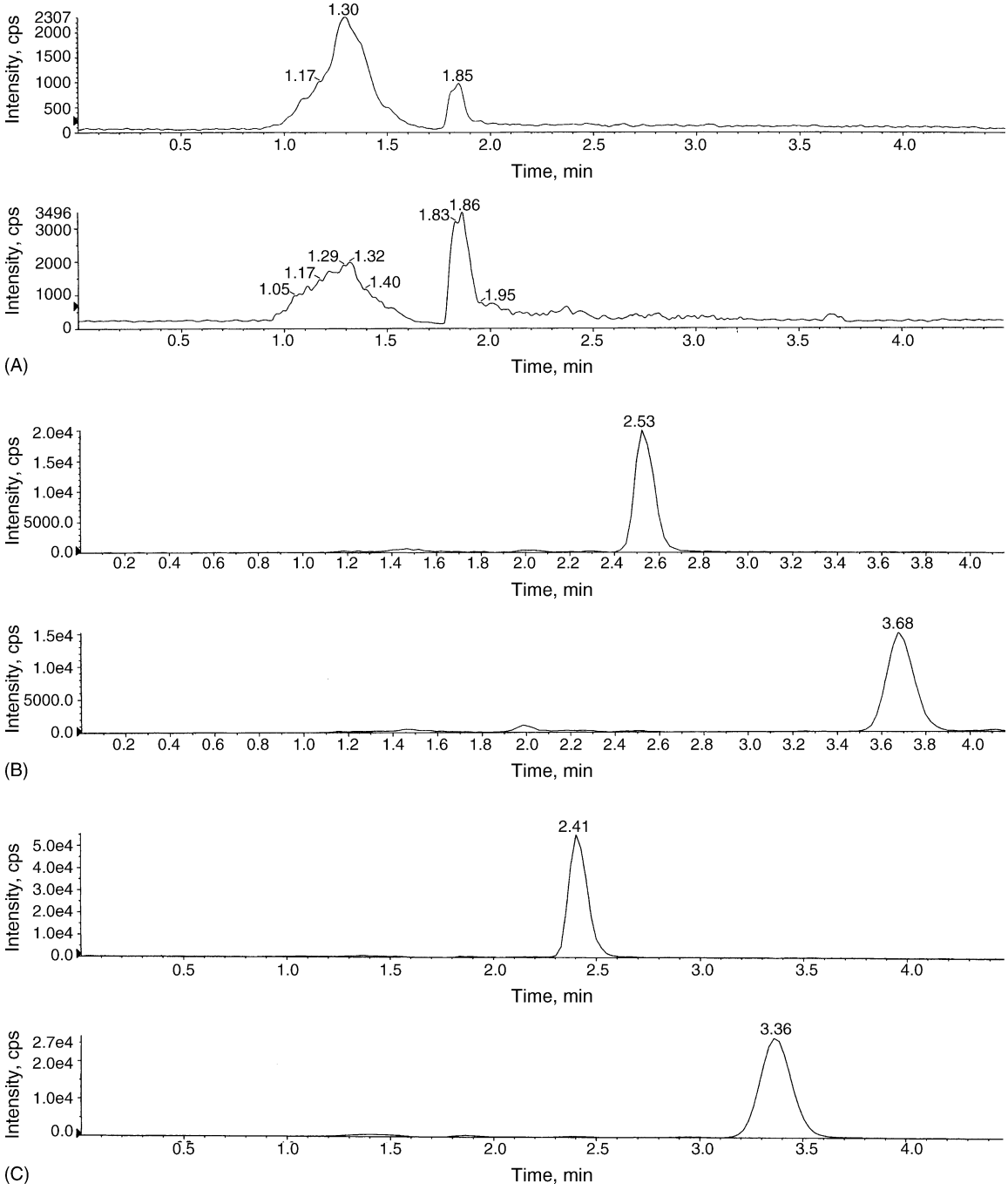

Fig. 2. Representative chromatograms from (A) a blank rat plasma; (B) a blank rat plasma spiked with standard Astragaloside IV (500 ng/ml) and the internal standard (1 μg/ml); and (C) a rat plasma sample 120 min after intravenous administration of 0.75 mg/kg Astragaloside IV. In each group, the upper panel is the selective ion peak of digoxin + Na^+ and the lower panel is the selective ion peak of Astragaloside IV + Na^+.

