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A Validated RP-HPLC Method for Simultaneous Determination of Daidzein, Genistein, Formononetin and Biochanin A from *In Vitro* Cultured Cells of *Trifolium pratense* L. (Fabaceae)

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ABSTRACT

The objective of present study is to develop and validate a simple, precise, rapid, specific and accurate reverse phase HPLC method for the simultaneous determination of daidzein, genistein, formononetin and biochanin A obtained from in vitro cultured cells of Trifolium pratense L. Chromatographic separation was achieved on reverse phase octadecylsilane C18 column with isocratic elution. The mobile phase consisted of a mixture of ammonium acetate and methanol (40:60) pumped at a flow rate of 1.0 ml. min^{-1}. The effluents were monitored at 254 nm with a UV detector at ambient temperature. Validation was carried out according to International Conference on Harmonisation (ICH) guidelines with respect to linearity, sensitivity, accuracy, precision, robustness and ruggedness. Good linearity was observed (r^2 > 0.998) over the test concentration range of the four isoflavones. Precision, accuracy, limit of detection and limit of quantification obtained were within acceptable range in each case. The method was robust and specific for the four isoflavones. The results indicate that the developed method is simple, precise, rapid, specific and accurate, and would be suitable for the quantification of daidzein, genistein, formononetin and biochanin A obtained from in vitro cultured cells of Trifolium pratense L.

Keywords: RP-HPLC, Simultaneous determination, Isoflavones, cell culture, *Trifolium pratense* L.

INTRODUCTION

Isoflavones are the heterocyclic phenols which are structurally related to estrogenic steroids of mammals, and are therefore, known as phytoestrogens. In plants, they are mostly in the form of glycosides [1]. They have the ability to activate mammalian estrogenic receptors [2], hence are widely used as an alternative treatment in hormone replacement therapy, as well as for the prevention of several chronic diseases, such as breast and prostate cancers, cardiovascular and osteoporosis incidences, and heart and bone diseases [3-5].

Isoflavones are predominantly found in leguminous plants, including *Trifolium Pratense* L. (red clover) which is indigenous to Europe and North America. Daidzein, genistein, formononetin and biochanin A are the most abundant active isoflavone ingredients found in red clover extracts [6-10]. It is necessary to determine these active compounds in order to ensure their reliability and repeatability in pharmacological and clinical research, understand their bioactivities and possible side effects as well as enhance product quality control [11].

For quality control of plant products, high performance liquid chromatography (HPLC) is a popular analytical method as it is accurate, precise and not limited by the volatility or stability of the sample compounds [12-14]. Although there are several reports on the methods of determination of the content of one or two of the active isoflavones, to the best of our knowledge, simultaneous determination of the four isoflavones has not been

undertaken. Furthermore, it is difficult to separate the four isoflavones due to their similar chemical structures (see Fig 1) [15-19].

The objective of the present study was to develop a new reversed phase HPLC method for the simultaneous quantitative determination of the four major isoflavones obtained from *in vitro* cultured cells of *Trifolium Pratense* L., namely, daidzein, genistein, formononetin and biochanin A.

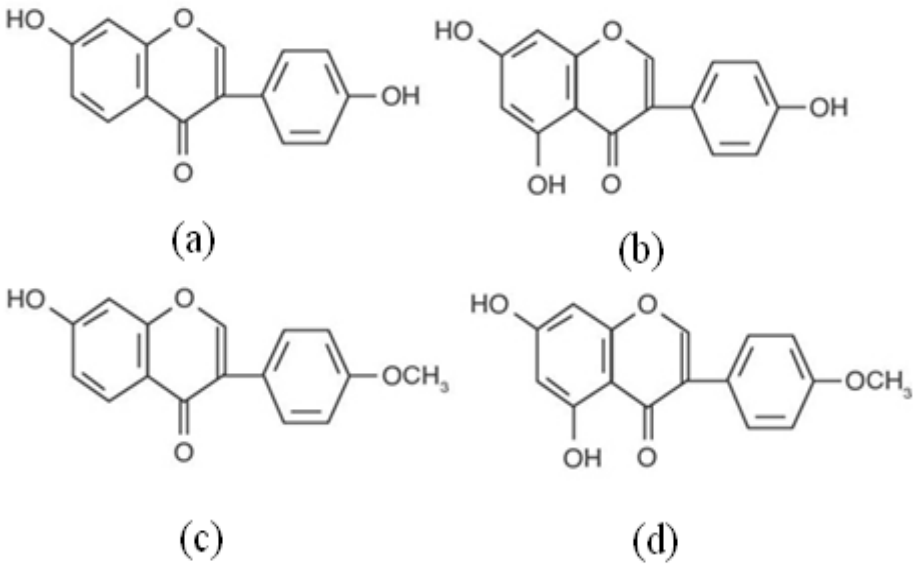

Figure 1: Chemical structures of the four major active isoflavones of *Trifolium Pratense* L. (a) Daidzein (b) Genistein (c) Formononetin and (d) Biochanin A

