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Development and validation of a RP-HPLC method for quantification of isoflavone aglycones in hydrolyzed soy dry extracts

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Abstract

Isoflavones are widely used as an alternative treatment to hormone replacement therapy and also for prevention of several chronic diseases, including cancers. Genistein, daidzein and glycitein are the most abundant isoflavone aglycones found in soy extracts, where they also occur as glycosides. This paper describes the development and validation of an isocratic reversed-phase HPLC (RP-HPLC) method for the analysis of isoflavone aglycones, released after acid hydrolysis of soy dry extracts, used as pharmaceutical raw material. The quantification was carried out in a C_{18} endcapped column, using a mobile phase composed of 0.1% acetic acid and methanol (52:48), at a flow rate of 1.0 ml/min and diode array detection (DAD) at 254 nm. The method showed to be linear ($r^2 > 0.99$), precise (R.S.D. < 2%), accurate (recovery of 98.88% for daidzein and 98.12% for genistein), robust and specific.

Keywords: Isoflavones; Aglycones; Acid hydrolysis; RP-HPLC; Genistein; Daidzein; Glycitein

1. Introduction

The use of isoflavones as an alternative to conventional hormone replacement therapy has increased in the last years, due to their estrogenic activity and low adverse effects. Isoflavones present phenolic groups in a position resembling the structure of 17-β-estradiol and other synthetic estrogens. This structural similarity has been postulated to explain the capacity of isoflavones in binding to estrogen receptors, thus acting as weak estrogens [1,2]. Isoflavones are believed to have a potential preventive action against most prevalent chronic diseases in actuality, including hormone-related cancers, osteoporosis and cardiovascular diseases, as well as in the alleviation of menopausal symptoms [3].

Genistein and daidzein are the most abundant isoflavone aglycones found in soy extracts, whereas glycitein is a minor compound. The corresponding glycosides also occur in soy extracts, named genistin, daidzin and glycitin, respectively [4]. Soy dry extracts have widely been used as pharmaceutical raw materials for manufacturing of capsules and tablets of isoflavones, used for treatment of menopausal symptoms. Several methods for the analysis of soy isoflavones have been published, including the quantification in soybeans, foodstuff and biological matrices [5–7]. On the other hand, only a few methods are available for assaying isoflavones in pharmaceutical products [8]. The quantitative determination of the glycosides and aglicones individually requires reference compounds for each isoflavone, what, besides a high cost, also implies in long analysis time and difficulty in optimizing the chromatographic conditions. To overcome these difficulties, some methods are based on the assay of the aglycones released after enzymatic hydrolysis [9,10], acid hydrolysis [11,12] or saponification [13,14].

Hence, the aim of this study was to develop and validate a simple HPLC method to separate and quantify genistein, daidzein and glycitein in soy dry extracts, after acid hydrolysis. The validated method was also applied for the analysis of soy dry extracts samples, employed as pharmaceutical raw material in Brazil.

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2. Experimental

2.1. Reagents and materials

Genistein, daidzein and glycitein reference compounds were purchased from ChromaDex (Santa Ana, CA, USA). Soy dry extracts standardized to contain 40% of total isoflavones, from six different suppliers, were kindly donated by drugstores from Belo Horizonte, MG, Brazil. Distilled water purified in a Millipore (Bedford, MA, USA) system was used in the analysis. Methanol, acetonitrile and acetic acid (HPLC grade) were purchased from Merck (Darmstadt, Germany), whereas ethanol and hydrochloric acid (analytical reagent grade) were obtained from Tedia (Fairfield, OH, USA).

2.2. Instrument and analytical conditions

The HPLC analysis were carried out on a Hewlett Packard HP-1100 system, composed of a quaternary pump, an auto sampler, a photodiode array detector (DAD) and a HP ChemStation software. The column used was a reversed-phase C_{18} endcapped Lichrospher (250 × 4.6 mm I.D.; 5 μm particle size) from Merck (Darmstadt, Germany), at 30 °C. UV-photodiode array detection was performed at 254 nm. UV spectra from 190 to 400 nm were on line recorded for peak identification. The injection volume was 20 μl. The separation of aglycone isoflavones was evaluated in different mobile phase compositions, at a flow rate of 1.0 ml/min. Organic solvents tested were acetonitrile and methanol, and the aqueous solvents were pure water and aqueous solutions of acetic acid (0.1 and 0.2%) and trifluoroacetic acid (0.05%). Different proportions of these solvents were evaluated and, for each condition, pH of the mobile phase, retention factor (k), resolution (R) and tailing factor (T) were determined. In order to determine k, t_0 was obtained using a solution of 0.01% (w/v) $NaNO_3$ diluted in the mobile phase. The optimized mobile phase was composed of 0.1% acetic acid and methanol (52:48).

