Quantitative Evaluation of Radix Astragali through the Simultaneous Determination of Bioactive Isoflavonoids and Saponins by HPLC/UV and LC-ESI-MS/MS

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The three major active isoflavonoids (calycosin-7-*O*-β-glucoside, isomucronulatol 7-*O*-β-glucoside, formononetin) and two main saponins (astragaloside I, astragaloside IV) in an extract of Radix Astragali were determined using rapid, sensitive, reliable HPLC/UV and LC-ESI-MS/MS methods. The separation conditions employed for HPLC/UV were optimized using a phenyl-hexyl column (4.6 × 150 mm, 5 μm) with the gradient elution of acetonitrile and water as the mobile phase at a flow rate of 1.0 mL/min and a detection wavelength of 230 nm. The specificity of the peaks was determined using a triple quadrupole tandem mass spectrometer equipped with an electrospray ionization (ESI) source that was operated in multiple reaction monitoring (MRM) in the positive mode. These methods were fully validated with respect to the linearity, accuracy, precision, recovery and robustness. The HPLC/UV method was applied successfully to the quantification of three major isoflavonoids in the extract of Radix Astragali. The results indicate that the established HPLC/UV and LC-ESI-MS/MS methods are suitable for the quantitative analysis and quality control of multi-components in Radix Astragali.

Key Words : Radix, HPLC/UV, LC-ESI-MS/MS, Isoflavonoids, Saponins

Introduction

Radix Astragali is the dried root of *Astragalus membranaceous* (Fisch.) Bge. and *Astragalus membranaceous* (Fisch.) Bge. var. *mongolicus* (Bge.) Hsiao (Leguminosae). This plant has been used widely in traditional Chinese medicine to tonify the "Qi" (vital energy) and is prescribed to treat symptoms such as fatigue, lack of appetite, frequent colds, spontaneous sweating and shortness of breath.[1] Pharmacological studies have revealed it to have antiviral,[2] hepatoprotective,[3] antioxidant,[4] cardiovascular protective[5] and immunostimulant activity.[6] In addition, Radix Astragali has been reported to increase the resistance to the immunosuppressive effects of chemotherapy.[7] Isoflavonoids, triterpene saponins and polysaccharides are the bioactive constituents most often associated with the biological activity of Radix Astragali.[8] Among these, isoflavonoids have been chosen as marker compounds for quality evaluations using HPLC/UV[9,10] due to the existence of chromophores for UV detection, or by LC-MS.[11,12] On the other hand, triterpene saponins, such as astragaloside IV in Radix Astragali, have been used for quality control in Chinese Pharmacopoeia.[13] Several analytical methods have been developed for the determination of saponins in Radix Astragali. However, due to the lack of chromophores, most of these have been performed using thin-layer chromatography (TLC),[14] high performance liquid chromatography and evaporative light scattering detection (HPLC-ELSD),[15,16] precolumn derivatization high performance liquid chromatography[17] and LC-MS with solid phase extraction (SPE).[18] Recently, the simultaneous determination of six isoflavonoids and four saponins of Radix Astragali by HPLC-DAD-ELSD was reported.[19] However, more rapid, sensitive, and reliable analytical and quantitative method for the quality control of Radix Astragali is necessary. Therefore, the aim of this study was to establish a rapid and sensitive method for the simultaneous analysis of three isoflavonoids (calycosin-7-*O*-β-glucoside, isomucronulatol 7-*O*-β-glucoside, formononetin) and two saponins (astragaloside I, astragaloside IV) (Figure 1) of Radix Astragali using HPLC/UV and LC-ESI-MS/MS methods.

UV detection is a convenient and sensitive method for evaluating the isoflavonoids in Radix Astragali. However, it is difficult to detect saponins in Radix Astragali using a UV detector because of the lack of chromophore groups and the poor absorption of UV radiation. Therefore, LC-ESI-MS/MS methods were used because they are powerful methods for rapidly identifying and elucidating the multiple-ingredients of Chinese medicine on account of their low detection limit, high specificity and structure elucidation.[20] This study developed a rapid and sensitive analytical and quantitative method to simultaneously analyze the three isoflavonoids and two saponins in Radix Astragali using high performance liquid chromatography and tandem mass spectrometry. The proposed method was validated[21,22] according to the linearity, accuracy, precision, recovery and robustness. This

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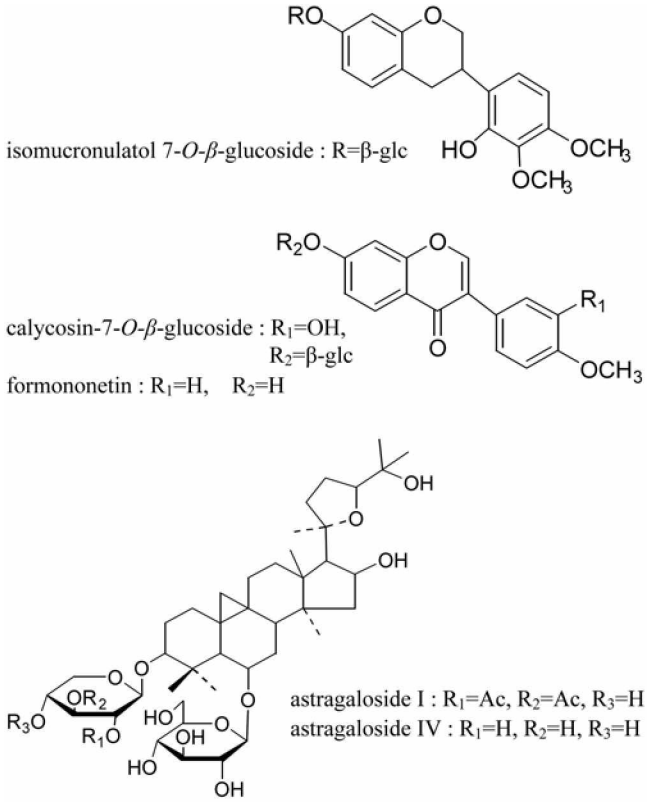

Figure 1. Molecular structures of the studied three isoflavonoids and two saponins in Radix Astragali.

method was found to be suitable for the quality control of Radix Astragali extracts and its pharmaceutical preparation.

