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Quantitation of astragaloside IV in rat plasma by liquid chromatography–tandem mass spectrometry

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Abstract

A simple, sensitive and specific liquid chromatography–tandem mass spectrometry method (LC–MS–MS) had been developed and validated for the quantitation of astragaloside IV (AGS-IV)-an active constituent of *Radix Astragali* in rat plasma. Assay method was developed by a series of operations described as below. The plasma proteins were precipitated with acetonitrile and digoxin was used as the internal standard (I.S.). The sample solution containing astragaloside IV and the I.S. were obtained and subsequently injected into a LC–MS–MS system following by a gradient elution at a slow flow rate combined with a valve diversion during the liquid chromatography. Chromatographic separation was achieved on a C4 (2.1 mm × 10 mm) column with a gradient mobile phase comprised of 90% methanol in water and 10 mM ammonium acetate buffer. The analytes were detected with a PE Sciex API 3000 mass spectrometer using turbo ion spray with positive ionization. Ions monitored in the multiple reaction-monitoring (MRM) modes were *m*/*z* 785.5 (precursor ion) to *m*/*z* 143.2 (product ion) for AGS-IV and *m*/*z* 781.2 (precursor ion) to *m*/*z* 243.3 (product ion) for digoxin (I.S.). The method was validated over a linear range of 1–1000 ng/ml. The low limit of quantitation was 1.0 ng/ml. Results from a 3-day validation study demonstrated that the developed method possessed good precision (CV% values were between 5.9 and 7.6%) and accuracy (96.5–102.1%) across the calibration range. The recoveries were 91 and 90% for astragaloside IV and I.S., and no significant matrix effects were observed. QC samples were stable when kept at room temperature for 4 h, at −20 °C for 4 weeks, and after three freeze/thaw cycles.

Keywords: *Astragalus membranaceous*; Astragaloside IV

1. Introduction

Radix Astragali is one of the most commonly used traditional Chinese medicines, which was prepared from the roots of *Astragalus membranaceus* and *Astragalus membranaceus* var. *mongolicus* (Leguminosae). Pharmacological tests showed that it possessed hepatoprotective, antioxidative, antiviral, antihypertensive and immuostimulant activities [1–3]. Astragaloside IV (AGS-IV), a 9, 19-cycloartane-type triterpene glycoside [4], has been regarded as one of the characteristic and active constituent of *Radix Astragali*. It was reported in recent studies that AGS-IV has neuroprotective, cardiovascular protective, anti-inflammatory and immune stimulating effects [5–9]. But up to now, little is known about the pharmacokinetics of AGS-IV.

Analytical methods for the determination of AGS-IV included thin-layer chromatography (TLC) [10], high performance liquid chromatography and evaporative light scattering detection (HPLC–ELSD) [11,12], precolumn derivatization HPLC [13] and LC–MS with a solid phase extraction (SPE) [14]. However, there are some limitations of these methods: (1) sample preparation procedure is complicated and could be labor-intensive; (2) the selectivity and specificity of these methods are limited and the biological sample assay could not be adequately performed; and (3) long run time and large sample volume are usually required. In order to investigate the pharmacokinetics profile of AGS-IV, a fast, sensitive

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and specific assay method needs to be developed to obtain a better estimate of the pharmacokinetics of this compound. As a part of our continuation work of absorption, distribution, metabolism and excretion studies for traditional Chinese medicines [15–18], here we described a LC–MS–MS assay with multiple reaction monitoring (MRM) for the measurement of AGS-IV in rat plasma. LC–MS–MS using MRM is expected more specific and sensitive than single-ion monitoring (SIM) LC–MS assay.

2. Experimental

2.1. Reagents and chemicals

AGS-IV (purity>95.0%) was provided by the Pharmaceutical Research Institute of Chengdu Diao Pharmaceutical Company (Chengdu, China). Digoxin (Sigma, USA) (purity>99.0%) was used as the internal standard. Methanol and acetonitrile (HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). In-house deionized water further purified with a Milli-Q Reagent Water System (Bedford, MA, USA). Drug–free heparin treated rat plasma was provided by Aventis Pharmaceutical Inc. (Bridgewater, NJ, USA). Other chemicals are all of analytical grade.