2.3. Preparation of standard solutions of aglycones

2.3.1. Stock solutions

Approximately 10 mg each of genistein, daidzein and glycitein reference compounds were accurately weighed in volumetric flasks of 10 ml. Methanol (7 ml) was added and the resulting solution was sonicated for 10 min. The volume of the flask was adjusted to 10 ml with methanol. These stock solutions were stored at 4 °C.

2.3.2. Standard solution

One millilitre each from the stock solutions of genistein, daidzein and glycitein were accurately transferred to a volumetric flask (25 ml) and filled up with the mobile phase.

2.4. Hydrolysis conditions

Hydrolysis efficiency was evaluated at five different concentrations of HCl ethanolic solution (1.0, 1.5, 2.0; 2.5 and 3.0 M), during 120 min. Around 50 mg of soy dry extract were accurately weighed in a 100 ml volumetric flask and HCl ethanolic solution (80 ml), at the described concentrations, was added. After sonication for 5 min, the solution was submitted to steam bath during 20, 40, 60, 80, 100 or 120 min. After cooling, the flask was filled up and 5 ml were transferred to a volumetric flask of 25 ml. The volume was adjusted to 25 ml with mobile phase.

2.5. Validation

2.5.1. Linearity

A standard solution containing daidzein (25 μg/ml), genistein (15 μg/ml) and glycitein (2 μg/ml) was prepared. Aliquots of this solution (4, 10, 20, 30 and 40 μl) were injected onto the HPLC equipment, in triplicate, and a calibration curve was obtained in relation to the mass of each aglycone injected, in two consecutive days. The corresponding linear range was of 5–50 μg/ml for daidzein, 3–30 μg/ml for genistein and 0.4–4 μg/ml for glycitein. Calibration curves were also obtained with a standard solution containing 2.5 μg/ml of daidzein and 1.5 μg/ml of genistein, in a similar way, to assay the linearity for aglycone traces. In this case, the linear range was 0.5–5 μg/ml for daidzein and 0.3–3 μg/ml for genistein. The obtained data were submitted to regression analysis and correlation coefficients were calculated for each aglycone (Exel®). Curves obtained in two consecutive days were statically compared.

2.5.2. Precision

The intra-day precision was evaluated by analyzing six samples, at 100% of the test concentration ($n = 6$). Similarly, the inter-day precision was evaluated in two consecutive days ($n = 12$). The concentration of each aglycone in soy dry extract was determined and the relative standard deviation (R.S.D) was calculated.

2.5.3. Accuracy

Stock solutions of daidzein and genistein, at three different concentration levels, were added to soy dry extracts before the hydrolysis. At each level, samples were prepared in triplicate and the recovery percentage was determined.

2.5.4. Specificity

Purity of the chromatographic peaks obtained for daidzein, genistein and glycitein was evaluated using the DAD detector, by recording the corresponding UV spectra at different points in chromatograms obtained for hydrolyzed samples of soy dry extract.

2.5.5. Robustness

Six sample solutions were prepared and analyzed under the established conditions and by variation of the following analytical parameters: chromatographic column batch, pH of the mobile phase and hydrolysis time. The aglycone contents and R.S.D were determined for each condition. The obtained data was submitted to statistical analysis (Student's t-test, applied at 0.05 significance level).

2.5.6. Detection and quantitation limits

Diluted standard solutions were injected onto the HPLC equipment, in quintuplicate, at decreasing concentrations (0.10–0.01 μg/ml). The limit of detection was defined as the concentration for which a signal-to-noise ratio of 3 was obtained. Quantitation limit was determined as the standard solution concentration, which produced peak areas with R.S.D bellow 2.0%.

2.6. Analysis of soy dry extract samples

Seven commercial samples of soy dry extract from six different suppliers were analyzed using the validated method. For the analysis, 50 mg of soy dry extract were accurately weighed in a volumetric flask (100 ml) and 3.0 M HCl ethanolic solution (80 ml) was added. The solution was sonicated for 5 min, then led in a steam bath for 40 min. After cooling, the flask was filled up and 5 ml were transferred to a volumetric flask (25 ml). The volume was adjusted to 25 ml with the mobile phase.

