Determination of Astragaloside IV in *Radix Astragali* (*Astragalus membranaceus* var. *monghulicus*) Using High-Performance Liquid Chromatography with Evaporative Light-Scattering Detection

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Abstract

A reverse-phase high-performance liquid chromatographic method is developed for the determination of astragaloside IV, a characteristic constituent in *Radix Astragali.* Samples are analyzed by means of a reverse-phase column (Zorbax Eclipse XDB C_{18}) using acetonitrile and water under gradient conditions as the mobile phase for 30 min. An evaporative light-scattering detector is used and set at an evaporating temperature of 43°C with a nebulizing gas (compressed air) pressure of 3.4 bar. The detection limit (signal-to-noise ratio > 5) of astragaloside IV is 40 ng on-column.

Introduction

Radix Astragali is prepared from the roots of certain species of plants from the genus *Astragalus* (Leguminosae) and has been used as a tonic, analgesic, antiseptic, and antisudorific in Chinese traditional medicine (1). It possesses hepatoprotective, antioxidative, antiviral, antihypertensive, and immunostimulant activity, and these activities are well-documented and reviewed (2–4). Astragaloside IV (AGS-IV) (Figure 1), a cycloartane-type triterpene glycoside, has been regarded as one of its characteristic and active constituents. The identification and quantitation of AGS-IV has been carried out by thin-layer chromatography (TLC) (5). Although the TLC method has been extensively employed in the quality assurance (QA)/quality control (QC) of *Radix Astragali* and the products containing it (5), the method presents a challenge for sensitivity and selectivity. Direct reverse-phase (RP) high-performance liquid chromatography (HPLC) with UV detection could be used for the analysis of AGS-IV in *Radix Astragali* samples. However, the detection of AGS-IV using UV is well-known for its insensitivity because of the weak chromophoric functionality of AGS-IV in the 200–210-nm region. A precolumn derivatization method has been proposed that allows an efficient detection using HPLC–UV with a detection limit (LOD) of 40 ng for AGS-IV on the column (6). Nevertheless, there is a drawback in the application of the derivatization method (i.e., extensive sample preparation and analysis time). It would be useful to develop an alternative method for the routine analysis of *Radix Astragali*, which is more efficient.

As a mass detection method, evaporative light-scattering detection (ELSD) is based on the nebulization of liquid chromatography (LC) column effluent into droplets by the nebulizing gas and the entrance of the resulting vapor into a temperature-controlled evaporator tube in which the evaporation of mobile phase takes place. The resulting "clouds" of solid microparticles are then directed towards a narrow light beam. As a result, light is scattered by these microparticles and measured using a photomultiplier or photodiode. A plot of detector response versus analyte concentration is sigmoidal, and the peak area (I) is related to the sample size and shape (but not the chemical identity of the

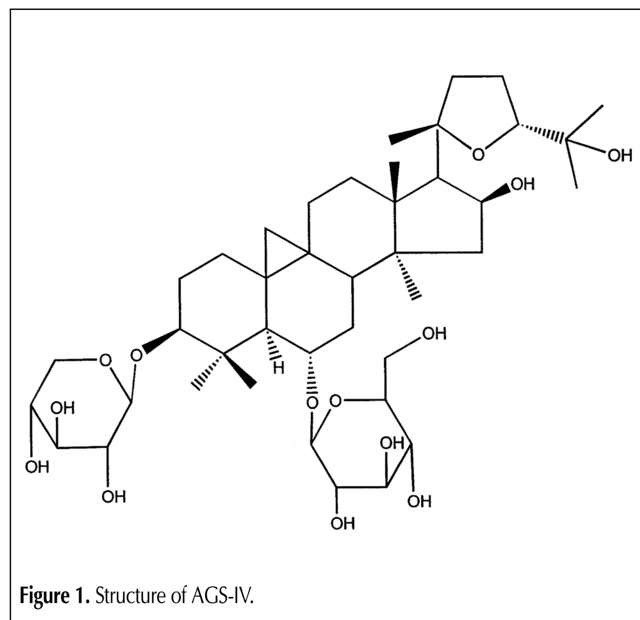

Figure 1. Structure of AGS-IV.