2.2. Standard and quality control (QC) solutions preparation

A stock solution of AGS-IV and I.S. (Fig. 1) were prepared by dissolving the reference compound in acetonitrile to obtain a solution at 1.0 mg/ml separately and stored at −20 °C. A working solution at 1000 ng/ml of I.S. was prepared by appropriate dilution of the stock solution in acetonitrile and stored at +4 °C before use.

Median working solutions of AGS-IV were prepared from stock solution by appropriate dilution in acetonitrile-water (1:1, v/v), at 100, 10 and 1 μg/ml daily before preparing the standards in blank plasma. The standards were prepared by serial dilution the median working solution in blank plasma to 1 ml. The final standard concentrations in rat plasma were 1, 5, 10, 25, 50, 100, 250, 500 and 1000 ng/ml, respectively.

A 100 μg/ml quality control was prepared from a separate weighing of AGS-IV in acetonitrile: water (1:1, v/v) prior to use. Dilutions were performed to prepare the low, medium and high levels of 2, 20 and 200 ng/ml together with the dilution of QC (2000 ng/ml) in rat plasma.

2.3. Sample collection and preparation

A volume of 0.3 mL blood sample was collected into heparinized tubes (final concentration about 20 IU/mL) at each time point from rat carotid catheter after administration. The volume removed was always replaced with an equal volume of heparinized (10 IU/mL) saline after the sample was collected. Blood samples were centrifuged for 5 min at 4500 × *g* under 4 °C. The plasma were harvested and transferred into tubes in a 96-well plate format and frozen at −20 °C until thawed for bioanalysis.

The rat plasma samples were thawed to room temperature. The rat plasma (25 μl) were transferred into a microcentrifuge tube (1.7 ml) and mixed with 50 μl of I.S. working solution to precipitate the plasma protein by the acetonitrile contained in the I.S. working solution. The tubes were vortex mixed at setting 10 for 1 min and centrifuged at 10,000 × *g* for 5 min. The supernatant (50 μl) was transferred into an autosampler vial and mixed with 50 μl of water before LC–MS–MS analysis. Standard working solutions and QC working solutions prepared simultaneously through the same procedure before it was injected into the LC–MS–MS system.

2.4. LC–MS–MS instrumentation

The liquid chromatograph was an HP 1100 LC system equipped with a binary pump and a CTC A200S Autosam-

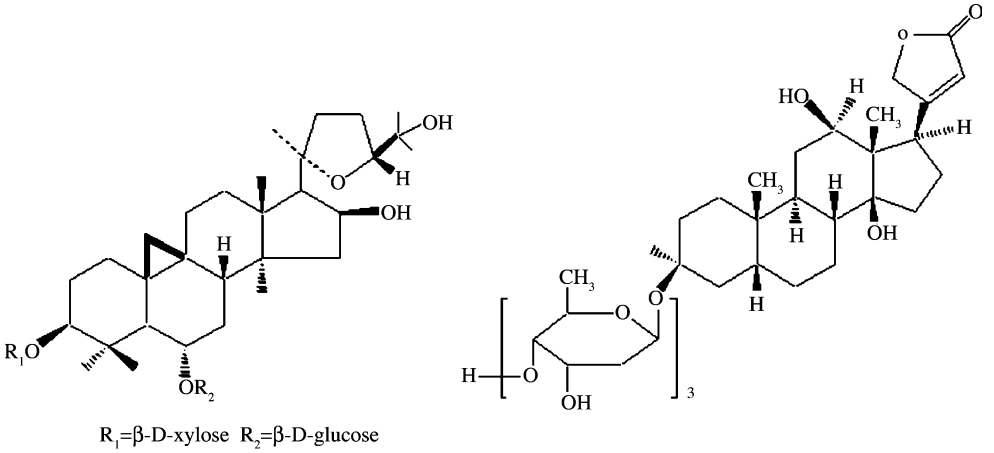

Fig. 1. Chemical Structures of: (A) AGS-IV and (B) Digoxin.

pler unit equipped with a Valco valve diverter obtained from Leap Technologies (Chapel Hill, CT, USA), the injector needle and valve were flushed in between sample injections with a water/methanol (75:25, v/v). Separation was carried out on a Phenomenex® Jupiter C-4 column, 50 mm × 2 mm, 5 μm (Torrance, CA, USA) equipped with a Varian® MetaGuard 10 mm × 2 mm, 5 μ, C-4 guard column (Torrance, CA, USA). The column was eluted at 0.2 mL/min with a binary gradient comprised of 90% methanol in water (A) and 10 mM ammonium acetate buffer, pH 7.0 (B). Starting conditions were 40% A and 60% B. The A concentration was increased to 95% in 0.1 min and then held at that concentration for 3 min. The starting condition (40% A) was restored over 0.1 min and the column flushed for additional 3 min before the next injection. Valve diversion was performed as 0–1.5 min to waste, 1.5–4.5 min to MS–MS detector.

