A Precolumn Derivatization High-Performance Liquid Chromatographic Method with Improved Sensitivity and Specificity for the Determination of Astragaloside IV in *Radix Astragali*

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Abstract

A reversed-phase high-performance liquid chromatographic method is developed for the determination of astragaloside (AGS) IV, which is known as the active constituent of *Radix Astragali*. The method uses precolumn derivatization with benzoyl chloride to form the benzoyl ester of AGS IV quantitatively and is carried out with a wide-ranging concentration (0.004–0.080 mg/mL) of the derivatized AGS IV. The eluent consists of 90% methanol, 4% tetrahydrofuran, 6% water, and 0.2% triethylamine, with vitamin D_3 added as the internal standard.

Introduction

Traditionally, *Radix Astragali* is prepared from the roots of certain species of *Astragalus* and has been used as a tonic, analgesic, antidipsetic, and antisudorific in traditional Chinese medicine (1). It possesses remarkable anti-inflammatory, antimicrobial, hypotensive, and immune-enhancing activities. Astragaloside IV (AGS IV) has been regarded as one of its characteristic and active constituents (molecular structure illustrated in Figure 1). According to one source (2), the identification and assay of AGS IV are to be carried out by thin-layer chromatography. Although direct high-performance liquid chromatography (HPLC) is viable for the simultaneous separation, identification, and assay of AGS IV, it has not been satisfactory, because the hydroxyl group of AGS IV only shows absorption near the vacuum UV (203 nm) and lacks sufficient specificity. Thus, precolumn derivatization of AGS IV was investigated and applied for an improvement of the sensitivity and specificity of AGS IV detection.

Derivatization is often used to improve the sensitivity and specificity of HPLC. Purposes of the derivatization are to tag a conjugated system to the molecule of the compound of interest so that a bathochromic effect is produced and to improve its chromatographic separation. For example, derivatization has been used successfully for the analysis of many hydroxyl-group-containing constituents such as amino acids, amino alcohol, and phenols in biomaterial (3–9). However, the use of this type of an approach in order to develop an assay of such a constituent in herbs is complicated.

There are usually several constituents that can be benzoylated in an herb sample. Even though ideally there is only one such constituent, it may not be an easy task to benzoylate it quantitatively and rapidly in order to begin developing an assay of it, especially when its molecule contains more than one hydroxyl group and its content in the herb is low.

In order to achieve the objective of developing an assay for the determination of AGS IV in *Radix Astragali*, the conditions for quantitative benzoylation and chromatographic separation were investigated (10–13). Derivatization with several reagents was carried out, but finally benzoyl chloride was adopted because of its efficiency and cheapness. Although many compounds of high

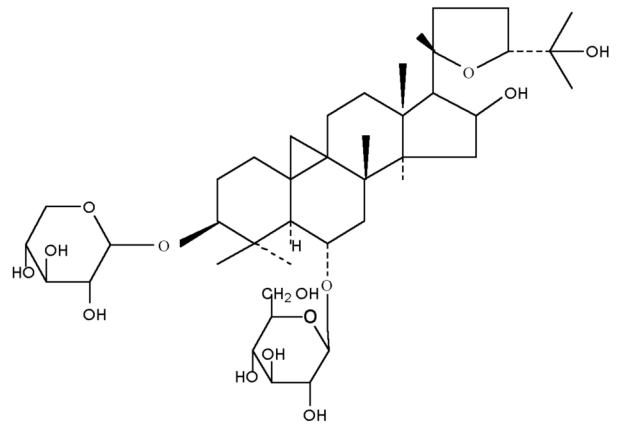

Figure 1. Molecular structure of AGS IV.

purity had been tested as an internal standard for the chromatographic separation of the benzoyl derivative of AGS IV, vitamin D_3 was selected by chance. The method that was developed proved adequate for the identification and quantitation of AGS IV in *Radix Astragali*.

