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Analysis of soy isoflavone plasma levels using HPLC with coulometric detection in postmenopausal women

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ABSTRACT

A reliable chromatographic method for the determination of soy isoflavones (genistein, daidzein and glycitein) using a coulometric detection has been developed and applied to analyse plasma of postmenopausal women. The chromatographic separation was performed on a C18 reversed phase column with a mobile phase composed of acetonitrile–phosphate buffer mixture. Coulometric detection was carried out at +0.500 V. A careful and rapid solid phase extraction procedure on hydrophilic/lipophilic cartridges was chosen for plasma sample purification with and without hydrolysis obtaining good extraction yield values for all the analytes (>90.0%). The enzymatic hydrolysis step was necessary for the determination of the total amount of soy isoflavones. The limit of quantitation was 0.5 ng mL^{-1} for genistein and 0.25 ng mL^{-1} for daidzein and glycitein. The method was found to be precise and accurate. Thus, the proposed method is suitable for the analysis of soy isoflavones (free and total amounts) in plasma of postmenopausal women under treatment with the SoymenGN® dietary supplement.

1. Introduction

Clinically, menopause represents the physiological condition associated with the permanent stopping of monthly menstruation and the appearance of unpleasant symptoms, such as insomnia, hot flushes, tachycardia and bone loss [1]. For several years, the hormone replacement therapy (HRT) has traditionally been used for treatment of menopausal diseases [2], preventing discomfort caused by diminished circulating estrogen and progesterone hormones. Recently, a large, randomized, controlled trial (the Women's Health Initiative) found that women undergoing HRT with estrogens, whether used or not used in combination with a synthetic progestin, had an increased risk of stroke, endometrial and breast cancer and venous tromboembolism [3], indicating that the health risks of hormone replacement exceeded benefits. Thus, alternative therapies which include natural products offer an attractive choice to provide healthy benefits for postmenopausal women. In the last few years, dietary supplements or formulations containing soy extracts have been used for the treatment of menopausal symptoms [4]. In fact, soy contains isoflavonoid phytoestrogens, which possess a chemical structure resembling that of estradiol-17β (Fig. 1a), a potent mammalian estrogen. The most common isoflavones are: genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, GNS, Fig. 1b), daidzein (7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, DDZ, Fig. 1c) and glycitein (7-hydroxy-3-(4-hydroxyphenyl)-6-methoxy-4H-1-benzopyran-4-one, GLC, Fig. 1d). The isoflavones appear to have both estrogenic and antiestrogenic effects, like selective estrogen receptor modulators (SERMs), depending on the target tissue [5]. Isoflavonoid phytoestrogens generally are present as glycosides in the human diet and are biotransformed to their corresponding aglycones and sugar moieties by enteral microorganisms [6]. Once absorbed, the aglycones are subject to hepatic glucuronidation and are also substrates for sulfotransferases.

A number of epidemiological studies and animal experiments have indicated that phytoestrogens could play a role in the prevention of several hormone-dependent diseases, such as breast and prostate cancer, menopausal diseases and osteoporosis [7,8]. For this reason several analytical techniques are reported in the literature for the measurements of soy isoflavones in biological fluids; recent papers include liquid chromatography (HPLC) with UV or Diode Array (DAD) [9,10], CoulArray [11,12] and amperometric [13,14] detection and with mass spectrometry (MS) [15,16,17]. Sample pretreatment is usually carried out by means of liquid–liquid [11,16,17] and solid phase extraction (SPE) [9,10,14,15] procedures. The coulometric detector, that represents a variant among electrochemical detectors, combines high sensitivity and selectivity if compared with other detectors such as

Abbreviations: GNS, genistein; DDZ, daidzein; GLC, glycitein; IS, internal standard; SPE, solid phase extraction; HLB, hydrophilic/lipophilic balance; HRT, hormone replacement therapy; SERMs, selective estrogen receptor modulators; RSD, relative standard deviation; LOQ, limit of quantitation; LOD, limit of detection.