MATERIALS AND METHODS

Chemicals and reagents

Reference standards of daidzein, genistein, formononetin and biochanin A were purchased from Sigma Aldrich, USA. The purity of all reference standards was over 99%. HPLC grade ammonium acetate and methanol were obtained from S. D. Fine Chemicals Pvt Ltd, Mumbai, India. All the organic solvents and chemicals used for extraction were of analytical grade and obtained from S. D. Fine Chem. Pvt Ltd. Double distilled and deionized water was used throughout the study.

Sample preparation

Isoflavones were extracted from *in vitro* cultured cells of *Trifolium pretense* L. using ethanol as the extraction solvent. About 500 mg of the wet cultured cells (accurately weighed) was dispersed in 2.5 ml of ethanol, sonicated for 10 min and shaken for another 30 min. Thereafter, 0.5 ml of 5M hydrochloric acid was added and shaken for 10 min. To this mixture, 2.0 ml of ethanol was added and shaken for 30min followed by centrifugation at 1000 g. The supernatant was passed through 0.45µ filter, and an aliquot (1 ml) of the supernatant was injected into the HPLC for analysis.

Standard solutions

A stock solution of the reference standards - daidzein, genistein, formononetin and biochanin A - was prepared by dissolving 10 mg of each compound (accurately weighed) in 10 ml of ethanol in a volumetric flask. Various concentrations of the standard solution ranging from 2.5 – 15 µg ml^{-1}, were prepared by diluting serially with ethanol.

Instrumentation and chromatographic conditions

Waters HPLC system equipped with a binary pump, auto-sampler and Waters 2487 dual wavelength absorbance detector was used for analysis. All separations were performed on an octadecylsilane C18 column (15 cm x 4.6 mm, 5 µm, W.R. Grace, USA). The mobile phase used was a isocratic of ammonium acetate and methanol (40:60) at a flow rate of 1 ml. min^{-1}. Quantification was performed with a UV detector (Waters, USA) at 254 nm. Injection volume was 20µL with a 12-min interval between sample injections. The column was kept at ambient temperature. The data were analyzed using Empower 2 softwar (version 2. Massachusetts, USA).

Validation of the method

Validation of the analytical method was carried out according to the International Conference on Harmonization guidelines [20]. The method was validated for linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), robustness and ruggedness as described below.

Linearity, Limit of detection (LOD) and limit of quantification (LOQ)

Linearity was obtained with six levels of concentrations of each compound within the range 2.5 to 15 µg. ml^{-1}. Analysis was performed in triplicate. The calibration curves were plotted according to the linear regression analysis of the integrated peak areas (*y-axis*) versus concentrations (*x-axis*, µg. ml^{-1}) for the four isoflavones.

The limits of detection and quantification were calculated by the method based on the standard deviation (σ) and the slope (S) of the calibration plot, using the formulae, LOD = 3.3σ/S and LOQ = 10σ/S, *respectively*.

Accuracy

Method accuracy was calculated by spiking six samples of red clover with standard stock solutions at the expected concentration of analytes. Two injections of each preparation were made and the theoretical amount of analytes in the sample preparations and the average percentage analytes recovered in the spiked solutions were calculated.

Precision

Precision was evaluated by repeated injection of the sample solution (6 times). Intra- and inter-day variability was determined by analysis of the average amount of standards in quality control samples prepared by standard solutions on three different days. The quality control samples were prepared as a single batch on the same day at each concentration, and then divided into aliquots that were stored at -20 °C until analysis. The quantity of each component was determined by their respective calibration curve. The inter-day reproducibility test was carried out on three different days and the results are expressed as relative standard deviation (RSD).