With this derivatization method, a single product resulted, and its molecular weight was determined by fast atom bombardment-mass spectrometry (FAB-MS). Benzoylation at the hydroxyl group on the D-ring was assumed tentatively, but this remains to be confirmed because the fragmentation patterns the derivative were determined by a more delicate MS procedure of the derivative.

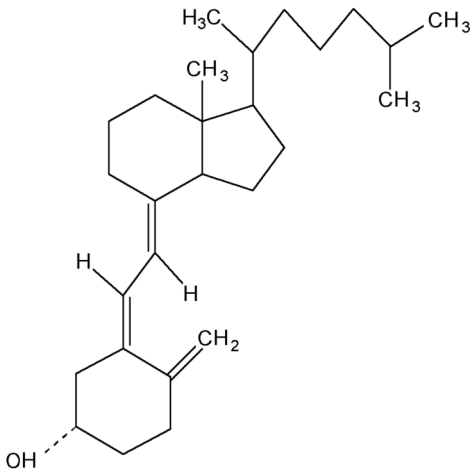

Figure 2. Molecular structure of vitamin D_3.

Table I. Content of AGS IV in Select Species of *Radix Astragali* from Different Habitats in China

Sample	Species	Origin	AGS IV (%)
1	*A. mongholicus* (Bge.) Hsiao.	Tuling, Shanxi	0.10
2	*A. mongholicus* (Bge.) Hsiao.	Datong, Shanxi	0.06
3	*A. mongholicus* (Bge.) Hsiao.	Huai'an, Hebei	0.10
4	*A. mongholicus* (Bge.) Hsiao.	Lijiadi, Hebei	0.09
5	*A. mongholicus* (Bge.) Hsiao.	Shadifang, Hebei	0.10
6	*A. mongholicus* (Bge.) Hsiao.	Beiqi, Sichuan	0.04
7	*A. mongholicus* (Bge.) Hsiao.	Neimenggu	0.03
8	*A. mongholicus* (Bge.) Hsiao.	Changchengou, Shanxi	0.10
9	*A. membranaceus* (Fisch.) Bge.	Changchun, Jilin	0.05
10	*A. membranaceus* (Fisch.) Bge.	Ji'nan, Shandong	0.02
11	*A. membranaceus* (Fisch.) Bge.	Huairentang, Beijing	0.04
12	*A. membranaceus* (Fisch.) Bge.	Cuijiawan, Shanxi	0.02
13	*A. membranaceus* (Fisch.) Bge.	Baijiajian, Shanxi	0.08
14	*A. membranaceus* (Fisch.) Bge.	Shuangyang, Jilin	0.07
15	*A. membranaceus* (Fisch.) Bge.	Shuangyang, Jilin	0.13
16	*A. membranaceus* (Fisch.) Bge.	Gangzi, Jilin	0.11
17	*A. membranaceus* (Fisch.) Bge.	Taishang, Shandong	0.09
18	*A. membranaceus* (Fisch.) Bge.	Goudong, Shandong	0.04
19	*A. membranaceus* (Fisch.) Bge.	Panshi, Jilin	0.02
20	*A. membranaceus* (Fisch.) Bge.	Gengxin, Jilin	0.17
21	*A. membranaceus* (Fisch.) Bge.	Qitamu, Jilin	0.10
22	*A. membranaceus* (Fisch.) Bge.	Zaolin, Shandong	0.04
23	*A. membranaceus* (Fisch.) Bge.	Mudanjiang, Heilongjiang	0.07
24	*A. chryopterus*	Zaolin, Shandong	0.007
25	*Hedysarum polybotrys* Hand.-Mazz.	Tianshui, Gansu	0
26	*A. ernestii* Comb.	Emei, Sichuan	0.08
27	*A. ernestii* Comb.	Litang, Sichuan	0.005
28	*A. ernestii* Comb.	Chengli, Sichuan	0.06
29	*A. ernestii* Comb.	Kangding, Sichuan	0.01

Experimental

Materials and reagents

Astragalus membranaceus (Fisch.) Bge. (membranous milkvetch root), *Astragalus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao (Mongolian milkvetch root), and other species of *Radix Astragali* all came from different habitats in China. The standard AGS IV (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China) and vitamin D_3 (Merck, Darmstadt, Germany) (molecular structure shown in Figure 2) that were used were both of analytical grade. Pyridine (redistilled over potassium hydroxide and stored over anhydrous sodium hydroxide); benzoyl chloride, chloroform, anhydrous sodium hydroxide, tetrahydrofuran, *n*-butanol, methanol, and triethylamine (all of analytical grade); methanol (HPLC grade); and redistilled water were all used as constituents of the mobile phase.