Experimental

Materials and reagents. The powder of the dried roots of Radix Astragali, three isoflavonoids reference compounds (calycosin-7-*O*-*β*-glucoside, isomucronulatol 7-*O*-*β*-glucoside, formononetin) and two saponins (astragaloside I, astragaloside IV) were kindly supplied by the Natural Product Research Institute, Seoul National University (Seoul, Korea). Acetonitrile, methanol and water used were of chromatographic grade (J.T. Baker, Philipsburg, MT, USA). All other reagents used were of analytical reagent grade or better.

Preparation of standard solutions. The reference compounds, calycosin-7-*O*-*β*-glucoside, isomucronulatol 7-*O*-*β*-glucoside, formononetin, astragaloside I and astragaloside IV were weighed accurately (2 mg each) and dissolved in 50% acetonitrile to prepare 200 ppm stock solutions. For the internal standard, epi-aminotadalafil (in case of HPLC/UV) and byakangelicol (in case of LC-ESI-MS/MS) were weighed accurately (10 mg each) and dissolved in 50% acetonitrile to prepare 1,000 ppm stock solutions. The prepared stock solutions were degassed in an ultrasonic bath and filtered through a 0.45 μm membrane. These stock solutions were diluted with 50% acetonitrile to the concentrations required in the experiment. All the solutions were stored at 4 °C before analysis.

HPLC/UV analysis. The HPLC equipment was a Waters HPLC system (Waters, Milford, MA, USA) with Waters 600E pumps (Waters), a Waters 2487 UV detector (Waters), a Hitachi L-2200 autosampler (Hitachi High Technologies America, San Jose, CA, USA) and a 10 μL sample loop. Hypersil GOLD (4.6 × 250 mm, 5 μm, Thermo Electron Corp., Waltham, MA, USA), Onyx Monolithic (4.6 × 100 mm, Phenomenex, Torrance, CA, USA) and phenyl-hexyl (4.6 × 150 mm, 5 μm, Phenomenex, Torrance, CA, USA) columns were tested with the guard columns filled with the same stationary phase. The column temperature was set to 30 °C using a Jasco CO-965 column oven (Jasco, Wakayama, Japan). The mobile phase consisted of 20% acetonitrile in water (A) and 80% acetonitrile in water (B) using a gradient elution of 0-10% B at 0-2 min, 10-40% B at 2-15 min, 40-100% B at 15-20 min, 100-100% B at 20-25 min, 100-0% B at 25-35 min. The mobile phase was filtered under vacuum through a 0.45 μm membrane filter and degassed prior to use. The analysis was carried out at a flow rate of 1.0 mL/min with the detection wavelength set to 230 nm and the injection volume was 10 μL. The total run time was 35 min. The chromatograms were processed using an Autochro-2000 (Ver. 1.0.0).

LC-ESI-MS/MS analysis. The Alliance 2795 HPLC system (Waters) was coupled with the Waters Quattro micro API (Waters) equipped with an ESI source with a column temperature set to 30 °C equipped with a pump, an autosampler, a degasser, an automatic thermostatic column oven and computer software with a MassLynx (Ver. 4.1). The chromatographic separation was performed on a Capcell pak C18 (2.0 × 50 mm, 3 μm, Phenomenex) column and a precolumn filter (0.5 μm, Upchurch Scientific, Oak Harbor, WA, USA). A mixture of acetonitrile and water (40:60, v/v %) was used as the mobile phase. The flow rate and the injection volume were 0.2 mL/min and 10 μL, respectively.

Detection was performed by mass spectrometry in the multiple reaction monitoring mode (MRM). Ultrahigh purity argon (Ar) and high purity nitrogen (N_2) were used as the collision and the nebulizing gases, respectively. In order to tune the mass spectrometer, a 1 μg/mL solution of the reference compounds dissolved in 50% acetonitrile was pumped continuously into the ESI source at a rate of 10 μL/min using a syringe pump. The ionized chamber was set at desolvation gas flow, source temperature and desolvation temperature of 700 L/hr, 120 °C and 350 °C, respectively. The optimal collision energy and precursor ion-product ion transitions for the multiple reaction monitoring experiment, based on peak area, were optimized.

Analytical method validation

Selectivity: HPLC/UV and LC-ESI-MS/MS conditions were determined so there would be no overlap of the bioactive compounds from the extract of Radix Astragali and good peak separation.

Linearity: The calibration curves were made by diluting the stock solutions with 50% acetonitrile. A reference solution of the three isoflavonoids (calycosin-7-*O*-*β*-glucoside, isomucronulatol 7-*O*-*β*-glucoside, formononetin) at concen-

trations of 200-10,000 ng/mL was analyzed by HPLC/UV. while the reference solution of the bioactive compounds (calycosin-7-*O*-β-glucoside, formononetin, astragaloside I, astragaloside IV) was measured at 50-750 ng/mL by LC-ESI-MS/MS. The regression equations were calculated in the form of $y = ax + b$, where y and x correspond to the peak area and compound concentration, respectively.