was linear over the quantities ranges from 10 to 5000 ng/ml in 0.5 ml rat plasma, and 5–5000 ng/ml in 0.5 ml dog plasma. The calibration curves were found to be linear and could be described by the regression equations: $Y = 0.0017X + 0.123$ ($R^2 > 0.9999$) for rat plasma (Fig. 4A), and $Y = 0.0018X + 0.1807$ ($R^2 > 0.9999$) for dog plasma samples (Fig. 4B), in which Y was peak area ratio of Astragaloside IV over the internal standard, and X was the agent recovered in ng/ml in plasma.

The lowest quantitation limit of Astragaloside IV was 10 ng/ml in 0.5 ml rat plasma and 5 ng/ml in 0.5 ml dog plasma. This sensitivity has proven useful in the analysis of pharmacokinetic data of both rats and dogs treated Astragaloside IV. In order to study tissue distribution and excretion

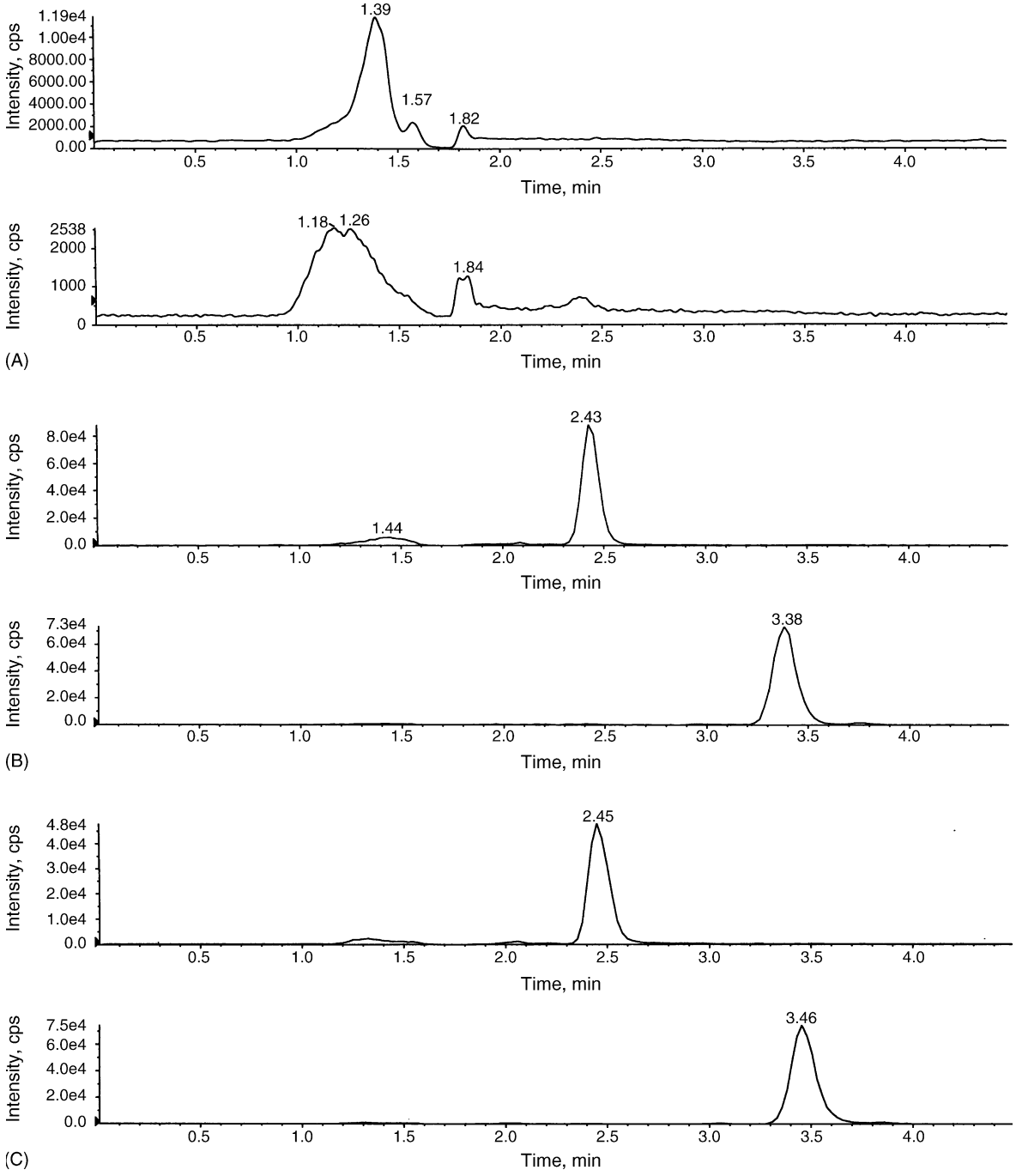

Fig. 3. Representative chromatograms from (A) a blank dog plasma; (B) a blank dog plasma spiked with standard Astragaloside IV and the internal standard; and (C) a dog plasma sample 10 min after intravenous administration of 0.5 mg/kg Astragaloside IV. In each group, the upper panel is the selective ion peak of digoxin + Na^+ and the lower panel is the selective ion peak of Astragaloside IV + Na^+.

pathway of Astragaloside IV, the standard curves for Astragaloside IV in various tissues and biological fluids have been evaluated. The results are summarized in Table 1. The standard curve slopes vary widely across tissues, which may be due to differential recovery of analyte and internal standard across tissues. The results indicate that there is feasible LC/MS/MS method for tissue distribution and excretion studies in rats.

