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Research Article

RP-HPLC-UV Method for Simultaneous Determination of Nine Isoflavonoids in Huangqi-Gegen Herbal Pair

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Abstract: A new simple and efficient RP-HPLC-UV method was developed for the simultaneous analysis of nine isoflavonoids in Huangqi-Gegen herbal pair. Effective chromatographic separation of these components was achieved on a Kromasil C_{18} column (4.6×250 mm, i.d.; 5 μm) with gradient elution of methanol and 0.2% formic acid aqueous solution at a flow rate of 1.0 mL/min in 60 min. Detection was performed at 250nm. The method was proved to be linear in the ranges of 81.28-406.4, 670.4-3352, 136.96-684.8, 33.376-166.88, 22.848-114.24, 10.176-50.88, 31.04-155.2, 44.544-222.72 and 26.56-132.8 ng for the nine isoflavonoid: 3'-hydroxypuerarin, puerarin, daidzin, calycosin-7-O-β-D-glucoside, genistin, ononin, daidzein, calycosin and formononetin, respectively. The average recoveries were 99.27, 102.38, 98.46, 103.06, 101.29, 99.71, 102.28, 97.89 and 100.78 % respectively; RSD was 1.79, 2.02, 1.44, 1.37, 1.26, 1.81, 1.29, 0.97 and 1.77 % respectively. The developed method was successfully applied for the quantitative analysis of constituents in huangqi gegen herbal pair.

Keywords: Active constituents, HPLC, huangqi-gegen, isoflavonoids, pair medicine

INTRODUCTION

Astragali Radix (Huangqi in Chinese) and Puerariae lobatae Radix (Gegen in Chinese), commonly used as traditional Chinese medicines, are officially listed in the Chinese Pharmacopoeia (The Pharmacopoeia Commission of the People's Republic of China, 2010). Huangqi, the dried root of Astragalus membranaceus. var. mongholicus (Bge.) Hsiao or Astragalus membranaceus (Fisch.) Bge, is widely used as an immune modulator and cardioprotector (Rios and Waterman, 1997). The constituents of huangqi most often associated with its pharmacologic activities are isoflavonoids, saponins, polysaccharides. Calycosin, calycosin-7-O-beta-D-glucoside, formononetin and ononin, being main isoflavonoids, are displayed beneficial effects such as anti-oxidation, anti-ischemia, anti-osteoarthritis and antiviral activities (Fan *et al*., 2003; Huh *et al*., 2010; Zhu *et al*., 2009). Gegen, the dried root of Pueraria lobata (Willd.) Ohwi, is commonly used to relieve fever and dysentery and for the treatment of cardiovascular diseases such as hypertension, myocardial infarction and arrhythmia (Jiang *et al*., 2005). Its chemical constituents are mainly isoflavonoids, such as 3'-hydroxypuerarin, puerarin, daidzin, daidzein, genistin and genistein (Si *et al*., 2006). More recent studies revealed that Gegen isoflavonoids have significant biological effects in improving blood circulation, preventing cardiovascular diseases (Chen *et al*., 2007). Among these isoflavonoids, puerarin, daidzin, daidzein, genistin and genistein have been found to possess cardiovascular tonic effects (Chang *et al*., 2008), antioxidation (Bebrevska *et al*., 2010), inhibit formation of advanced glycation end products (Kim *et al*., 2006), protective of diabetic retinopathy (Teng *et al*., 2009), brain neurocyte (Pan and Li, 2008) and hepatic failure (Kim *et al*., 2009). Pharmaceuticals containing Huangqi-Gegen have long clinical application. Yufengningxin tablets has long been used to treat hypertension, coronary disease, angina pectoris, neck and nape pain (The Pharmacopoeia Commission of the People's Republic of China, 2010).

There are quite a few HPLC methods with either MS or PAD or UV detection reported in the literature for the identification and quantification of components in single herb of either Huangqi (Qi *et al*., 2006; Yu *et al*., 2007) or Gegen (Lin *et al*., 2005; Prasain *et al*., 2007). From a review of the literature, it was noticed that the efficiency of the assay for quality control of pharmaceuticals is highly dependent upon the selection of the representative markers. As reported in most of the

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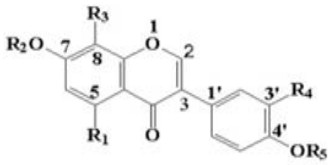
Table 1: Structure of nine isoflavones in Huangqi-Gegen herbal pair