Ruggedness and robustness

The ruggedness of the system was determined by carrying out the experiment on different instruments - Waters HPLC and Shimadzu HPLC - using different operators and different columns of a similar type, Octadecylsilane C18 and Kromacil C18 column. The average percent recovery for the two systems/columns/analysts was noted.

In order to evaluate the robustness of the proposed method, the influence of small deliberate variations of the method parameters in the determination of the four isoflavones was examined. The factors varied were the wavelength, flow rate, mobile phase (proportion of methanol) and volume of injection. Each factor was changed at three levels (−1, 0 and 1). One factor at a time was changed to estimate the effect. The robustness of the method was evaluated at a concentration level of 20 µg. ml^{-1} for each isoflavone.

Application of the developed HPLC method

The contents of four different isoflavones extracted from the *in vitro* cultured cells of red clover were determined with the test method. The contents of the four isoflavones were calculated with the regression equations obtained from their calibration curves.

RESULTS

Figure 2 illustrates the separation of the mixture of all four phytoconstituents. The mixture of ammonium acetate and methanol (40:60) gave optimum chromatographic separation of the four isoflavones.

The detection wavelength of 254 nm was chosen because daidzein, genistein, formononetin and biochanin A have good absorption and sensitivity at this wavelength. The resolution was good with a resolution value > 1.5. The calibration graphs obtained for the four isoflavones within the concentration range of 2.5 - 15µg. ml^{-1} showed good linear relationship with a correlation coefficient (r^2) of 0.9982 for daidzein, 0.9991 for genistein, 0.9989 for formononetin, and 0.9997 for biochanin A (see Table 1). The LOD and LOQ values for the four isoflavones (see Table 1) indicate high sensitivity of the method.

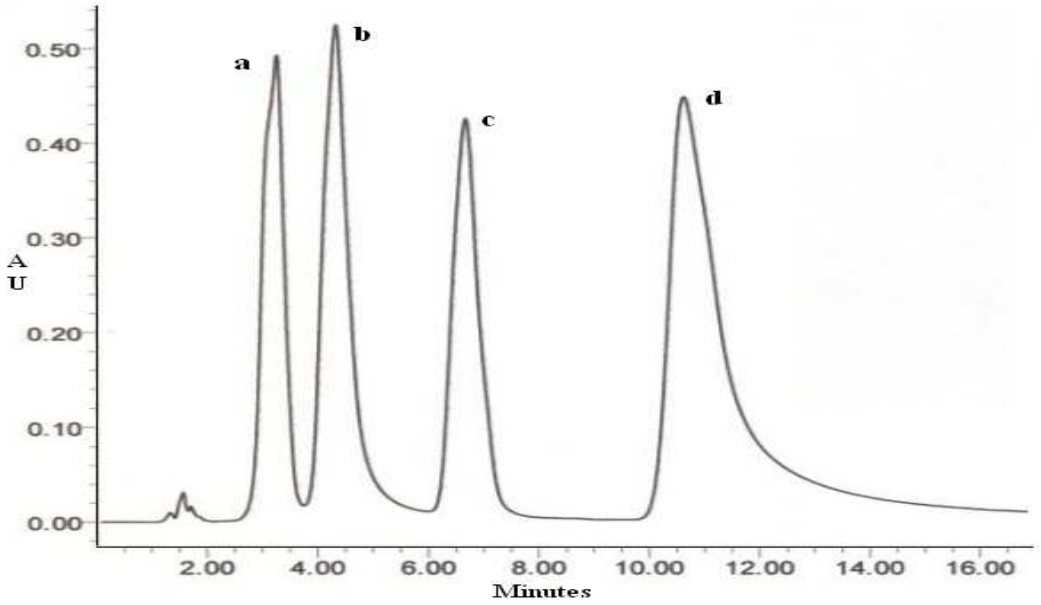

Figure 2: A typical chromatogram of the four isoflavones; (a) daidzein, (b) genistein, (c) formononetin and (d) biochanin A

Table 1: Linearity and sensitivity data for the four isoflavones in red clover

	Daidzein	Genistein	Formononetin	Biochanin A
RT (min)	3.26	4.326	6.492	10.391
Linear regression equation	50160x + 20378	14752x + 3422	40987x + 5085	39627x + 943
r2	0.9982	0.9991	0.9989	0.9987
LOD*	2.11	0.82	0.81	0.95
LOQ*	0.69	1.52	1.28	0.67

Note: *RT – retention time; r^2 = correlation coefficient; * =% of RSD*

The accuracy of the method was determined from recovery data. The recovery data for proposed method was satisfactory with RSD values < 2 %, which indicates excellent accuracy of the method (see Table 2). The inter-day and intra-day precision data for the four isoflavones were good, with RSD values < 2 % (see Table 3).