Apparatus

The HPLC system consisted of a Shimadzu (Tokyo, Japan) LC-10AD pump, DGU-4A degasser, SPD-10A ultraviolet (UV)–visible detector, CTO-10A column oven, 20-µL injection loop, and LC workstation for data collection.

Preparation of standard solution

A 0.8124-mg/mL stock solution of standard AGS IV in pyridine was prepared. A stock solution of vitamin D_3 (0.3880 mg/mL) in methanol was kept at 4°C.

Preparation of sample solution

A 0.5-g dried sample powder of *Radix Astragali* was extracted with 100 mL of methanol (4 h, 80°C) in a Soxhlet's extractor. The solvent was evaporated to dryness, and the residue was dissolved in 20 mL of *n*-butanol. The *n*-butanol solution was extracted with 6 mL of 1% NaOH. A triplicate wash removed some constituents such as flavonoids and polysaccharides in the sample. The *n*-butanol phase was washed with 5 mL of distilled water twice. After the final water wash, the organic phase was transferred to a 100-mL stoppered conical flask, evaporated to dryness, and redissolved with 1 mL pyridine.

Derivatization procedure

Two milliliters of chloroform and 0.4 mL of benzoyl chloride were added to the sample solutions.

Each mixture was shaken thoroughly for 1 min and stored at 4°C for 12 h. The mixture was evaporated to dryness, 1 mL of the internal standard solution was added to each sample, and then methanol was added to increase the volume to 10 mL.

Chromatographic conditions

The separation column was a Spherisorb ODS C_{18} (250- × 4.4-mm i.d., 5-μm particle size) equipped with a 20- × 4-mm ODS C_{18} precolumn. The eluent consisted of 90% methanol, 4% tetrahydrofuran, 6% water, and 0.2% triethylamine. The flow rate was 0.8 mL/min, the column temperature was kept at 25°C, and the wavelength was set at 230 nm.

Validation study

A series of standard solutions—0 (the derivatization blank), 0.04, 0.08, 0.16, 0.32, 0.48, 0.64, and 0.80 mg/mL—of AGS IV was prepared by diluting the 0.8-mg/mL stock solution with pyridine. All of the standard AGS IV as well as different *Radix Astragali* sample solutions prepared in the same way were derivatized with benzoyl chloride. All of the solutions were filtered through a 0.45-μm microporous filter, and 20 μL of the filtered solution was injected into a chromatograph in triplicate. The calibration curve was constructed by plotting the peak-area ratio of AGS IV to the internal standard against the concentration of AGS IV. The analysis of *Radix Astragali* samples was based upon this curve.

Results and Discussion

Optimization of the reaction conditions

The reaction temperature, time, and amount of pyridine all had effect on the yield of the derivatization. In order to obtain the optimal conditions, many experiments were performed. The reaction was carried out at different temperatures for various durations of time. The optimum yield of the product was obtained when the temperature of the reaction was maintained between 4°C and 20°C. The effect of the reaction time was also examined by allowing the reaction to proceed from 1 to 12 h. The results indicated that a reaction time of 12 h was necessary in order to obtain optimal yield of the derivative. Holding the parameters of temperature and time constant, it was determined that a 2.5-fold volume ratio of pyridine to benzoyl chloride produced the best signal-to-background (S/B) ratio. The results showed that too little of pyridine failed to complete the reaction, and too much reagent increased the amount of a background product, which was coeluted with the derivative causing the S/B ratio to decrease. Therefore, a 2.5-fold volume ratio excess of pyridine to benzoyl chloride was adopted for future derivatization.