The MS–MS system consisted of a PE-Sciex (Foster City, Canada) API 3000 equipped with a TurboIonSpray interface. Ionization of analytes was carried out in positive ionization mode with the settings of the electrospray interface: source temperature, 350 °C; ion source voltage, 5000 V. The flows of nebulizer, curtain and collision gases (nitrogen) were optimized to maximize the signal intensity *S*/*N* ratio. Quantitative determination of unchanged AGS-IV was performed by using multiple reaction monitoring (MRM) mode, in order to measure the following ion transitions: *m*/*z* 785.5 $[M + H]^+ \rightarrow$ *m*/*z* 143.2 (collision energy, 21 eV) was monitored for AGS-IV; *m*/*z* 781.6 $[M + H]^+ \rightarrow$ *m*/*z* 243.3 (collision energy, 22 eV) was monitored for digoxin.

2.5. Method validation

2.5.1. Calibration curve and quantification of samples

The nine-point calibration curve was constructed by plotting peak area ratio (*y*) of AGS- IV to the internal standard versus AGS-IV concentrations (*x*). The regression parameters of slope, intercept and correlation coefficient were calculated by weighted ($1/x^2$) linear regression in Analyst 1.1 software used in Sciex API 3000. The concentrations of calibration standards, analyzed in duplicate, were back calculated. Concentrations for the QC samples were calculated from the resulting peak area ratios and the regression equation of the calibration curve.

2.5.2. Accuracy and precision

Accuracy and precision were evaluated by determining the AGS-IV concentration in six lots of QC samples at three level concentrations and one level of dilution QC samples. Dilution QC samples were assayed to ensure that dilution of study samples did not affect accuracy and precision. The dilution QC samples were diluted 1:4 with blank rat plasma prior to analysis and processed as other QC samples. Six lots of samples at each concentration were analyzed in three separate days. The accuracy of the assay was expressed as (mean observed concentration/nominal concentration) ×100%. Intra- and inter-day precision was obtained by one-way analysis of variance (ANOVA) testing and was expressed as percent coefficient of variation (%CV). Acceptable accuracy was within ±15% and the intra- and inter-day precision was ≤15% CV at every concentration.

2.5.3. Matrix effect and recovery

Matrix effect was evaluated by comparing the absolute peak areas of the post-spiked standards with those of the neat standards prepared in injection solvent. On the other hand, the matrix effect was also evaluated by performing accuracy and precision determination from QC samples in six lots of control plasma (see intra-day validation in Section 2.5.2). Recovery was determined by comparing the absolute peak areas of the pre-spiked standards with those of the post-spiked standards. The pre-spiked standards were prepared by the procedure described in Section 2.3. The post-spiked samples were the drug-free control plasma prepared by the same procedure and then spiked with working standard stock solutions. The concentrations of 25, 100 and 600 ng/ml for AGS-IV and 100-ng/ml concentrations for I.S. were prepared for matrix effect and recovery assessment.

3. Results and discussion

3.1. Chromatography and specificity

Under the developed chromatographic conditions, the retention times for AGS-IV and I.S. were, approximately 3.53 and 2.95 min, respectively. The system's dead volume was around 1 min elution and first 1.5 min was switched to waste. Therefore the analyte and I.S. were relatively far away from the general ions. Gradient elution was necessary in order to reduce retention time and avoid excessive broadening of the peaks. $[M + Na]^+$ ions were present in the full scan spectrum of AGS-IV and digoxin, with higher relative abundance, but no product ion was found by varying the collision gas pressure and the collision energy. Therefore, it was decided to monitor the $[M + H]^+$ and its product ions in order to promote the specificity. No evidence of the mass peak corresponding to the aglycone (*m*/*z* 491) was found in the mass spectrum of AGS-IV, indicating this analyte to be stable at the adopted ionization conditions.