No	Components	R_1	R_2	R_3	R_4	R_5
1	3'-Hydroxyl puerarin	H	H	Glc	OH	H
2	Puerarin	H	H	Glc	H	H
3	Daidzein	H	Glc	H	H	H
4	Calycosin glycosides	H	Glc	H	OH	Me
5	Genistin	OH	Glc	H	H	H
6	Ononin	H	Glc	H	H	Me
7	Daidzein	H	H	H	H	H
8	Calycosin	H	H	H	OH	Me
9	Formononetin	H	H	H	H	Me

literature for Huangqi and Gegen, it was readily established that calycosin-7-O-beta-D-glucoside is present in the highest quantity in Huangqi (Wu *et al*., 2005), whilst daidzin is also found in a large quantities in Gegen. Therefore, the above reported assay, which did not include these active components, could not serve as a sufficient quality control tool for pharmaceuticals containing both Huangqi and Gegen. A simple and efficient assay method capable of detecting multiple active components from both herbs is essential. For an objective and scientific assessment of the quality of Huangqi-Gegen decoction and other Chinese medicines containing Huangqi-Gegen pair medicines, this study established a method for the determination of nine isoflavonoids by RP-HPLC-UV.

MATERIALS AND METHODS

Materials: Shimadzu LC 10AT vp plus high-performance liquid chromatography, SHIMADZU SPD 10A vp plus UV detector, SHIMADZU CBM 10A vp plus ChemStation; Sartorius electronic balance (Germany, d 0.01 mg); polarimeter (WXG 4, Shanghai Pudong Physical Optics Instrument Factory); methanol HPLC grade water for the preparation of pure water, other reagents were of analytical grade; puerarin reference was purchased from the National Institutes for food and drug Control, genistin and other reference were from Shanghai Tauto biotech, calycosin-7-O-β-D-glucoside reference was provided by the Shanghai Chinese chemical reference substance Ltd., 3'-hydroxy-puerarin and daidzein reference (Kim *et al*., 2009; Teng *et al*., 2009) by the laboratory-made (melting point, specific rotation, UV, MS and NMR is consistent with the literature, purity>96%), the structure of nine isoflavones in Table 1. Huangqi and Gegen herbs, purchased from Guangzhou University of Chinese Medicine Pharmacy Ltd., were identified by Professor weimin Li.

Preparation of the test solution: Reference preparation: Dissolve appropriate amount of reference substance, in methanol in a 25 mL volumetric flask and mix well. Measure accurately this stock solution, dilute with mobile phase at initial proportion, the concentration of the working solution was 20.32, 167.60, 34.24, 8.34, 5.71, 6.54, 7.76, 11.14, 24.80 and 6.64 μg/mL (1-9), respectively.

Table 2: The mobile phase gradient elution procedure

Time/min	Mobile phase A/%	Mobile phase B/%
0 ~ 15	25	75
15 ~ 30	25→45	75→55
30 ~ 45	45	55
45 ~ 50	45→25	55→75
50 ~ 60	25	75

Test preparation: According to the proportion of Huangqi-Gegen decoction prescription, weighed 20 g of huangqi pieces, 10 g of gegen pieces, add 10 times of water, reflux extraction two times, each time for 1 h, the combined extracts were concentrated under reduced pressure and constant volume at 250 mL, shake and filtrate (0.45 μm).

Chromatographic conditions and system suitability: Kromasil 100-5 C_{18} (4.6 × 250 mm, id., 5 μm) column, pre-column (Security Guard, C_{18}, 4.0×3.0 mm, Phenomenex); using methanol as mobile phase A, 0.1% citric acid solution as mobile phase B, gradient elution (Table 2); flow rate of 1.0 mL/min, column temperature was 25°C, the detection wavelength was 250 nm, the injection volume was 10 μL. Theoretical plates of the test solution chromatogram with each corresponding absorption peaks were not less than 7000 and peaks separation greater than 2.0.

Methodological studies: Limits of detection and quantification: The Limits of Detection (LOD) and Quantification (LOQ) for each studied component were defined as the lowest concentration of component in the diluted standard solution producing a signal-to-noise ratio (S/N) of 3:1 and 10:1, respectively.

Calibration curves: Working solutions containing all nine references were prepared by diluting the stock

Table 3: The linear relationship of isoflavones

	y = ax+b (y = Peak area, x = Injection Quality, ng)		
No	a	b	R^2
1	3269.8	9273.800	1.0000
2	4846.4	-1490266	0.999 9
3	33740	-6075100	0.999 6
4	3088.2	-16236.70	1.0000
5	2966.4	-1417400	0.999 8
6	6478.9	-2749200	0.999 9
7	4327.2	-252.4200	0.999 9
8	2898.3	3075.1000	0.999 8
9	3548.2	1417.4000	0.999 9

Table 4: The linear relationship of isoflavones

No	The linear range/ng	LOD/ng	LOQ/ng
1	81.28-406.4	19.24	63.50
2	670.4-3352	19.74	65.80
3	136.96-684.8	21.40	68.48
4	33.376-166.88	8.040	25.72
5	22.848-114.24	6.280	20.98
6	26.176-130.88	6.540	20.68
7	31.04-155.2	8.550	25.50
8	44.544-222.72	9.88	30.52
9	26.56-132.8	7.32	22.80

solution to proper concentration in order to draw calibration curves. Each calibration curve contained six different concentrations and was performed in triplicate:

Precision tests: Precision drawing reference solution, into the liquid chromatograph, six replicates.