3. Results and discussion

3.1. Chromatographic separation

The chromatographic parameters were initially evaluated for a standard solution of genistein (40 μg/ml). Retention factor, tailing factor and pH of the mobile phase were determined for different proportions of ACN and the aqueous solvents. The pH of the mobile phase clearly affected the symmetry of genistein peak. A decrease in pH value increased the peak symmetry and decreased *k*. The use of water and ACN (67:33) as mobile phase resulted in a tailing factor (2.795) higher than the value acceptable in literature [15], probably due to the ionization of the phenolic hydroxyl groups present in the compound. Acidification of both the aqueous and organic phases with acetic or trifluoroacetic acid promoted ionization suppression and, consequently, improved peak symmetry. The use of trifluoroacetic acid in the aqueous phase was more efficient than acetic acid in reducing peak asymmetry.

The second stage of method development comprised the separation of the three aglycones. A standard solution containing genistein, daidzein and glycitein, at the concentration of 40 μg/ml each, was analyzed in different compositions of mobile phases and the chromatographic parameters were assayed in each condition (Table 1). A mobile phase composed of 0.05% aqueous trifluoroacetic acid and ACN (67:33) did not promote an adequate separation between daidzein and glycitein peaks. In order to improve resolution, *k* was increased by diminishing the proportion of acetonitrile in the mobile phase (28%). This condition showed an adequate resolution ($R = 2.250$), however the run time increased from 14 to 22 min. Hence, selectivity of the mobile phase was altered, by changing acetonitrile to methanol. Better resolution and tailing factor were obtained, but the pH value of the mobile phase was still low (2.4). Substitution of trifluoroacetic acid for acetic acid in methanol provide a similar resolution, with a pH value (3.7) not so aggressive to the column stationary phase. The baseline separation of all aglycones was achieved within 19 min, employing the optimized mobile phase composition of 0.1% aqueous acetic acid solution and methanol (52:48), pH 3.7. In this condition, daidzein eluted at 10.52 min ($k = 6.53$), glycitein at 11.83 min ($k = 7.47$) and genistein at 17.38 min ($k = 11.44$).

Most of the methods described in literature for separation and quantification of isoflavones apply gradient elution, with a long run time, also requiring column equilibrium before subsequent injections [16–18]. In the present work, a method using isocratic conditions was developed, showing a short overall run time (19 min). It is faster than the existing gradient methods, has a low cost for routine analyses and the pH value of the mobile phase is not particularly aggressive to the stationary phase. The use of an endcapped column reduces the influence of the residual silanol groups present in the stationary phase, affording better symmetry and resolution between the peaks.

3.2. Optimization of hydrolysis

Isoflavones are alternatively analyzed as aglycones due to the high cost of the reference compounds or because some of them are not commercially available. In this work, the glycosides were hydrolyzed with hydrochloric acid solutions at different concentrations, in order to release the aglycones. Hydrolysis efficiency was evaluated by the increase in aglycone concentrations in the reactional middle and also by the reduction of the peak areas corresponding to the glycosides. As depicted in Fig. 1, the hydrolysis was complete after 40 min, using a 3.0 M HCl ethanolic solution. At this condition, the aglycones reached the highest content and the peaks corresponding to the glycosides were not detected. In the chromatogram obtained for a sample of soy dry extract (Fig. 2a), peaks of the glycosides can be seen at the beginning of the chromatogram, besides peaks corresponding to daidzein and genistein. After hydrolysis (Fig. 2b), the glycoside peaks completely disappeared and only those corresponding to the aglycones are detected. Glycitein peak could only be seen after hydrolysis, once

Table 1
Chromatographyc parameters for daidzein, glycitein and genistein at different mobile phase composition

Mobile phase composition	Resolution daidzein/glycitein (*R*)	Tailing factor (T) of genistein	Mobile phase pH
0.05% Trifluoroacetic acid:ACN (67:33)	1.014	1.283	2.2
0.05% Trifluoroacetic acid:ACN (72:28)	2.250	1.302	2.2
0.05% Trifluoroacetic acid:methanol (52:48)	2.710	0.984	2.4
0.1% Acetic acid:methanol (52:48)	2.659	1.010	3.7