The results showed that a reaction temperature of 4°C, a 12-h reaction time, and a 2.5-fold ratio of pyridine to benzoyl chloride were the optimal conditions for the derivatization, which has been further ascertained by an orthogonal design test.

Method evaluation

Typical chromatograms of the blank solution, the standard AGS IV, and two samples of *Radix Astragali* are shown in Figure 3. The calibration curve of the peak-area ratio (y) versus the concentration (x, mg/mL) was linear; $y = 2.822x + 2.757 \times 10^{-3}$, $R^2 = 0.9999$ ($n = 7$). The linear range for the determination of AGS IV was 0.004–0.080 mg/mL. The limit of detection was 0.002 mg/mL. The average recovery of AGS IV was 94.7% ($n = 5$). Repeatability of the assay was 1.3% ($n = 5$). The derivative products were stable during storage for three months at 4°C.

Characterization of the derivatization product

The FAB-MS was used to characterize the derivative formed from the reaction between benzyl chloride and AGS IV. The data showed an *m/z* value of 888, which corresponded with the monobenzoyl derivative of AGS IV.

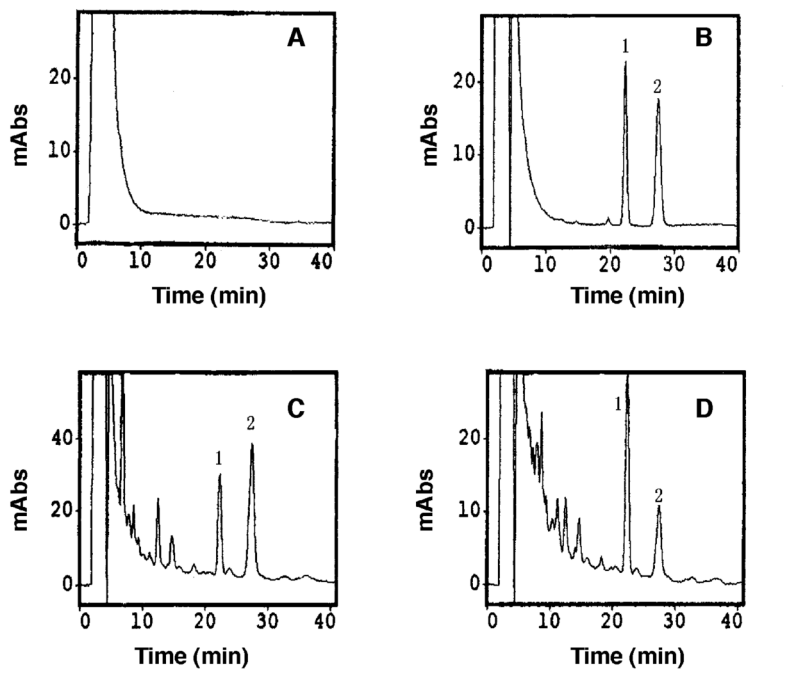

Figure 3. Chromatograms of derivatized AGS IV: blank solution (A), standard AGS IV (B), a sample from Baijiajian (C), and a sample from Cuijiawan (D). Peak 1 is vitamin D_3 and peak 2 is AGS IV.

Conclusion

Benzoylation of AGS IV can be proceeded quantitatively with a sufficient excess of the reagent. The chromatographic property of AGS IV was improved by the derivatization in order for it to be determined sensitively by HPLC with a UV detector. It was applied to the analysis of a large number of *Radix Astragali* samples successfully (the data obtained is shown in Table I).

The benzoylation of benzoyl chloride in pyridine is a well-established reaction for the derivatization of glycoside-possessing hydroxyl groups in the precolumn operation of HPLC for herbs. The condition of the reaction is mild and may not destroy the chemical constituents in herbs. Benzoylation improves the sensitivity and specificity. This

approach should be suitable for the determination of other chemical constituents in complex systems.

Acknowledgments

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