The Presence of Three Isoflavonoid Compounds in *Psoralea corylifolia*

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Abstract

The optimization of a high-performance liquid chromatographic method to determine three isoflavonoids (daidzein, genistein, and biochanin A) in the fruit of *Psoralea corylifolia* is developed and validated. Dried psoralea fruit powder is extracted with aqueous methanol followed by the hydrolysis of the analytes' conjugated glycosides with hydrochloric acid. The HPLC assay is performed on a reverse-phase C_{18} column with gradient elution using acetonitrile and 10% acetic acid as the mobile phase at a flow rate of 0.8 mL/min. Flavone is used as the internal standard and the substances are detected at 260 nm. Calibrations are linear (correlation coefficient ≥ 0.995) for all three analytes. The limits of detection are 0.01 µg/mL for daidzein and genistein and 0.1 µg/mL for biochanin A. The overall intra- and interassay precision range from 2.5% to 4.9% and from 0.5% to 4.7%, respectively. The method proved to be sensitive, specific, accurate, and precise for the determination of daidzein, genistein, and biochanin A in *Psoralea corylifolia*.

Introduction

Isoflavone is one of the classes of phytoestrogen with a chemical structure similar to estradiol found predominantly in legumes and has been postulated to exert biologic effects in humans (1–2). Genistein (the major isoflavonoid compound) has been found to have an estrogenic effect preventing bone resorption and promote increasing bone density (3–5). A synthetic isoflavone (ipriflavone) with daidzein as a major metabolite may prevent bone loss in patients with osteoporosis (6–8). Thus, the estrogenic effects of isoflavone may be a factor affecting the rates of osteoporosis and fracture risk. The dried ripe fruit of *Psoralea corylifolia* is an important ingredient in Chinese herbal prescriptions classified as tonics and supporting herbs (9). A rachitic rat fed with psoralea fruit extract revealed significant increases of serum inorganic phosphorus and bone calcification, indicating its efficacy as a remedy for bone fractures, osteomalacia, and osteoporosis (10). Therefore, it is possible that the herb may contain an estrogen-like compound that exerts such a bone-remodeling function. The purpose of our investigation is to identify compounds (possibly with an estrogenic effect) that may play a role in bone remodeling. This study focuses on developing a high-performance liquid chromatographic (HPLC)–UV method to simultaneously identify three isoflavonoid compounds in psoralea fruit. We report in this study that the developed method is superior to a previously reported method in identifying the analytes from complex compositions in the dried fruit of *Psoralea corylifolia* and was successfully applied to the quantitation of genistein, daidzein, and biochanin A in the herb.

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Experimental

Chemicals and materials

Methanol, ethanol, hydrochloric acid, glacial acetic acid, acetonitrile (ACN), trifluoroacetic acid (TFA), and butylated hydroxytoluene (BHT), were of analytical grade or HPLC grade and obtained from Merck KGaA (Darmstadt, Germany). Daidzein and genistein were purchased from ICN Biomedical, Inc. (Costa Mesa, CA), and biochanin A and flavone were obtained from Sigma Chemicals Co. (St. Louis, MO). Dried psoralea fruit (*Psoralea corylifolia* L.) was obtained locally from Sunten Pharmaceutical Co., Ltd. The herb was imported from China, and the identity was reconfirmed by the manufacturer.

Sample preparation

Twenty-five milligrams of the dried and milled psoralea fruit was suspended in 500 µL of 80% methanol (containing 0.1 µg/µL of flavone as an internal standard and 0.05% BHT as an antioxidant) in a seal-tight capped tube. In parallel, the herb sample was spiked with 20 µg of daidzein, genistein, and Biochanin A, respectively, prior to extraction. The samples were incubated in a shaker incubator. After a 1-h extraction at 80°C, the mixture was centrifuged and the supernatant was collected as an aqueous methanol-based extract. The methanol extract was then adjusted

with concentrated hydrochloric acid to obtain a 2M HCl hydrolysis mixture. Acid hydrolysis was carried out at 80°C in a shaker incubator. After 2 h of incubation, the hydrolysate was cooled and centrifuged. The supernatant was filtered through a 0.45-µm syringe filter (Gelman, Ann Arbor, MI), and 20 µL of the sample was subjected to HPLC analysis.