Precision and accuracy: In order to confirm the reproducibility, the intra- and inter-day precision were estimated by analyzing five replicates containing the standard compounds at four different concentrations in a single day and repeating this analysis for five days, respectively. The acceptance criteria for intra- and inter-day accuracy and intra- and inter-day precision were < 15% for both bias and C.V., except at the limit of quantification (LOQ), for which accuracy and precision should be < 20%.

Limits of detection and quantification: The limit of detection (LOD) is defined as the concentration of the standard solution with a signal-to-noise ratio > 3 (S/N > 3). The limit of quantification (LOQ) is defined as the concentration of standard solution with a signal-to-noise ratio > 10 (S/N > 10). The acceptance criteria for the intra-day and inter-day accuracy and precision were < 20% for both the bias and C.V.

Robustness: The robustness test is considered to be a part of the validation method associated with precision. The purpose of this test was to identify the possible sources of error in the established method that might occur due to the changes in the specified internal conditions. Robustness evaluates the influence of changes in the internal factors of the established analytical procedure, such as the variations in column temperature, flow rate and mobile phase composition, providing an indication of its reliability during normal use. In this paper, the influence of small variations on the internal parameters of this method, such as the column temperature, flow rate and mobile phase composition were examined in triplicate for the each standard compound.

Quantitative determination by HPLC/UV

Recovery: For the preparation of the crude extract, the powders of the dried roots of Radix Astragali were sieved through a 30 mesh. The recovery test was executed by mixing a powdered root sample (400 mg) with three control levels (1 μg/mL, 2 μg/mL, 3 μg/mL) of the reference compounds. The mixture was then extracted by sonication with 10 mL of 70% ethanol at room temperature for 1 hr. The extract solution was filtered through a 0.45 μm membrane. The HPLC/UV analysis experiments were performed in triplicate for each control level. The data was compared with those from the standard solution (assuming 100% recovery) and extracted sample (400 mg powered root samples without the reference compounds)

Quantitative determination: The powdered dried roots of eight Radix Astragali from Korea and ten Radix Astragali from China were sieved through a 30 mesh. The quantitative determination was performed by mixing the powdered root sample (400 mg) with 10 mL of 70% ethanol. Sample preparation was performed as mentioned above.

Results and Discussion

HPLC/UV analysis

Optimization of chromatographic conditions: The HPLC conditions were selected by the requirement for obtaining the chromatograms with a better resolution of the adjacent peaks within a short retention time. The chromatographic conditions established in this study produced baseline separation of the three isoflavonoids in Radix Astragali in a single run detected at 230 nm. However, this method failed to detect the two saponins due to the lack of chromophores. The gradient elution of 20% acetonitrile in water and 80% acetonitrile in water at various ratios was used as the mobile phase. The ODS column, monolithic column and phenyl-hexyl columns were examined to determine which was most suitable. The run time of the ODS column was almost 100 min at 1.0 mL/min. The run time of the monolithic column was 40 min at 3.0 mL/min with good separation of the 70% ethanol extract of Radix Astragali. The phenyl-hexyl column showed good separation with the shortest total run time. Figure 2 shows the representative HPLC/UV chromatograms of the three isoflavonoids and the 70% ethanol extract of Radix Astragali. The chromatographic peaks were identified by comparing the retention time and UV spectra of the compounds in the extract with those of the standard compounds, which were eluted in parallel with a series of mobile phases.

Selectivity: The retention time of the three isoflavonoids and I.S. in the extract of Radix Astragali were 5.1 min (calycosin-7-*O*-β-glucoside), 12.1 min (isomucronulatol 7-

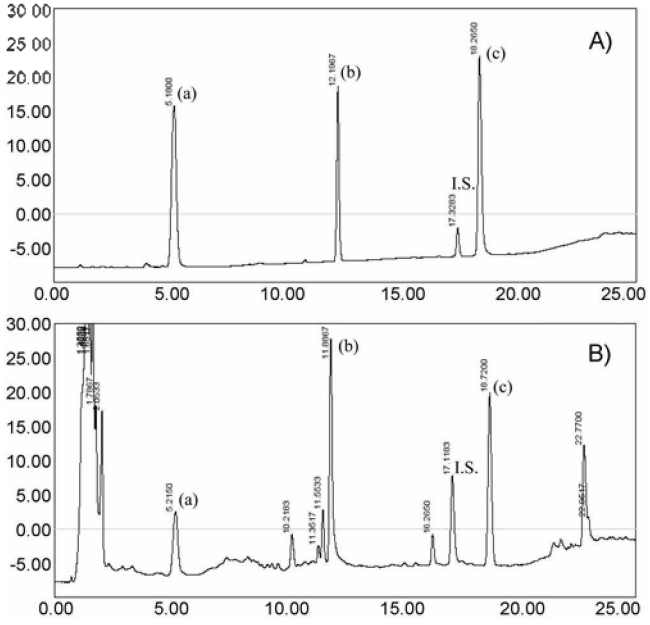

Figure 2. Chromatograms of the three isoflavonoids in Radix Astragali using the developed HPLC method. The chromatographic conditions are described in experimental section. A) Chromatogram of the reference compounds. B) Chromatograms of the 70% ethanol extract of Radix Astragali. The indicated peaks from a to c corresponds to (a) calycosin-7-*O*-β-glucoside, (b) isomucronulatol 7-*O*-β-glucoside and (c) formononetin, respectively. I.S. represents the internal standard, epi-aminotadalafil.