Table 2: Recovery data for the isoflavones obtained by the proposed HPLC method

Analyte	Amount added (µg. ml^{-1})	Mean amount detected (µg)	Recovery (n=6)	
			Mean	RSD (%)
Daidzein	15	14.97	99.8	1.23
Genistein	15	15.06	100.4	1.38
Formononetin	15	14.98	99.86	1.42
Biochanin A	15	14.80	98.66	1.31

Table 3: Intra- and inter-day precisions of the proposed HPLC method

Analyte	Intra-day variation (n=6) RSD %)	Inter-day variation (n=3*3 days, RSD %)
Daidzein	1.22	1.93
Genistein	1.82	1.56
Formononetin	0.97	0.86
Biochanin A	1.15	1.11

The ruggedness data are shown in Table 4. Ruggedness signifies the reproducibility of the method under different conditions. Robustness indicates the ability of the method to remain unaffected by small changes in parameters such as flow rate, temperature and organic phase ratio (Table 5). The contents of the constituents were not adversely affected by these changes as evident from the RSD values less than 2, indicating that the method is rugged and robust.

Table 4: Ruggedness of the proposed HPLC method

	Recovery (% of RSD)			
Test conditions	***Daidzein***	***Genistein***	***Formononetin***	***Biochanin A***
System 1 (waters)	1.1	1.94	1.86	2.03
System 2 (shimadzu)	1.29	1.68	1.95	1.72
Analyst 1	2.09	1.57	1.29	1.67
Analyst 2	1.89	1.62	1.33	1.46
Column 1 (ODS C18)	1.08	0.76	0.87	1.94
Column 2 (Kromacil C18)	0.98	1.19	1.09	1.47

Table 5: Robustness of the proposed HPLC method

Chromatographic change		**Recovery (% of RSD)**			
Factor	***Level***	***Daidzein***	***Genistein***	***Formononetin***	***Biochanin A***
Wavelength					
250	-1	0.79	0.89	1.59	1.47
254	0	0.6	1.21	1.05	1.81
258	1	1.61	1.5	1.18	1.77
Mobile phase					
45:55	-1	1.26	1.16	1.19	1.39
40:60	0	1.62	1.19	1.37	0.93
35:65	1	1.17	1.24	1.38	1.45
Flow rate					
0.8 $ml.min^{-1}$	-1	1.09	0.79	0.46	0.67
1.0 $ml.min^{-1}$	0	1.01	1.76	0.68	0.89
1.2 $ml.min^{-1}$	1	1.29	0.7	1.17	1.71
Injection volume					
15 µl	-1	1.19	0.45	1.98	1.72
20 µl	0	1.54	1.3	0.87	1.7
25 µl	1	1.72	1.28	1.19	1.51

Analysis of isoflavone extract

Using the proposed method, the four isoflavones extracted from the *in vitro* cultured cells of *Trifolium pratense* L. were identified by comparing the retention times and UV spectra of the samples with those of the authentic standards. The data are shown in Table 6. A satisfactory chromatographic separation of four isoflavones from *invitro* cultured cells of *Trifolium pratense* L. was thus achieved.

Table 6: Assay data (n = 3) for the four isoflavones extracted from the *in vitro* cultured cells of red clover (*Trifolium pratense* L.)

Sample	**Daidzein**	**Genistein**	**Formononetin**	**Biochanin A**
1	6.77	2.64	4.58	1.64
2	6.89	2.22	4.49	1.48
3	6.53	2.84	4.31	1.79

DISCUSSION

The development of HPLC methods for the determination of various chemical compounds has received considerable attention over the years because of their reliability in quality control and pharmaceutical analysis. Simultaneous determination of different analytes with similar chemical structures, in a single run under similar chromatographic conditions, is usually a difficult task. In red clover, the four isoflavones are commonly known to co-exist which makes it difficult to separate them from each other in a single run.