Six blank plasma samples from six lots of rat plasma were processed with and without the internal standard to evaluate presence of interfering peaks. The degree of interference was assessed by inspection of MRM chromatograms. No significant interfering peaks from the plasma were found at the retention time and in the ion channel of either the analyte or the I.S. Fig. 2 showed the typical chromatograms obtained from plasma sample and blank plasma spiked with I.S.

3.2. Recovery and matrix effect

The recovery and the matrix effect on LC–MS–MS detection were evaluated for both analytes and the internal stan-

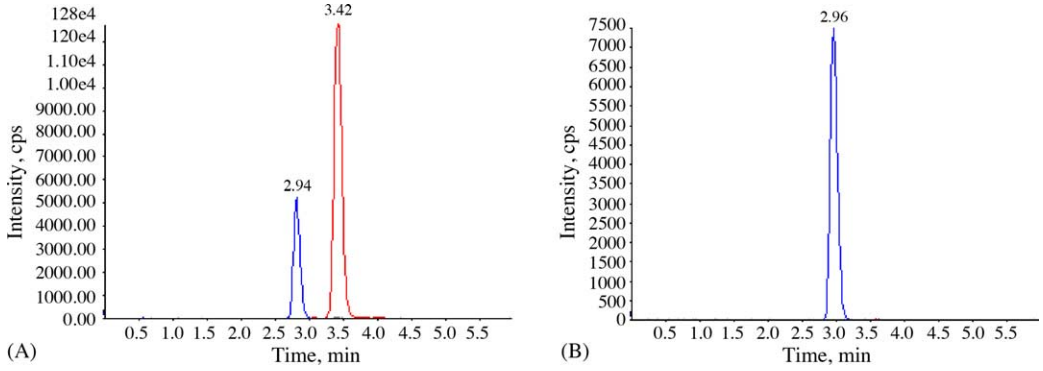

Fig. 2. Typical chromatogram at *m/z* 785.5 → 143.2 or *m/z* 781.6 → 243.3 obtained from a plasma sample spiked with 1000 ng/ml of Digoxin: (A) sample plasma and (B) blank plasma.

Table 1
Recovery and matrix effect of AGS-IV and I.S. in rat plasma

Nominal concentration ng/ml)	Mean peak area ($n = 5$)			Recovery (%) [C.V.%][d]	Matrix effect (%) [C.V.%][e]
	Pre-spiked[a]	Post-spiked[b]	Neat[c]		
AGS-IV					
25	44900	50600	52400	88.9 [9.4]	96.6 [2.7]
100	192000	208000	218000	92.4 [7.8]	95.5 [5.2]
600	1060000	1160000	1260000	91.8 [8.6]	92.0 [5.3]
I.S. ($n = 15$)					
100	14600	16200	17900	90.1 [8.1]	90.2 [3.0]

[a] Pre-spiked is the standard spiked into control plasma before prepared.
[b] Post-spiked is the standard spiked in the prepared control plasma.
[c] Neat is the standard in injection solvent.
[d] Calculated as [(mean pre-spiked peak area/ mean post-spiked peak area) ×100]%.
[e] Calculated as [(mean post-spiked peak area/ mean neat peak area) ×100]%.

dards. Five replicates were used for AGS-IV and each of the nominal concentrations of 25, 100 and 600 ng/ml, which generated 15 replicates of I.S. at 100 ng/ml working concentration. The mean recoveries were 88.9, 92.4 and 91.8% for AGS-IV and 90.1% for I.S., respectively (Table 1). Less than 10% matrix suppression was observed (Table 1). Based on this result and the intra-day precision and accuracy values obtained from six different lots of plasma samples (Table 2), the matrix effect should not have a significant impact on assay performance.

3.3. Linearity, accuracy and precision

A nine-point calibration standard curve ranging from 1 to 1000 ng/ml of AGS-IV was performed in duplicate in four analytical runs. The average percentage deviation of the 72 standards across all four runs for all nine standards was −1.47%, along with the maximum percentage deviation observed was −14.5%. The regression coefficients (R^2) for the four runs were greater than 0.99 (data not shown). The accuracy and precision results obtained for six lots of QC samples were shown in Table 2. The intra-day precision was within 7.6% and the inter-day precision was within 5.9%. The assay accuracy was 96.5–102.1% of the nominal values.