Table 1. Results of linear regression analysis in the determination of the three isoflavonoids using the HPLC/UV method

Analytes	Regression equation	Correlation coefficient (r^2)	LOD (ng/mL)		LOQ (ng/mL)	
			Conc.	S/N	Conc.	S/N
Calycosin-7-*O*-*β*-glucoside	$y = 0.001x - 0.0798$	0.9999	50	3.00	200	13.25
Isomucronulatol 7-*O*-*β*-glucoside	$y = 0.0006x + 0.0035$	0.9983	50	3.29	200	11.09
Formononetin	$y = 0.0009x - 0.0785$	1.0000	50	3.16	200	10.32

In the regression equation, $y = ax - b$, y refers to the peak area, and x refers to the concentration of the reference compound (ng/mL). LOD is the limit of detection, and LOQ is the limit of quantification.

O-*β*-glucoside), 17.3 min (I.S.) and 18.2 min (formononetin), and all peaks were well separated.

Linearity: The linearity of the peak area ratio with respect to the concentration was examined under optimal HPLC/UV conditions and is described as a regression equation. The standard curves were linear over the concentration ranges from 200 to 10,000 ng/mL. The regression equation was calculated to be $y = 0.001x - 0.0798$ for calycosin-7-*O*-*β*-glucoside, $y = 0.0006x + 0.0035$ for isomucronulatol 7-*O*-*β*-glucoside and $y = 0.0009x - 0.0785$ for formononetin, in where y and x correspond to the peak area and concentration of each compound (ng/mL), respectively (Table 1). Each coefficient of determination (r^2) was > 0.9983, as determined by least square analysis, suggesting good linearity between the peak areas (y) and the compound concentrations (x) over a wide concentration range.

Precision and accuracy: Table 2 shows the precision and accuracy of the three isoflavonoids from Radix Astragali determined in the intra- and inter-day trials (n = 5). The results show intra-day and inter-day precision of 0.70-9.39% and 2.55-19.76%, respectively. The intra-day and inter-day accuracy of the three isoflavonoids are 91.6-110.0% and 94.3-107.1%, respectively. This indicates that the developed HPLC method is accurate and precise for the simultaneous evaluation of the three active isoflavonoids in Radix Astragali.

Limits of detection and quantification: The LODs (S/N > 3) of the three isoflavonoids were 50 ng/mL, which indicated high sensitivity under these HPLC conditions. In addition, the LOQs (S/N > 10) of the three isoflavonoids were 200 ng/mL. This shows that the acceptance criteria for the intra- and inter-day accuracy and intra- and inter-day precision were < 20% for both the bias and C.V..

Robustness: The robustness was determined in order to evaluate the reliability of the established HPLC methods. Briefly, the experimental conditions, such as the column temperature, flow rates and composition of the mobile phase were purposely altered. The area precision (C.V.), relative retention time (RRT), peak tailing factor (T) and theoretical

Table 2. Intra-, inter-day precision and accuracy of the bioactive components in Radix Astragali using HPLC/UV method (n = 5)

Analytes	Conc. (ng/mL)	Intra-Day		Inter-Day	
		Precision (C.V. %)	Accuracy (%)	Precision (C.V. %)	Accuracy (%)
Calycosin-7-*O*-*β*-glucoside	200	7.40	108.3	14.13	103.6
	500	6.08	91.6	7.25	95.2
	1000	3.75	94.4	3.97	99.1
	10000	1.66	93.5	4.77	101.0
Isomucronulatol 7-*O*-*β*-glucoside	200	9.39	108.0	19.76	101.1
	500	4.71	101.7	4.51	100.0
	1000	5.01	100.0	5.14	97.9
	10000	4.09	95.5	4.15	99.9
Formononetin	200	3.32	110.0	7.29	107.1
	500	5.41	97.5	2.55	97.4
	1000	0.70	97.6	2.62	94.3
	10000	2.78	94.9	3.20	95.4

Table 3. The robustness test for the HPLC/UV method (n = 3)

Analytes	Conditions		C.V.	RRT	T	N
Calycosin-7-*O*-*β*-glucoside	Temperature (°C)	30	0.91	0.31	0.95	2256
		35	7.06	0.31	1.15	2382
		40	1.90	0.30	1.16	2295
	Flow rate (mL/min)	0.9	0.42	0.33	0.93	3344
		1.0	0.91	0.31	0.95	2672
		1.1	0.39	0.31	1.06	2361
	Gradient Profile*	(a)	7.11	0.28	1.08	2511
		(b)	0.91	0.31	0.95	2256
		(c)	2.75	0.33	1.10	2555
Isomucronulatol 7-*O*-*β*-glucoside	Temperature (°C)	30	2.41	0.71	1.01	39343
		35	7.96	0.71	1.23	37300
		40	4.21	0.71	1.14	34280
	Flow rate (mL/min)	0.9	1.15	0.71	1.15	40966
		1.0	2.41	0.71	1.01	39343
		1.1	3.88	0.68	1.26	29890
	Gradient profile*	(a)	3.42	0.65	1.31	39609
		(b)	2.41	0.71	1.01	39343
		(c)	3.48	0.74	1.16	60681
Formononetin	Temperature (°C)	30	2.02	1.05	1.52	40809
		35	2.42	1.05	1.27	49660
		40	3.22	1.04	1.31	36051
	Flow rate (mL/min)	0.9	1.96	1.06	1.56	49816
		1.0	2.02	1.05	1.52	40809
		1.1	1.08	1.06	1.35	43260
	Gradient profile*	(a)	3.85	1.05	1.44	44132
		(b)	2.02	1.05	1.52	40829
		(c)	2.49	1.05	1.25	55097