Repeatability tests: Precision drawing test solution into the liquid chromatograph, repeated six times.

Stability tests: Stability was tested with a sample solution at room temperature and analyzed at 0, 2, 4, 8, 12 and 24 h.

Recovery tests: According to the proportion of Huangqi-Gegen decoction prescription, weigh the same batch of Huangqi Pieces 2 g, Pueraria Herbal 1 g, 6 copies. Accurately weighed, add precision to a considerable amount of the reference solution, the solvent evaporated, with the same approach to measuring, calculating recoveries.

Application: The optimized method was applied to analyse the contents of nine components in Huangqi-gegen decoction, the samples solutions were prepared as described above. The content HPLC analysis of each compound analysed was calculated from the corresponding calibration curve.

RESULTS AND DISCUSSION

Methodological studies: Limits of detection and quantification, Calibration curves: All calibration curves were constructed from peak areas of the reference standards versus their concentrations (Table 3 and 4).

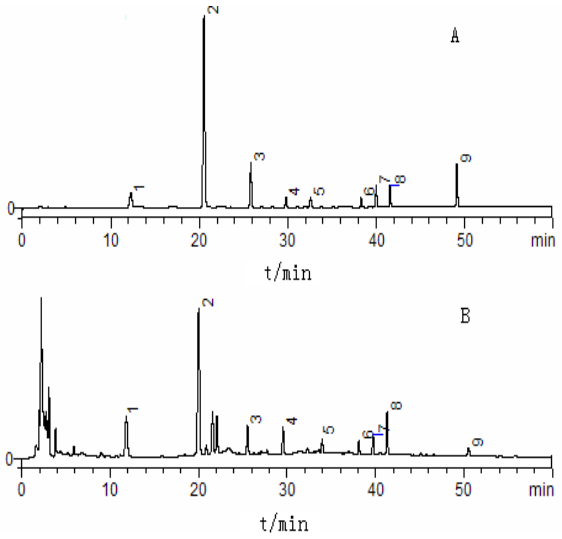


Fig. 1: Representative HPLC-UV (250 nm) chromatograms of: (A) refrence, (B) Sample. Peak number: (1): 3'-hydroxypuerarin; (2): puerarin; (3): daidzin; (4): calycosin-glucoside; (5): genistin; (6): ononin; (7): daidzein; (8): calycosin; (9): formononetin

Table 5: The linear relationship of isoflavones

	Different regions		
No	Shanxi-hunan	Shanxi-guangdong	Neimong-hunan
1	1.94±0.160	0.92±0.110	1.88±0.140
2	16.13±0.27	11.01±0.21	15.92±0.24
3	3.24±0.130	1.17±0.110	3.31±0.140
4	0.83±0.070	0.86±0.060	1.17±0.080
5	0.57±0.060	0.24±0.030	0.61±0.070
6	0.54±0.070	0.51±0.060	0.81±0.080
7	0.64±0.050	0.37±0.030	0.72±0.060
8	0.82±0.040	0.79±0.030	0.97±0.060
9	0.27±0.020	0.31±0.030	0.66±0.040

Precision tests: resulting peak area RSD was 0.94, 1.13, 0.77, 1.81, 0.93, 1.31, 0.98, 1.04 and 1.17% (1-9), respectively. The results showed good precision instruments.

Repeatability tests: resulting peak area RSD was 0.97, 1.15, 0.87, 1.89, 0.91, 1.25, 0.67, 0.93 and 1.04 % (1-9), respectively. The results showed good reproducibility.

Stability tests: The resulting peak area RSD were 1.34, 1.27, 1.03, 1.99, 1.39, 1.83, 1.17, 0.94 and 1.43 % (1-9), respectively. The results showed stable within 24 h.

Recovery tests: the results of each reference standard average recoveries was 99.27, 102.38, 98.46, 103.06, 101.29, 99.71, 102.28, 97.89 and 100.78%; RSD was 1.79, 2.02, 1.44, 1.37, 1.26, 1.81, 1.29, 0.97 and 1.77 % (1-9), respectively.

Application: The results are shown in Fig. 1 and Table 5.

CONCLUSION

In this study, RP-HPLC-UV method for the simultaneous determination of the content of nine isoflavones from Huangqi-Gegen decoction, the method of high sensitivity, specific and the mobile phase systems and sample preparation method is simple, rapid, accurate and effective. These provided for effectively control the quality of Huangqi-Gegen decoction and Chinese medicines containing Huangqi-Gegen an experimental basis.

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