Equipment and chromatographic conditions

HPLC analyses were carried out on a "GOLD system" chromatograph from Beckman (Fullerton, CA). The system consisted of the solvent delivery module Model 126 and the UV detector Model 166. The column configuration consisted of a Nova-Pak C_{18} (4 µm, 3.9- × 150-mm i.d.) reversed-phase column coupled with a Nova-Pak C_{18} (4 µm, 3.9- × 20-mm i.d.) guard column (both from Waters, Milford, MA).

Two types of gradient elution were carried out with the flow rate at 0.8 mL/min and column temperature at 20°C; the systems were (A) ACN and (B) 10% acetic acid in water (v/v). The separation profile for gradient type I was 23%, 70%, 70%, and 23% A in B at 0, 8, 8, and 15 min, as previously described (11). The solvent system for gradient type II was the same as type I, except both A and B were conditioned with 0.1% TFA (v/v) and the gradient profile for separation was optimized as 15%, 15%, 25%, 70%, 70%, and 15% A in B at 0, 2, 2.5, 14.5, 17.5, and 25 min. The substances were detected at 260 nm.

Method validation

Ethanol stock solutions containing daidzein, genistein, biochanin A, and flavone (the internal standard) were prepared and then diluted to appropriate concentration ranges for the construction of calibration curves. Each calibration curve was performed in triplicate with ten different concentrations by plotting the standard concentration of each analyte as a function of the peak area.

The experiment was performed three times (interassay) with five HPLC analyses (intra-assay) in each experiment. The concentration of each analyte was obtained from a correspondent calibration curve. The percent recovery of an individual analyte was determined as the concentration difference of the standard spiked and original samples divided by the known quantity of the spiking analyte and was considered as a measure of accuracy. The coefficient of variation (CV) was taken as a measure of precision. Finally, the content of isoflavone in dried psoralea fruit was calculated as the concentration of each analyte multiplied by the total volume of the hydrolysis extract divided by the known quantity of sample analyzed.

Results and Discussion

Optimization of elution gradient

The major variables affecting the HPLC separation for specific compounds are flow rate, eluent composition, and mode of elution. In this study, the flow rate was constant at 0.8 mL/min and the column temperature maintained at 20°C. The elution behavior was determined using two gradient profiles by measuring the retention times of three isoflavonoid compounds from the authentic and psoralea extract. The chromatogram resulting from the analysis of an authentic standard by a previously described gradient (11) showed a poorly defined peak shape

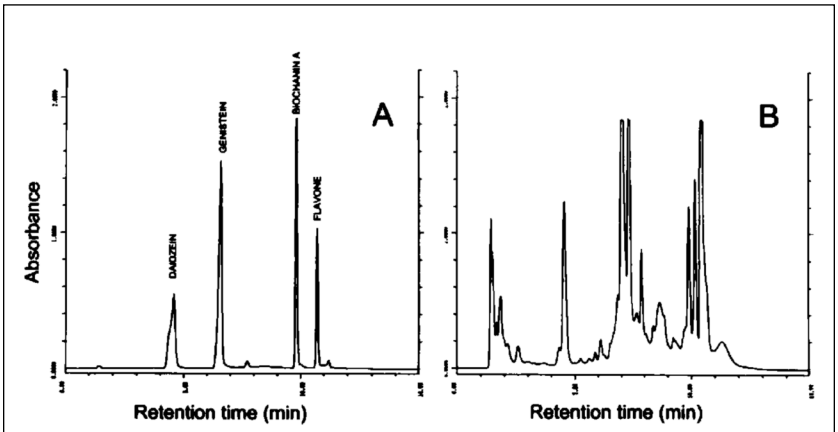

Figure 1. Representative chromatograms of (A) authentic isoflavone standards and (B) the acid-treated psoralea fruit extract separated by gradient type I.