C.V. represents the area precision, RRT represents the relative retention time, T represents the peak tailing factor and N represents the theoretical plate number. *: Gradient profile is as follows: (a) 10-30% B at 2-15 min (b) 10-40% B at 2-15 min (c) 10-50% B at 2-15 min and the others are same as shown in the above text.

plate number (N) were then evaluated. The changes in column temperature and flow rates were 35 ± 5 °C, and 1.0 ± 0.1 mL/min. respectively. All the results listed in Table 3 show the average of at least three independent experiments. The area precision (C.V.) was < 7.96% and the other parameters were maintained so there would not be any interference with the other peaks for the Radix Astragali extract.

LC-ESI-MS/MS Analysis

Optimization of chromatographic separation conditions: For LC-ESI-MS/MS analysis, a Waters 2795 HPLC system was connected to Waters Quattro micro API system. The best conditions for ionizing the active constituents in Radix Astragali in the mass spectrometer were obtained with ESI positive ionization mode using a mixture of acetonitrile and water (40:60, v/v %) as the mobile phase. However, it was difficult to detect isomucronulatol 7-*O*-*β*-glucoside in Radix Astragali using LC-ESI-MS/MS due to a lack of ionization. Figure 3 shows the mass spectra produced by LC-ESI-MS/MS under the above mentioned chromatographic conditions. In the mass spectra, the $[M+H]^+$ of the two isoflavonoid and $[M+Na]^-$ of the two saponins can be easily observed. In the MS^2 spectra, the fragment ions indicating the loss of glucose, xylose and CH_3OH were observed.

The molecular ions of calycosin-7-*O*-*β*-glucose, formononetin, astragaloside I, astragaloside IV and byakangelicol were fragmented at collision energy of 25, 38, 90, 90 and 26 eV, respectively. Figure 4 shows the multiple reaction monitoring (MRM) chromatograms of calycosin-7-*O*-*β*-glucose at *m/z* 285.0 from the MS/MS scan precursor ion at *m/z* 446.9, formononetin with *m/z* 268.9 → 197.0, astragaloside I