The aim of this study was to develop a reverse phase HPLC method with isocratic elution for the quantification of daidzein, genistein, formononetin and biochanin A in the extract obtained from *in vitro* cultured cells of *Trifolium pratense* L. under similar chromatographic conditions and in the shortest possible time. The method was validated for its performance according to ICH guidelines in terms of linearity, range, accuracy, precision, LOD, LOQ, robustness and ruggedness for the four isoflavones. The developed method was demonstrated to be simple, rapid and statistically validated for its accuracy. The advantages of the method lie in the simplicity of sample preparation and low cost of the reagents used. The proposed HPLC conditions ensured sufficient resolution and precise quantification of the compounds. Statistical analysis data were the indicative of satisfactory precision and reproducibility.

CONCLUSION

This developed RP-HPLC method for simultaneous determination of four isoflavones showed high precision, sensitivity and accuracy. The method has the advantage of short retention times, economical and readily available mobile phase, and good resolution of peaks. The run time is relatively short, which would enable rapid quantification of many samples in routine and quality-control analyses of various extracts containing any combination of the four isoflavones, viz, daidzein, genistein, formononetin and biochanin A. The developed method complies with the stipulated values for ICH guidelines.

REFERENCES

[1] E. Middleton (Jr), C. Kandaswami, In: J.B. Harbourne (Ed.), The impact of plant flavonoids on mammalian biology: Implications for immunity, inflammation and cancer, The Flavonoids: Advances in research (Chapman & Hall, London, **1993**) 619.
[2] D.L. Barton, C.L. Loprinzi, S.K. Quella, J.A. Solan, M.H. Veeder, J.R. Egner, P. Fidler, P.J. Stella, D.K. Swan, N.L. Vaught, P. Novotny, *J. Clin. Oncol.*, **1988**, 16, 495-500.
[3] C.Y. Hsieh, R.C. Santell, S.Z. Haslam, W.G. Helferich, Cancer Res., **1998**, 58, 3833-3838.
[4] S.M. Potter, J.A. Baum, H. Teng, R.J. Stillman, N.F. Shay, J.W. Erdman(Jr), *Am. J. Clin. Nutr*., **1998**, 68, 1375S.
[5] F. Brouns, *Food Res. Inter.*, **2002**, 35, 187.
[6] F. Zonta, P. Bogoni, P. Masotti, G. Micali, *J. Chromatogr. A.*, **1995**, 718, 99.
[7] P. Delmonte, J.I. Rader, *J. AOAC. Int.*, **2006**, 89, 1138.
[8] H. Ha, Y.S. Lee, J.H. Lee, H. Choi, C. Kim, *Arch. Pharm. Res.*, **2006,** 29, 96.
[9] Z. Yu, X. Gao, Y. Zhao, K. Bi, *J. Chromatogr. Sci*,. **2006,** 44, 272.
[10] M. Chen, J. Shi, L. Wang, X. Geng, *Se. Pu.*, **2006**, 24, 489.
[11] Y.Z. Liang, P. Xie, and K. Chan, *J. Chromatogr. B.*, **2004**, 812, 53.
[12] P. Jandera, V. Skeifíkova, L. Rehova, T. Hajek, L. Baldrianova, G. Skopova, V. Kellner, A. Horna. *J. Sep. Sci.*, **2005**, 28, 1005.
[13] M. Holcapek, M. Lisa, P. Jandera, N. Kabatova, *J. Sep. Sci.*, **2005,** 28, 1315.
[14] B.J. Klejdus, L. Vacek , J. Benesova, O. Kopecky, V. Lapcikand and J. Kuban. *Anal. Bioanal. Chem.*, **2007**, 389, 2277.
[15] F. Occhiuto, R.D. Pasquale, G. Guglielmo, D.R. Palumbo, G. Zangla, S. Samperi, A. Renzo, C. Circosta, *Phytother. Res.*, **2007**, 21, 130.
[16] Y.J. Moon, M.E. Morris, *Mol. Pharm.*, **2007**, 4, 865.
[17] S.L. Pumford, M.M. Morton, A. Turkes, K. Griffiths., *Ann. Clin. Biochem.*, **2002**, 39, 281.
[18] K. Mitani, S. Narimatsu, H. Kataoka, *J. Chromatogr. A.*, **2003**, 986, 169.
[19] P. Delmonte, J.L. Rader, *J. AOAC. Int.*, **2006,** 89, 1138.
[20] International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use, "Validation of analytical procedure: Methodology. ICH Q2(R1). Geneva (**1996**).