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residual particles passing through the light beam) by the following relationship:

$$I = am^b \quad \text{Eq. 1}$$

where b is the slope of the response line, m is the mass of the compound injected, and a is the response factor. As a result, plots of the peak area versus the analyte concentration with logarithmic coordinates are linear. ELSD has been applied to a wide range of UV-transparent analytes including lipids (7,8), peptides (9), carbohydrates (10), and botanical bioactive compounds (11). We recently reported the determination of a marker compound, 24 (*R*)-pseudoginsenoside F_{11}, in North American ginseng by using HPLC–ELSD (12). It is interesting that the ELSD is not a technique that is widely known nor used in the QA/QC of herbal products. Based on a project with the aim to evaluate the application of the ELSD in the QA/QC of dietary supplements, this study describes the quantitative analysis of AGS-IV in *Radix Astragali* in a single run by HPLC–ELSD using gradient elution.

Experimental

Materials and reagents

Pharmacopoeial *Radix Astragali* was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). An AGS-IV reference standard was provided by the Institute for the Advancement of Chinese Medicine, Hong Kong Baptist University (Kowloon, Hong Kong), which was originally purchased from the National Institute for the Control of Pharmaceutical and Biological Products. Methanol and acetonitrile (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ). Deionized water was obtained with an in-house Nanopure water system (Barnstead, Newton, MA).

Apparatus

A Waters 2690 Alliance HPLC system (Waters Corporation, Milford, MA) equipped with a 996 photodiode-array UV detector, an online degasser, and an autosampler were used for solvent delivery and detection. After the UV detector, the column effluent was directed to a Sedex (Alfortville, France) 75 ELSD. The detector output was interfaced using a SATIN box to the Waters Millennium 2000 chromatographic manager system loaded on a Compaq (Houston, TX) 6400X/10000/CDS computer for data handling and chromatogram generation.

Preparation of standard solution

In a clean, dry 10-mL volumetric flask, the AGS-IV reference standard (2.0 mg) was accurately weighed and dissolved in methanol to make a stock solution. Calibration working standard solutions (4–200 µg/mL) were prepared by diluting the stock solution with methanol in appropriate quantities. Three controls were also positioned to be in the lowest, middle, and highest regions of the calibration curve (i.e., 50, 120, and 180 µg/mL). All working solutions were stored at –20°C and brought to room temperature before use.

Preparation of sample solution

Finely pulverized *Radix Astragali* roots (*Astragalus membranaceus* (Fish) Bge. var *monghulicus* (Bge.) Hsiao) were weighed (0.5 g) into a polytetrafluoroethylene-stopped 20-mL sample vial. Methanol (Fisher, HPLC grade, 18 mL) was added, and the mixture was shaken and then sonicated at 25–30°C for 30 min. After cooling, the mixture was filtered through filter paper (Whatman #40, Whatman Inc., Clifton, NJ) into a 250-mL round-bottom flask, and the residue was returned to the sample vial. Another 18 mL of methanol was added, and the mixture was sonicated at 25–30°C for 30 min. The extract was filtered through filter paper (Whatman #40) into the same round-bottom flask. This extraction procedure was repeated once more before washing the residue with methanol (3 × 15 mL) while on the filter. The combined methanol extracts were evaporated under reduced pressure at 35°C. The residue was redissolved and transferred with methanol to a 10-mL volumetric flask and diluted to volume with methanol. The sample solution was filtered through a 0.2-µm Whatman hydrophilic membrane filter into an HPLC sample vial just before HPLC–UV–ELSD analysis.

Chromatographic conditions

The chromatographic separations were carried out on a Zorbax Eclipse XDB C_{18} column (250- × 4.6-mm i.d., 5-µm particle size, PN#990967.902) (Agilent, Palo Alto, CA) protected by a Waters Delta-Pak C_{18} guard column (Waters Technologies Ireland, Ltd., Wexford, Ireland) and set at 20°C. The mobile phase used for the separation consisted of solvent A (water, deionized) and solvent B (acetonitrile). The elution profile was: 0 → 30 min, 20% → 58% B; 30 → 31 min, 58% → 90% B; 31 → 35 min, 90% B (washing out); 36 → 37 min, 90% → 20% B; 37 → 40 min, 20% B (reconditioning). All gradient steps were linear. The flow rate was set at 1.6 mL/min, the column temperature was fixed at 20°C, and the injection volume was chosen to be 10 µL. The peak identification was based on retention time and comparison with the injected authentic reference standard. The peak was detected consecutively with the UV and the ELSD. In UV, the detection wavelength was set at 203 nm. After the UV detector, the eluent was transferred to the ELSD with a gain of 11, the evaporating temperature was at 43°C, and the nebulizing gas pressure at 3.4 bar. Prior to each run, the HPLC–UV–ELSD system was allowed to warm up for 20–30 min and the pumps were primed using the protocol suggested by the manufacturer. Using a freshly prepared mobile phase, the baseline was monitored until stable before the samples were run.