3.4. Lower limit of quantitation

The lower limit of quantitation (LLQ) was evaluated by spiking AGS-IV at a concentration of 1 ng/ml with six different lots of drug-free rat plasma and assaying them as unknown samples against the standard curve and QC samples. The mean concentration of LLQ for six lots was 1.05 ng/ml. The mean deviations for all six LLQ samples were within ±4.5%, the maximum deviation of the predicted concentrations for

Table 2
Accuracy and precision for the assay method of AGS-IV in rat plasma

Nominal concentration (ng/ml)	Mean observed concentration (ng/ml)	Accuracy (%)	Inter-day precision (% CV)	Intra-day precision (% CV)	*N*	Number of runs
2	2.0	102.1	4.9	5.9	18	3
20	19.3	96.5	5.9	7.0	18	3
200	193.8	96.9	5.7	7.6	18	3
2000	1946.1	97.3	3.4	7.0	18	3

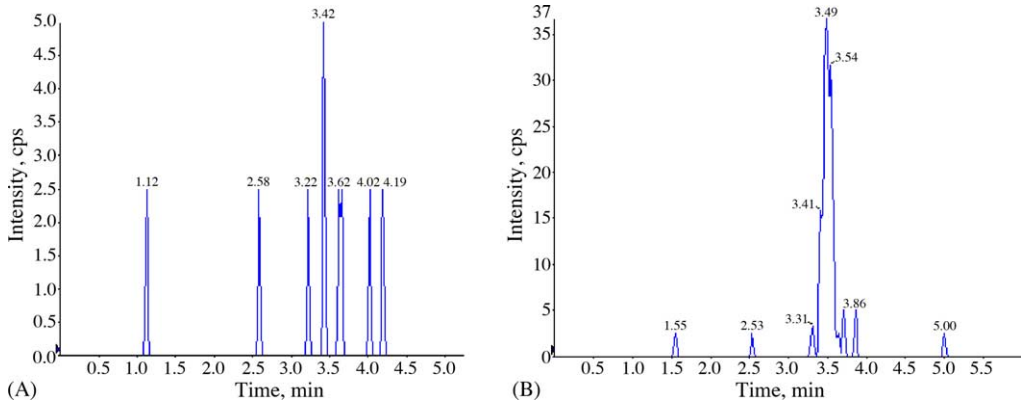

Fig. 3. Typical chromatogram at *m/z* 785.5 → 143.2 for AGS-IV obtained from: (A) rat plasma sample spiked with 500 ng/ml of Digoxin and (B) rat plasma contained AGS-IV at LLQ (1 ng/ml) and I.S. at 500 ng/ml of Digoxin.

all six LLQ samples was not more than 13.0% of the nominal value. A typical chromatogram at the LLQ was shown in Fig. 3. The deviations from the individual lots were within the acceptance criterion (≤20%) for the LLQ samples recommended by the FDA Guidance [19]. Given the fact that the analyte and its internal standard were structurally similar but differed in their proton affinity, the LLQ data obtained were acceptable.

3.5. Stability

The stability of AGS-IV in rat plasma was assessed by leaving the QC samples at two different concentrations (20, 200 ng/ml) at room temperature for 4 h. Freeze/thaw stability was assessed by thawed at room temperature and refrozen at −20 °C over three cycles and assayed. Freshly processed standard samples were used to quantitate all the QC samples. In rat plasma, AGS-IV was found to be stable for at least 4 weeks at −20 °C, for 4 h at room temperature and for three freeze/thaw cycles. Samples spiked with I.S. were stable for at least 48 h at room temperature.

4. Conclusions

To evaluate AGS-IV pharmacokinetics in rats, a LC–MS–MS method was developed and fully validated. The method provided a sensitive and reliable procedure for the determination of AGS-IV and could be applied to the pharmacokinetic studies of AGS-IV in rats and might be easily extended to other animal species. The current method offered a number of advantages over the existing methods, such as shorter analysis time, smaller sample volume (300 μl blood), amenable to serial sampling studies and devoid of extensive sample cleanup.

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