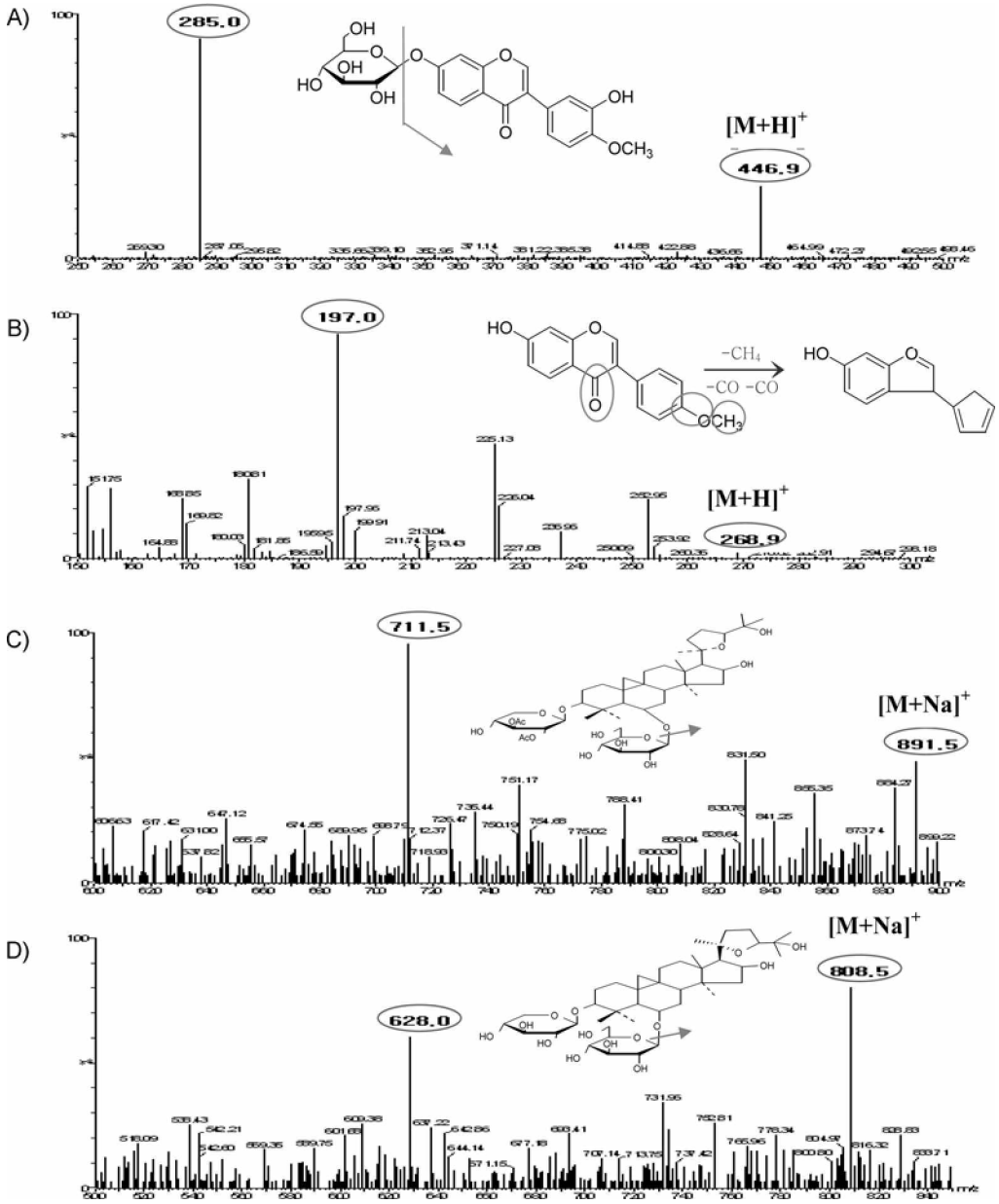

Figure 3. Mass spectra of A) calycosin-7-*O*-*β*-glucoside, B) formononetin, C) astragaloside I and D) astragaloside IV produced by LC-ESI-MS/MS.

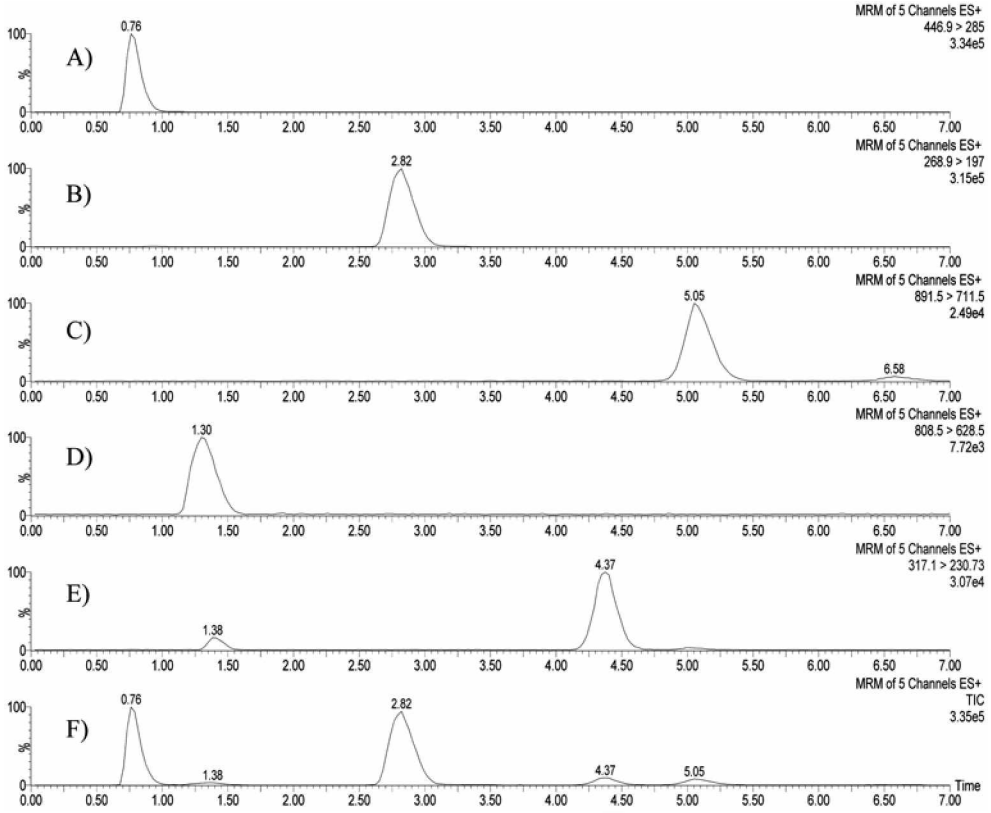

Figure 4. LC-ESI-MS/MS chromatograms of A) calycosin-7-*O*-*β*-glucoside, B) formononetin, C) astragaloside I, D) astragaloside IV, E) byakangelicol (I.S.) and F) total ion chromatogram (TIC).

with *m*/*z* 891.5 → 711.5, astragaloside IV with *m*/*z* 808.5 → 628.0 and byakangelicol (I.S.) with *m*/*z* 317.1 → 230.7. The peaks obtained for all compounds using the LC-ESI-MS/MS technique had good shapes with good separation. Under these conditions, two isoflavonoids, two saponins and an internal standard, byakangelicol were analyzed within 7 min.

Selectivity: The retention times for the extract of Radix Astragali were 0.76 min (calycosin-7-*O*-*β*-glucoside), 1.30 min (astragaloside IV), 2.82 min (formononetin), 5.05 min (astragaloside I) and 4.37 min (I.S.), and all peaks were well separated.

Linearity: The linearity of the peak area ratio with respect to the concentration using the developed LC-ESI-MS/MS method was examined and the results are shown as a regression equation. The standard curves were linear over the concentration ranges from 50 to 750 ng/mL. The regression equation was $y = 0.0114x + 0.4142$ for calycosin-7-*O*-*β*-glucoside. $y = 0.0170x + 2.0764$ for formononetin, $y = 0.0017x - 0.0576$ for astragaloside I and $y = 0.0005x - 0.0156$ for astragaloside IV, in where y and x correspond to the peak area and concentration of each compound (ng/mL), respectively. The high correlation ($r^2 > 0.9913$) indicates good linearity between the peak areas (y) and concentrations (x) over a relatively wide concentration range (Table 4).