3.4. Accuracy and sensitivity of the detection method

Astragaloside IV is extremely stable at −70 °C even after thawing at 37 °C. The overall accuracy of this method was 93–110% for both rat plasma and dog plasma (Table 2). The results obtained indicate that intra and inter-assay coefficients of variance (R.S.D.) in plasma were less than 15.03%. These results suggest that the procedures described

Table 1
Standard curves and extraction recovery of Astragaloside IV in plasma, urine, bile fluid and tissues of rats

Biological samples	Concentration ranges	Regression equation	Correlation coefficient	ER of Astragaloside	ER of digoxin
Plasma	10–5000 ng/ml	$Y = 0.0017X + 0.123$	0.9999	92.7 ± 2.26%	90.7 ± 4.52%
Urine	10–10,000 ng/ml	$Y = 1.0064X + 0.3468$	0.9994	84	83.2
Bile	10–10,000 ng/ml	$Y = 1.087X + 0.308$	0.9997	85.8	81.5
Fece	100–20,000 ng/ml	$Y = 0.5654X + 0.3454$	0.9979	74.0	71.6
Heart	5–1000 ng/0.3 g	$Y = 0.0032X + 0.0626$	0.9995	66.8	64.2
Kidney	5–1000 ng/0.3 g	$Y = 0.0048X + 0.2081$	0.9996	72.4	62.6
Muscle	5–1000 ng/0.3 g	$Y = 0.0028X + 0.0449$	0.9992	67.1	69.1
Adipose Tissue	5–1000 ng/0.3 g	$Y = 0.0042X + 0.0100$	0.9999	83.5	76.4
Ovary	5–1000 ng/0.3 g	$Y = 0.0034X - 0.0314$	0.9962	75.6	72.9
Testicle	5–1000 ng/0.3 g	$Y = 0.0043X + 0.2464$	0.9978	71.0	62.1
Stomach	5–1000 ng/0.3 g	$Y = 0.0034X + 0.0548$	0.998	63.6	62.7
Liver	5–1000 ng/0.3 g	$Y = 0.0021X + 0.1611$	0.9991	66.6	61.3
Skin	5–1000 ng/0.3 g	$Y = 0.0018X + 0.2017$	0.9956	84.4	77.9
Duodenum	5–1000 ng/0.3 g	$Y = 0.0031X + 0.0799$	0.9981	72.6	75.1
Brain	5–1000 ng/0.3 g	$Y = 0.0059X + 0.146$	0.9987	66.2	64.8
Spleen	5–1000 ng/0.3 g	$Y = 0.0045X + 0.1685$	0.9987	72.5	63.3
Lung	5–1000 ng/0.3 g	$Y = 0.0028X + 0.0121$	0.9989	63.8	65.9