Reproducibility

The precision and accuracy of the method were assessed by intra- and interday validations. The variation was evaluated by injecting three sets of controls (50, 120, and 180 µg/mL, $n = 3$) on three separate days. By substituting the peak area into the calibration curve equation from the same run, the measured concentrations were obtained. By comparing calculated and theoretical concentrations, the relative errors were obtained. The coefficient of variance was calculated by comparing the measured concentrations.

The recovery was assessed by adding 100 µg of the standard to 500 mg of *Radix Astragali* samples and extracted in a similar way as the previously mentioned sample. Triplicate analysis provided total AGS-IV, which was used to determine recovery.

Results and Discussion

Optimization of the ELSD parameters

In ELSD, a constant nebulization process is important for sat-

isfactory repeatability. Several factors have influence on the average diameter of the droplets and their distribution, which include density, viscosity, and liquid surface tension. Among these factors, the nebulizer gas flow rate affects the signal response most significantly. When the gas flow rate is too low, large droplets are formed, resulting in spikes and random noise. However, when the gas flow rate is too high, the droplets decrease in size, which results in a decreased signal response. The optimum nebulizer gas (compressed air) pressure in this work was determined to be 3.4 bar.

The evaporating temperature is also an important parameter affecting the signal response. At low temperature, solvent evaporation is not complete; at high temperature, the detector response is decreased, owing to the decrease in particle size by an improper vaporization of the nebulized analytes in the drift tube. The signal-to-noise ratio (S/N) was improved when the temperature was lowered to 43°C. Also, the gain in ELSD was set at 11 in order to obtain the best sensitivity.

Chromatography

Figure 2 shows the typical LC–UV and LC–ELSD chromatograms of AGS-IV with a retention time of approximately 16 min within a 30-min gradient elution. The LOD (S/N > 5) of the described method was observed for AGS-IV at 40 ng on-column in the current assay. Because the compound has no double bond in the molecule, it shows poor UV absorption with an LOD of 200 ng (S/N > 5) on-column. This verifies the advantage of ELSD over normal UV in the detection of UV transparent compounds.

Method validation

Linearity was examined by applying the calibration working standard solutions for three consecutive days. The calibration curve, log-transformed peak area (y) versus log-transformed concentration (x), was calculated according to the least-squares methods for AGS-IV tested with regression more than 0.998 (Figure 3):

$$y = 1.4178 + 1.6801x \quad \text{Eq. 2}$$

The intra- and interday reproducibility of the method was eval-

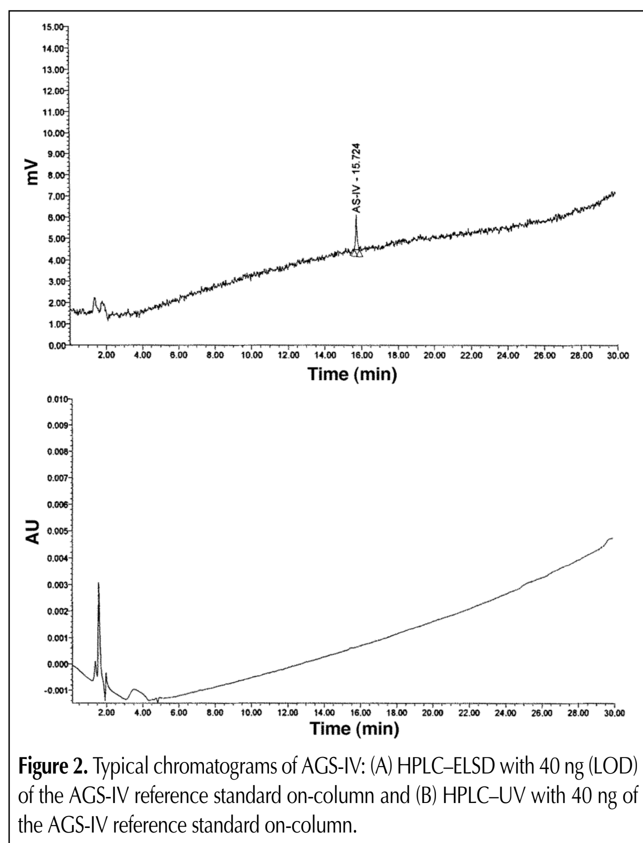

Figure 2. Typical chromatograms of AGS-IV: (A) HPLC–ELSD with 40 ng (LOD) of the AGS-IV reference standard on-column and (B) HPLC–UV with 40 ng of the AGS-IV reference standard on-column.