Precision and accuracy: Table 5 shows the intra-day and inter-day ($n = 5$) precision and accuracy of the two isoflavonoids and two saponins from Radix Astragali. The data shows that the intra-day and inter-day precision were 1.05-5.24% and 3.77-19.77%, respectively. The intra-day and inter-day accuracy of the two isoflavonoids and two saponins were 86.8-117.6% and 87.0-116.2%, respectively. This indicates that the developed LC-ESI-MS/MS method is accurate and precise for the simultaneous evaluation of the two isoflavonoids and two saponins in Radix Astragali.

Table 4. Results of linear regression analysis of the LC-ESI-MS/MS method

Analytes	Regression equation	Correlation coefficient (r^2)	LOD (ng/mL)		LOQ (ng/mL)	
			Conc.	S/N	Conc.	S/N
Calycosin-7-*O*-*β*-glucoside	$y = 0.0114x + 0.4142$	0.9949	1.25	3.97	50	35.52
Formononetin	$y = 0.0170x + 2.0764$	0.9980	1.25	3.66	50	32.70
Astragaloside I	$y = 0.0017x - 0.0576$	0.9920	1.25	3.66	50	15.32
Astragaloside IV	$y = 0.0005x - 0.0156$	0.9913	1.25	3.97	50	14.26

In the regression equation, $y = ax - b$, y refers to the peak area, x refers to concentration of the reference compound (ng/mL). LOD is the limit of detection and LOQ is the limit of quantification.

Table 5. Intra-, inter-day precision and accuracy of the bioactive components in Radix Astragali using LC-ESI-MS/MS method (n = 5)

Analytes	Conc. (ng/mL)	Intra-Day		Inter-Day	
		Precision (C.V. %)	Accuracy (%)	Precision (C.V. %)	Accuracy (%)
Calycosin-7-*O*-*β*-glucoside	50	1.65	86.8	18.66	90.8
	100	2.32	88.2	8.46	103.6
	250	2.45	90.2	13.56	87.9
	750	3.44	87.5	14.71	95.0
Formononetin	50	1.05	105.3	19.77	97.8
	100	2.18	98.5	6.77	99.9
	250	1.41	112.2	5.84	101.0
	750	4.53	98.1	13.16	93.4
Astragaloside I	50	2.71	116.2	7.74	116.2
	100	3.11	90.5	3.77	87.0
	250	2.98	109.9	4.98	93.6
	750	1.74	113.2	7.25	111.3
Astragaloside IV	50	3.51	117.6	7.48	115. 3
	100	2.64	95.2	6.49	91.2
	250	1.51	112.3	8.84	111.9
	750	5.24	108.1	9.73	100.5

Limits of detection and quantification: The LODs (S/N > 3) of the two isoflavonoids and two saponins were 1.25 ng/mL and 12.5 ng/mL, respectively, which indicates a high sensitivity under these LC-ESI-MS/MS conditions. The LOQs (S/N > 10) of the two isoflavonoids and two saponins were 50 ng/mL, which show that the acceptance criteria for intra- and inter-day accuracy and intra- and inter-day precision were < 20% for both the bias and C.V.

Robustness: The reliability of the established LC-ESI-MS/MS methods was examined by testing the robustness. Briefly, the experimental conditions, such as column temperature, flow rates and composition of the mobile phase, were altered purposely. The area precision (C.V.), relative retention time (RRT), peak tailing factor (T) and theoretical plate number (N) were evaluated. The changes in the column temperature and flow rates were 35 ± 5 °C and 200 ± 20 μL/min, respectively. The changes in the mobile phase were 40 ± 2% acetonitrile. All the results listed in Table 6 are the average of at least three independent experiments. The area precision (C.V.) was < 12.08% and the other parameters were maintained so there would not be any interference with the other peaks in the Radix Astragali extract. Based on these results, the LC-ESI-MS/MS method developed in this study was found to be acceptable for the simple, rapid and simultaneous determination of the two isoflavonoids and two saponins in Radix Astragali.

Quantitative determination by HPLC/UV

Recovery: The extraction recovery test was performed by extracting a known amount of the isoflavonoids from the Radix Astragali powder samples. A known amount of each standard compound at three different levels was mixed with the sample powder and extracted, as described in the experimental section. The % recovery of each standard ranged from 96.56 to 99.27%, and the RSD was < 3.0% (Table 7).

Table 6. Results of the robustness test of the LC-ESI-MS/MS method (n = 3)

Analytes	Analytical Condition		C.V.	RRT	T	N
Calycosin-7-*O*-*β*-glucoside	Temperature	30 °C	3.48	0.18	1.13	98
		35 °C	3.02	0.19	1.33	129
		40 °C	3.25	0.20	1.41	180
	Flow rate	180 uL/min	10.3	0.18	1.29	144
		200 uL/min	5.34	0.18	1.22	200
		220 uL/min	5.20	0.18	1.49	171
	Gradient Profile	38%	1.25	0.17	1.45	185
		40%	5.75	0.18	1.21	157
		42%	2.32	0.17	1.39	140
Formononetin	Temperature	30 °C	2.30	0.64	1.70	675
		35 °C	1.43	0.64	1.65	622
		40 °C	3.34	0.64	1.50	670
	Flow rate	180 uL/min	12.08	0.64	1.48	690
		200 uL/min	5.26	0.64	1.39	709
		220 uL/min	3.17	0.64	1.72	665
	Gradient profile	38%	0.24	0.64	1.37	775
		40%	3.08	0.64	1.41	683
		42%	1.87	0.64	1.61	631
Astragaloside I	Temperature	30 °C	2.65	1.16	1.28	1621
		35 °C	2.36	1.35	1.27	2224
		40 °C	0.34	1.53	1.46	2368
	Flow rate	180 uL/min	0.88	1.16	1.51	1978
		200 uL/min	1.29	1.17	1.58	1608
		220 uL/min	5.82	1.17	1.37	1952
	Gradient profile	38%	0.34	1.25	1.43	1436
		40%	2.46	1.16	1.39	1735
		42%	0.77	1.23	1.20	1260
Astragaloside IV	Temperature	30 °C	1.49	0.31	1.19	110
		35 °C	0.28	0.35	1.56	168
		40 °C	0.97	0.40	1.38	363
	Flow rate	180 uL/min	7.40	0.31	1.06	132
		200 uL/min	2.30	0.31	1.11	115
		220 uL/min	5.76	0.30	1.38	149
	Gradient profile	38%	2.59	0.30	1.52	88
		40%	6.22	0.30	0.91	122
		42%	5.30	0.30	1.52	95