Extraction recovery (ER) was performed at final concentration of 1000 ng/ml except 2500 ng/ml in plasma, bile fluid and fece ($N = 2$ except $N = 3$ for plasma).

as above are satisfactory with respect to both accuracy and precision.

3.5. Detection of Astragaloside IV in plasma following intravenous administration

The plasma Astragaloside IV concentrations within 8 h in rats following intravenous administration of 0.75 mg/kg Astragaloside IV and 12 h in dogs following intravenous administration of 0.5 mg/kg Astragaloside IV can be quantitated by the established LC/MS/MS method. A two compartment, first order pharmacokinetic model was used to describe the pharmacokinetics of Astragaloside IV following intravenous administration (Fig. 5). Astragaloside IV was moderately eliminated with systemic clearance of 3 ml/kg/min in rats and 4 ± 1 ml/kg/min in dogs, which is about 5.43 and 12.9% of hepatic blood flow [20], suggesting of low systemic clearance for Astragaloside IV. The elimination half-life was 97.96 min in male rats and 60.02 ± 8.39 min in male dogs. The volume of distribution at the central compartment was 0.20 l/kg in rats and 0.14 ± 0.07 l/kg in dogs, which are less than 0.6 l/kg of total body water, suggesting that there is limited distribution of Astragaloside IV in peripheral tissues. The values for AUC and Cmax in rats were 289.16 mg h/ml and 3.78 mg/ml, while the values for AUC and Cmax in dogs were 156.04 ± 7.34 mg h/ml and 4.39 ± 2.59 mg/ml, respectively. The detailed pharmacokinetics and tissue distribution of Astragaloside IV will be presented in a separate paper (Tables 3–5).

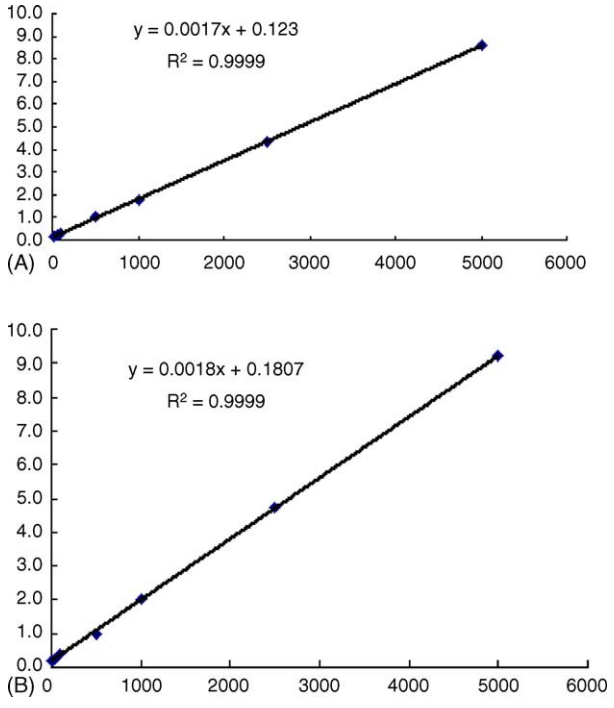

Fig. 4. Standard curves of Astragaloside IV in rat plasma (A) and dog plasma (B).