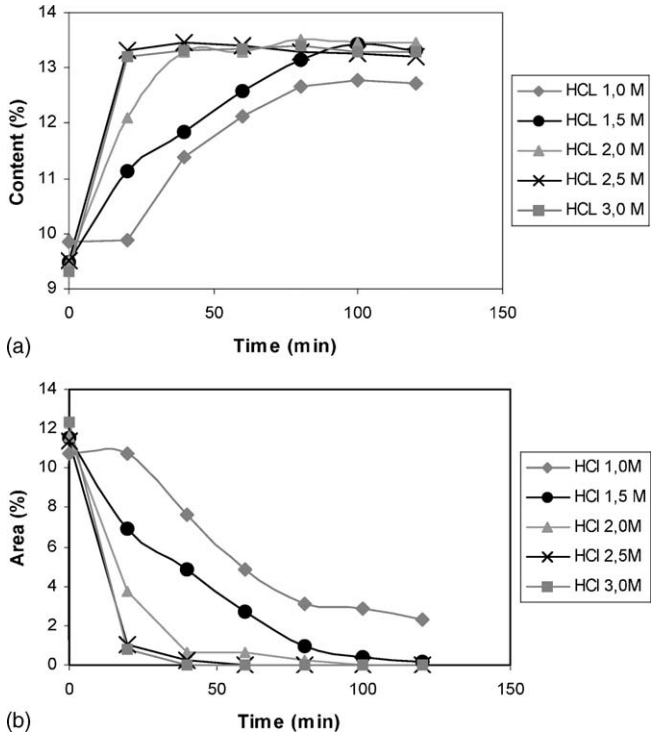

Fig. 1. Hydrolysis profiles of soy dry extract at five different concentrations of hydrochloric acid: (a) Genistein content versus hydrolysis time and (b) genistin area percentage versus hydrolysis time.

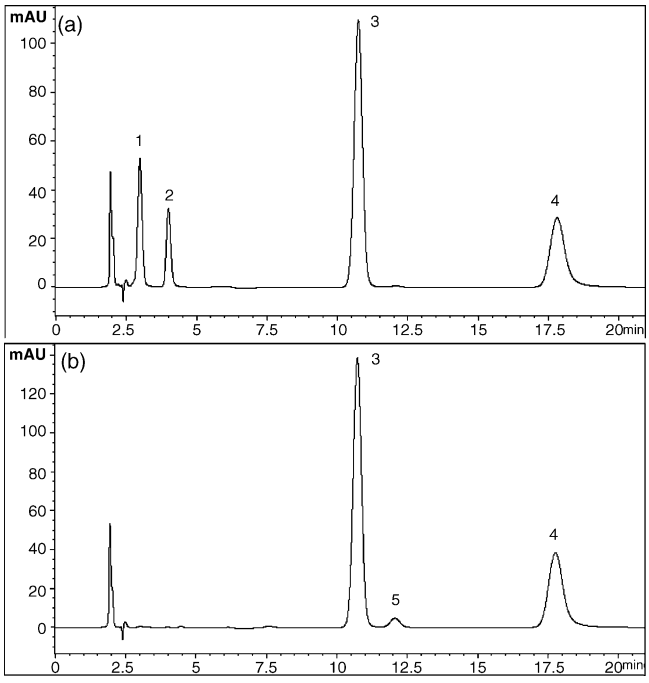

Fig. 2. Chromatogram obtained with 0.1% acetic acid and methanol (52:48) of (a) sample of soy dry extract before hydrolysis; (b) hydrolyzed sample of soy dry extract. Peak assignation: (1 and 2) isoflavones glycosides; (3) daidzein; (4) genistein and (5) glycitein.

glycosides derived from it are the minor compounds in soy dry extracts.

3.3. Validation

3.3.1. Linearity

A linear relationship was found between the areas and the injected mass for all the aglycones, at the assayed range (Table 2). Calibration curves obtained at two consecutive days, for each aglycone, were statically similar ($p < 0.05$).

3.3.2. Precision

In the intra-day precision analyses ($n = 6$), the mean contents of daidzein, genistein and glycitein were 26.00% (R.S.D. = 0.44%); 12.90% (R.S.D. = 0.56%) and 1.03% (R.S.D. = 0.96%), respectively. The values found in the inter-day precision ($n = 12$) were 25.83% (R.S.D. = 0.78%); 12.89% (R.S.D. = 0.52%) and 1.01% (R.S.D. = 1.66%) for daidzein, genistein and glycitein, respectively. The obtained R.S.D. (%) values, lower than 2.0%, attested the precision of the method.