C.V. represents the area precision. RRT represents the relative retention time, T represents the peak tailing factor and N represents the theoretical plate number

The average recovery is represented by the formula: R (%) = [(amount from the root sample spiked standard – amount from the root sample)/amount from the spiked standard] × 100.

Analysis of Radix Astragali: The developed HPLC/UV method was then applied to the simultaneous determination of the three major isoflavonoids, calycosin-7-*O*-*β*-glucoside, isomucronulatol 7-*O*-*β*-glucoside and formononetin, in the Radix Astragali extract. Figure 2 shows chromatogram of the reference compounds and the 70% ethanol extract of Radix Astragali. As shown in Figure 2b, there was good chromatographic separation of the isoflavonoids in the extract of Radix Astragali using the established method. Eighteen commercially available Radix Astragali samples were obtained from Korea and China. Table 8 summarized the concen-

Table 7. Results of the recovery test by HPLC/UV

Analytes	Spiked grade*	Recovery (%)			Recovery mean (%)	RSD (%)
		1	2	3		
Calycosin-7-*O*-*β*-glucoside	Low	95.66	94.72	99.29	96.56	2.41
	Middle	99.70	99.63	98.48	99.27	0.68
	High	99.48	98.68	99.33	99.16	0.42
Isomucronulatol 7-*O*-*β*-glucoside	Low	95.85	96.06	99.49	97.13	2.05
	Middle	99.40	98.76	97.64	98.60	0.89
	High	97.41	96.97	95.92	96.77	0.77
Formononetin	Low	99.62	96.59	98.47	98.22	1.53
	Middle	96.83	99.26	96.71	97.60	1.44
	High	97.43	97.84	99.74	98.34	1.23

*Low: 1 μg/mL, Middle: 2 μg/mL, High: 3 μg/mL

Table 8. Quantitative analytical results of the various Radix Astragali samples

No#	Mean concentrations of compounds (μg/g*)		
	Calycosin-7-*O*-*β*-glucoside	Isomucronulatol 7-*O*-*β*-glucoside	Formononetin
K1	207.02	198.06	37.54
K2	100.01	97.76	20.32
K3	44.64	84.20	27.21
K4	125.82	40.00	144.25
K5	215.95	223.44	37.90
K6	396.90	97.82	67.14
K7	98.49	77.68	43.56
K8	295.72	96.17	63.99
C1	116.81	64.16	83.09
C2	265.25	154.35	97.37
C3	169.62	47.13	69.56
C4	27.28	46.63	364.65
C5	75.51	43.32	255.77
C6	230.93	63.02	135.64
C7	193.84	18.81	85.26
C8	144.69	26.71	134.51
C9	127.23	28.70	331.10
C10	28.60	42.38	N.D.

*Refers to dry weight of Radix Astragali. N.D.: not detected. No#: "K" refers to Radix Astragali of Korea and "C" refers to Radix Astragali of China.

trations (μg/g). The data presented in Table 8 shows that the concentrations of three isoflavonoids, calycosin-7-*O*-*β*-glucoside, isomucronulatol 7-*O*-*β*-glucoside, and formononetin in the extract of Korean Radix Astragali were 44.64-396.90 μg/g, 39.97-223.44 μg/g, and 20.32-144.25 μg/g, respectively. The amounts of those isoflavonoids in the extract of Chinese Radix Astragali were 27.28-265.25 μg/g, 18.81-154.35 μg/g, and 69.56-364.65 μg/g, respectively. The formononetin was not detected in "C10" (number 10, the Radix Astragali of China).

Conclusions

Many papers have reported that isoflavonoids and saponins are the active constituents in Radix Astragali responsible for its biological activities. Therefore, techniques for the simultaneous quantitative and qualitative analysis of Radix Astragali are of great importance. This study examined the feasibility of HPLC/UV and LC-ESI-MS/MS methods in evaluating the quality and quantity of Radix Astragali. These methods successfully analyzed and quantified the three isoflavonoids and two saponins from Radix Astragali. The baseline separation of the three isoflavonoids and short analysis time were achieved using the HPLC/UV method. However, it was difficult to detect the saponins in Radix Astragali using a UV detector due to the lack of chromophore groups. The analysis of trace amounts was achieved by LC-ESI-MS/MS on account of its low detection limit and high specificity. In addition, the method showed good linearity, limit of detection, accuracy and precision. Overall, these simple, rapid, sensitive, reliable HPLC/UV and LC-ESI-MS/MS methods are suitable for the routine quantitative analysis and quality control of Radix Astragali.

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