In summary, we have developed a highly sensitive and accurate analytical LC/MS/MS method for quantitative

Table 2
Intra-assay accuracy of the determination of Astragaloside IV in plasma ($N = 5$)

Concentration (ng/ml)		Accuracy (%)
Added	Found	
Rats		
10	10.48 ± 1.76	104.84 ± 17.60
100	93.13 ± 5.18	93.13 ± 5.18
2500	2586.06 ± 113.63	103.44 ± 4.55
Dogs		
10	10.35 ± 0.59	103.49 ± 5.94
500	546.80 ± 4.50	109.36 ± 0.90
2500	2500.89 ± 16.81	99.91 ± 2.75

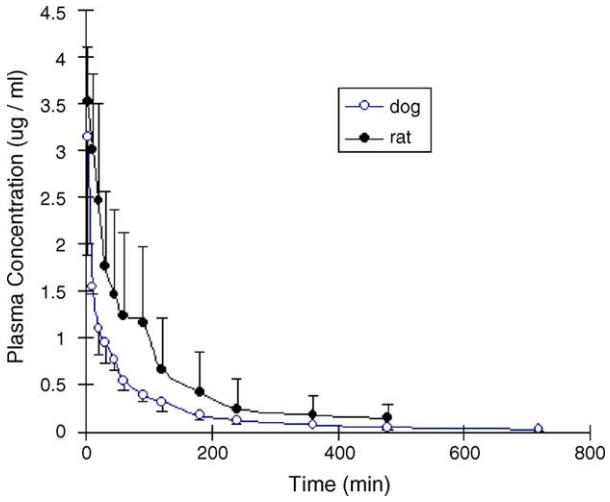

Fig. 5. Plasma Astragaloside IV concentration–time profiles following intravenous administration of 0.75 mg/kg Astragaloside IV to male rats ($n = 3$/timepoint) and 0.5 mg/kg Astragaloside IV to male dogs ($n = 3$).

detection of the novel cardioprotective agent Astragaloside IV in plasma, tissues and other biological fluids. The availability of this assay will now permit detailed pharmacokinetic studies of Astragaloside IV in both rats and dogs.

Table 3
Intra-assay and inter-assay precision of the determination of Astragaloside IV in plasma ($N = 5$)

	Concentration (ng/ml)		R.S.D. (%)
	Added	Found	
Rats			
Intra-Assay	10	11.05 ± 1.32	11.96
	500	524.02 ± 35.26	6.73
	2500	2470.32 ± 83.67	3.39
Inter-Assay	10	10.89 ± 1.64	15.03
	500	494.55 ± 15.70	3.18
	2500	2739.47 ± 158.80	5.80
Dogs			
Intra-Assay	10	9.72 ± 0.77	7.97
	500	455.70 ± 52.37	11.49
	2500	2520.72 ± 245.18	9.73
Inter-Assay	10	10.83 ± 1.20	11.10
	500	579.96 ± 47.42	8.18
	2500	2614.22 ± 79.34	3.03

Table 4
Stability of Astragaloside IV in Plasma after freeze-thawn ($N = 5$)

Concentration (ng/ml)	
Added	Found
Rats	
10	10.87 ± 0.69
500	480.46 ± 8.55
2500	2369.36 ± 161.31
Dogs	
10	11.22 ± 0.53
500	513.94 ± 18.05
2500	2485.84 ± 62.35

Table 5
Quality control of Astragaloside IV in plasma after single-blind experiment ($N = 5$)

Concentration (ng/ml)	
Added	Found
Rats	
10	9.82 ± 1.08
100	87.97 ± 4.73
2500	2551.19 ± 126.23
Dogs	
10	11.19 ± 1.07
100	110.34 ± 7.50
2500	2595.56 ± 56.58

Acknowledgements

The work was supported in part by the 863 Foundation of China (No. 2003AA2Z3507) and Scientific Foundation of Shanghai China (No. 03QMH1414, 04DZ19843, 04DZ19856 and 04DZ19857).

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