3.3.3. Accuracy

The accuracy of the method was investigated by means of a recovery experiment. A mean recovery ($n = 9$) of 98.88% (R.S.D = 0.90%) for daidzein and of 98.12% (R.S.D. = 0.74%) for genistein proved the accuracy of the method.

3.3.4. Selectivity

Spectral purity of the aglycones peaks was assayed by means of the DAD. Peak purity values of 99.86, 99.89 and 99.99% were obtained for daidzein, glycitein and genistein, respectively, in a hydrolyzed sample of soy dry extract. Purity was above 99.0% to all aglycones, showing that other compounds were not co-eluted in the analyzed peaks for the obtained chromatograms.

3.3.5. Robustness

Statistical analysis showed no significant difference between the analytical conditions established for the method and for

Table 2
Overview of the linearity data of the isoflavones aglycones

	Daidzein	Glycitein	Genistein	Daidzein traces	Genistein traces
Correlation coefficient	1.0000	0.9998	0.9999	1.0000	0.9992
Slope ± standard error	4918.4 ± 4.9	4878.5 ± 40.5	6706.7 ± 33.6	4847.4 ± 18.3	6419.8 ± 101.7
Intercept ± standard error	−11.326 ± 3.031	−6.261 ± 1.996	−59.720 ± 12.433	−0.187 ± 1.122	−8.087 ± 3.764
Standard error	3.60	2.37	14.78	1.33	4.48
Concentration range (μg/ml)	5–50	0.4–4	3–30	0.5–5	0.3–3
Number of points	5	5	5	5	5

Table 3
Content of isoflavone aglycones in soy dry extract samples

Sample	Mean content before hydrolysis (%)		Mean content after hydrolysis (%)			Total isoflavones contents after hydrolysis (%)
	DA	GE	DA	GL	GE	
1	22.70	9.59	26.14	1.10	12.79	40.03
2 a	45.53	ND	49.05	0.75	0.56	50.36
2 b	7.56	ND	25.28	6.44	2.37	34.09
3	22.46	7.84	28.24	1.26	10.36	39.86
4	22.15	7.70	26.21	1.08	11.22	38.51
5	22.25	8.20	28.13	1.29	9.93	39.35
6	43.25	ND	47.61	0.85	0.40	48.86

ND: not detected.

experiments where variations in some parameters were introduced. Hence, the method showed to be robust for different column batches, pH of the mobile phase in the range 3.5–3.9 and hydrolysis time from 35 to 45 min.

3.3.6. Detection and quantitation limits

Detection limits of 0.01, 0.02 and 0.03 μg/ml were obtained for daidzein, glycitein and genistein, respectively. Quantitation limits of 0.04, 0.07 and 0.10 μg/ml were found for daidzein, glycitein and genistein, respectively. The low values of detection and quantitation limits demonstrate the high sensitivity of the method.

3.4. Application of the validated method in the analysis of samples of soy dry extracts

Samples of commercial soy dry extract standardized to contain 40% of total isoflavones, employed as pharmaceutical raw material, were analyzed. They were furnished from six different suppliers, and two batches of the supplier 2 were analyzed. The aglycone contents obtained for each sample, before and after hydrolysis, are presented in Table 3. Before hydrolysis, only daidzein and genistein were detected in the extracts. The concentrations of these isoflavones increased after hydrolysis, due to the conversion of glycosides in the aglycone forms. In all assayed samples, glycitein was detected only after hydrolysis, due to its low content in the soy dry extract, as previously reported [4]. The isoflavones contents varied considerably, even among batches from the same supplier. In most of the assayed samples, the amounts of isoflavones found were not in accord with the labeled values. Those extracts are standardized to contain 40% of total isoflavones; however, the amounts of individual isoflavones are not controlled and might vary due to the influence of several factors, including soy variety, period of the crop and geographical location [4]. Since differences in the biological activity of the individual isoflavones are well reported, and genistein is considered to be the most active of them [19], these variations may have a serious impact on the therapeutic efficacy of soy extracts based products.

4. Conclusions

The developed method showed to be a suitable technique for the quantification of isoflavone aglycones after acid hydrolysis of soy dry extracts. Since it is a simple method, we expect that it might be used in the future for the quality control of soy dry extracts. Significant variations in total and individual isoflavone contents were observed for the assayed samples. These data indicate the necessity of standardization and control of the individual isoflavone contents in soy dry extracts used as pharmaceutical raw material, in order to assure the efficacy of treatments based on such products.

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