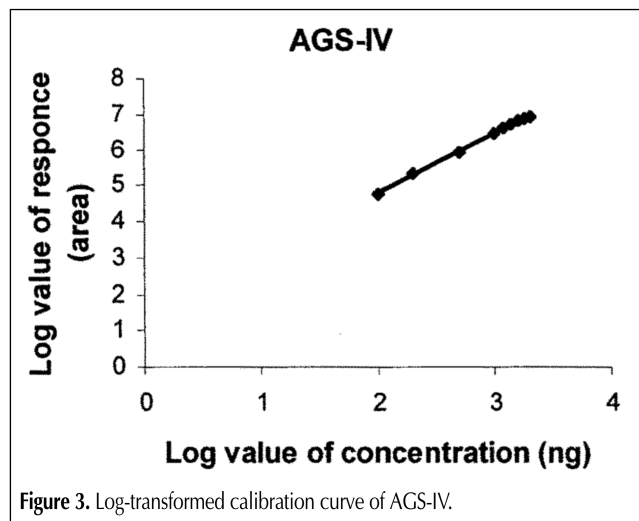

Figure 3. Log-transformed calibration curve of AGS-IV.

Table I. Reproducibility for Three Consecutive Days

Group	Spiked concentration (µg/mL)	Day	Measured concentration* (µg/mL)	%Coefficient of varience	%Relative error
QC-1	50	1	47.48 ± 0.52	1.10	–5.03
		2	48.58 ± 0.27	0.56	–2.83
		3	45.53 ± 0.50	1.10	–8.95
QC-2	120	1	118.83 ± 1.56	1.31	–0.97
		2	120.53 ± 1.78	1.48	0.44
		3	113.75 ± 1.49	1.31	

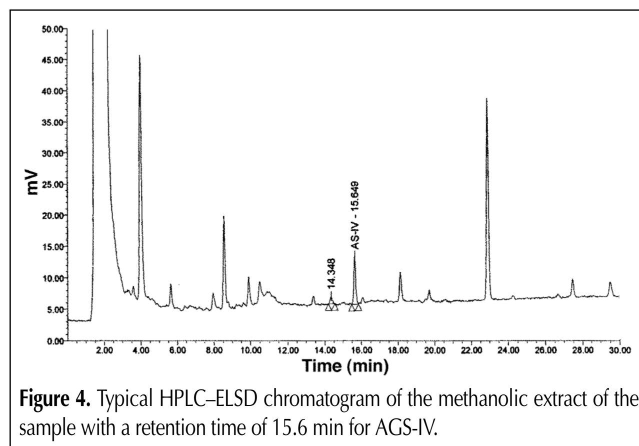

Figure 4. Typical HPLC–ELSD chromatogram of the methanolic extract of the sample with a retention time of 15.6 min for AGS-IV.

uated by analyzing a set of three controls (50, 120, and 180 µg/mL, *n* = 3) on three separate days (*n* = 3) and calculating the %RSD and relative errors. As shown in Table I, the %RSD and the relative errors were found to be less than 1.48% and 8.95%, respectively. The average recovery of AGS-IV was 95.8% (*n* = 3).

Sample analysis

As shown in Figure 4, three sets of Pharmacopoeial *Radix Astragali* samples were analyzed according to the method described previously. The average content of AGS-IV was found to be 0.016%. This compares favorably with a previous report (6) for *A. monghulicus* (Bge.) Hsiao from eight samples all grown in different locations with content ranging between 0.03% to 0.10%.

Conclusion

An HPLC method has been developed for the determination and quantitation of AGS-IV in *Radix Astragali* using an ELSD. With the method, AGS-IV was successfully quantitated using the calibration curve with a detection limit of 40 ng on the column.

Validation of the HPLC–ELSD method included inter- and intraday precision and accuracy. All of the validation parameters studied were found to have RSDs less than 8.95% and did not show any bias in a single direction.

The HPLC–ELSD method was found to be rapid, relatively inexpensive, straightforward, and reproducible.

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