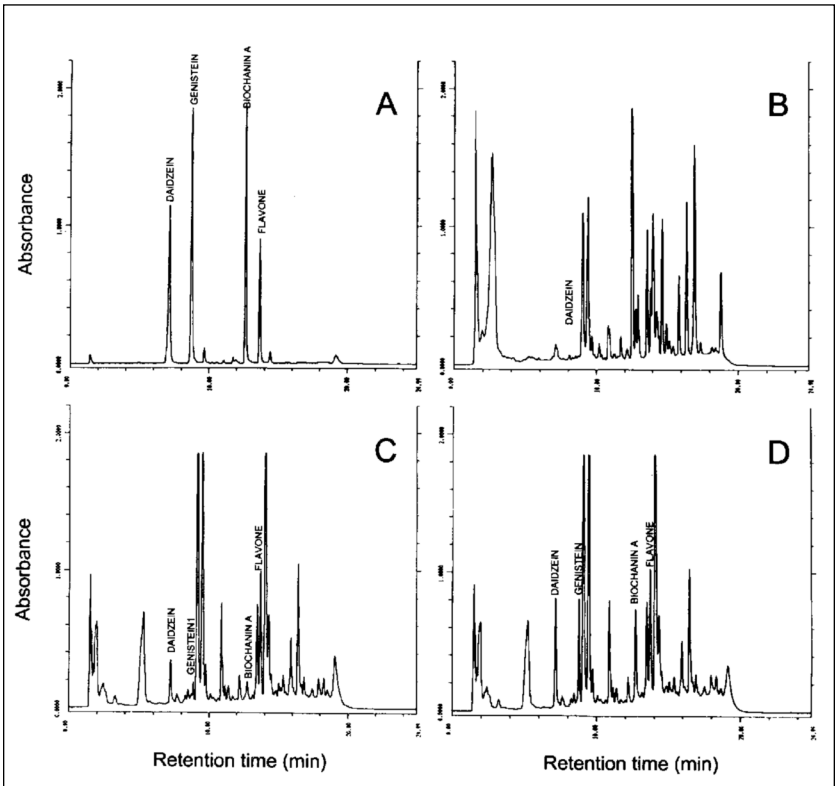

Figure 2. Representative chromatograms of the gradient type II separation of (A) authentic isoflavone standards, (B) the aqueous methanol extract of psoralea fruit, (C) the HCl-treated aqueous methanol extract, and (D) authentic standards spiked and acid-treated extract.

of daidzein (Figure 1A). This may be because of the ionization or deprotonation of the 7-hydroxyl group of the molecules resulting in a differential retention of the isomers on the stationary phase. Moreover, analytes were not resolved adequately from other components (Figure 1B). Thus, it was necessary to optimize the chromatographic conditions. For this purpose, the mobile phase was adjusted with TFA (0.1%) in order to prevent the ionization of daidzein and improve its peak shape (Figure 2A). Additionally, the optimized gradient (type II) resulted in the best resolution for the analytes. The optimized gradient included a gradient formation at 2 min with a sharp gradient (15–25% of A in 0.5 min). Under such conditions, the hydrophilic fraction eluted immediately during the initial phase of analysis, the retention time of the analytes were increased, and especially an improved peak shape of daidzein was seen (Figure 2C). The succeeding lengthened gradient elution (25–70% of A in 12 min) delayed the retention time and separated genistein and biochanin A from other components at a greater distance (Figure 2C). The resulting chromatogram showed that separation by gradient type II dramatically improved peak shape. Also, analytes from the psoralea extract were well-resolved, suggesting this method should be adequate to quantitate the isoflavonoid compound in the sample.

Calibration curves

The calibration curves were obtained with the ranges of 0.01 µg/mL to 1.5 mg/mL for daidzein and genistein and 0.1 to 1.5 mg/mL for biochanin A. The curves obtained were linear and reproducible, as evident by the correlation coefficients of 0.998, 0.996, and 0.995 for daidzein, genistein, and biochanin A, respectively. The lower limit of linear response that represents sensitivity or limit of detection was achieved at 0.01 µg/mL for daidzein and genistein and 0.1 µg/mL for biochanin A. The results demonstrated that the developed HPLC method showed good sensitivities for all analytes.

Quantitation of isoflavone content

The main isoflavones of biological interest generally occur in a complex mixture of glycosides and their malonyl esters in plants (12,13), thus it is appropriate to remove the conjugated glycosides prior to the analysis in order to obtain total isoflavone content in the herb. Because reproducibility and extraction yield are critical for the quantitation of the principal components analyzed in the herb, an internal standard (flavone) was added prior to extraction and authentic standards were spiked in parallel. In order to prevent an undesired hydrolysis of analytes in the herb, we conducted the sample preparation in two steps. First, psoralea fruit powder was extracted with aqueous methanol at 80°C for 1 h in the presence of BHT. Then, the aqueous methanol extract was collected and subjected to acid hydrolysis in 2M HCl at 80°C for 2 h. We found little degradation of the analytes as monitored by the internal standard, and less components were coextracted (data not shown). Most importantly, hydrolysis of the aqueous methanol extract dramatically reduced the number of components and also led to the easily detectable amounts of the aglycones (Figure 2B and 2C). All analytes were well-resolved with baseline separation (Figure 2C). Identification of the analytes in herbal extracts was further confirmed by comparison with the chromatogram of the standards spiked and acid-treated extract (Figure 2D).

The CV for the isoflavonoid compounds in the psoralea fruit extract were below 5% in both the intra- and interassay (Table I), which represents a reproducibility of this HPLC assay. Furthermore, the accuracy represented by the recoveries for daidzein, genistein, and biochanin A were 100.2%, 98.5%, and 99.7%, respectively, (Table I). Finally, the isoflavone contents were calculated as 797.4 ± 29.4 µg/g, 318.4 ± 15.0 µg/g, and 628.2 ± 27.9 µg/g for daidzein, genistein, and biochanin A, respectively, in dried psoralea fruit. Together, the precision and recoveries confirmed the validity of the developed analytical method, in particular because of the fact that excellent values for interassay precision and recovery were obtained, rendering the quantitated isoflavone content trustworthy.

Table I. Precision and Spiking Recovery of Isoflavone Analysis in Psoralea Fruit

	Intra-assay (n = 5)			Interassay (n = 3)		
Isoflavone	Isoflavone content*	%CV†	%Recovery‡	Isoflavone content*	%CV†	%Recovery‡
Daidzein			100.1			100.2
Original	19.2 ± 0.8	4.1		19.9 ± 0.7	3. 7	
Spiking§	39.3 ± 1.1	2.9		40.0 ± 0.7	1.7	
Genistein			99.1			98.5
Original	7.8 ± 0.3	3.4		8.0 ± 0.4	4.7	
Spiking	27.6 ± 1.3	4.9		27.7 ± 0.1	0.5	
Biochanin A			99.1			99.7
Original	15.0 ± 0.6	3.9		15.7 ± 0.7	4.5	
Spiking	34.8 ± 0.9	2.5		35.6 ± 0.9	2.4	

§ Daidzein, genistein, and biochanin A (20 µg each) was added to 0.025 g sample prior to extraction.
* Data represented the amount of isoflavone obtained from 600 µL extract of 0.025 g dried psoralea fruit.
† Standard deviation divided by the mean times 100.
‡ %Recovery = [(Spiking concentration – original concentration) / 20] × 100.

Conclusion

It can be seen from the chromatogram that the established gradient profile for authentic standards can be applied to analyze the natural sample without further adjustment (compare Figures 2A and 2C). The identity of an individual compound analyzed in the sample was confirmed in parallel by spiking the samples with standards as seen in Figure 2D. The results indicate that 2M HCl treatment is enough to break the *O*-glycoside bond with little degradation of the analytes. Acid hydrolysis also removed some interfering components and resulted in a better resolution (compare Figure 2B and 2C). The analytical method and sample preparation procedures developed in this study were successfully applied to quantitate the isoflavonoid compound in psoralea fruit and

may have potential application in the analysis of the isoflavonoid compound of other herbs.

Acknowledgments

We would like to thank Drs. H.H. Chang and T.H. Tsai for their work on the preparation of this manuscript. This research work was supported by a grant (CCMP89-RD105) from the Committee on Chinese Medicine and Pharmacy, Department of Health, Taiwan, and was also sponsored in part by the Chung Cheng Agriculture Science & Social Welfare Foundation, Taiwan.

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Manuscript accepted July 17, 2001.