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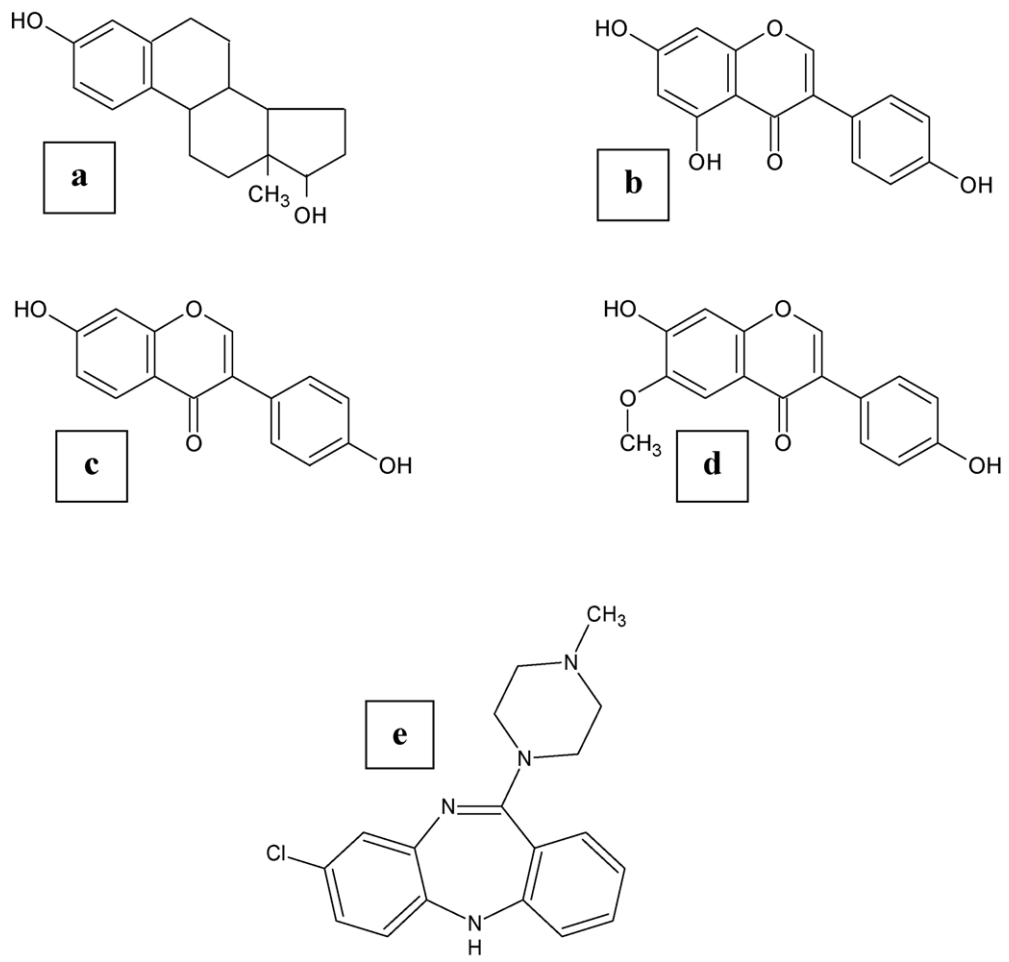

Fig. 1. Chemical structures of (a) estradiol-17β; (b) genistein (GNS); (c) daidzein (DDZ); (d) glycitein (GLC); and (e) clozapine, used as the internal standard (IS).

UV or DAD or amperometric ones; besides it is more advantageous than coularray detector being cheaper and more feasible. Thus, we developed an original HPLC method based on the use of a coulometric detector for the simultaneous determination of the soy isoflavones (free and total amounts). This method has been applied to plasma samples from postmenopausal women treated with the SoymenGN® dietary supplement, obtaining good results in terms of sensitivity and accuracy.

2. Materials and methods

2.1. Chemicals

Genistein, daidzein and glycitein, acetonitrile and methanol for HPLC, 85.0% (w/w) phosphoric acid, potassium dihydrogen phosphate, ascorbic acid, 99.85% (w/w) acetic acid and triethylamine were purchased from Sigma–Aldrich (St. Louis, MO, USA). Clozapine, used as the internal standard (IS, Fig. 1e), was kindly provided by Novartis Pharma (Basel, Switzerland). The type H-2 aqueous solution of β-glucuronidase/sulfatase enzymes from *Helix pomatia* (≥85,000 units mL^{-1}) was obtained from Sigma–Aldrich. Ultrapure water (18.2 MΩ cm), obtained by means of a MilliQ apparatus by Millipore (Milford, MA, USA), was used for the preparation of all the solutions. The *free* fraction of GNS, DDZ and GLC was defined as the free isoflavones obtained after the solid phase extraction procedure (see Section 2.5); the *total* GNS, DDZ and GLC was referred to the amount of each isoflavone after the treatment with both β-glucuronidase/sulfatase enzymes from *Helix pomatia* and SPE procedure (see Section 2.5).

2.2. Preparation of solutions

Primary stock solutions of GNS, DDZ, GLC and IS (1.0 mg mL^{-1}) were prepared by dissolving 2.0 mg of pure substance in 2.0 mL of methanol. Working standard solutions at different concentrations were prepared freshly every day by diluting primary stock solutions with the mobile phase. Stock solutions were stable for at least 1 month when stored at −20 °C. The enzyme mixture was made up freshly and contained 0.15 g of ascorbic acid in 10 mL of acetate buffer (pH 4.0; 0.2 M) and 500 μL of β-glucuronidase/sulfatase solution from *Helix pomatia*.

2.3. Sample collection

Blood samples (3 mL each) were obtained from four postmenopausal women (age range: 51–58 years), subject to the treatment with SoymenGN® (I.R.Med s.r.l., Verona, Italy) capsules, both "light" (40 mg of soy isoflavones; morning dosing) and "dark" (40 mg of soy isoflavones plus 5 mg of melatonin; evening dosing) ones. Four blood samples from each patient were included. Blood samples were drawn 0.5, 1.5, 3.0 and 4.0 h after the evening administration. All blood samples were stored in glass tubes containing ethylenediaminetetraacetic acid as the anticoagulant, then centrifuged (within 2 h from collection) at 4000 rpm for 15 min at 5 °C; the supernatant (plasma) was then transferred to polypropylene tubes and stored at −20 °C until analysis. A Hettich (Tuttlingen, Germany) Universal 32 R centrifuge was used. Blood samples from healthy volunteers (whose plasma was used as blank plasma) were treated in the same way.

2.4. Apparatus and chromatographic conditions

The chromatographic apparatus for the determination of both free and total isoflavones was composed of a Jasco (Tokyo, Japan) PU-1580 chromatographic pump and an ESA (Milford, MA, USA) Coulochem III coulometric detector, equipped with a high sensitivity analytical cell (model 5011 ESA) and a conditioning cell (model 5021 ESA). The high sensitivity analytical cell contained two flow-through working electrodes (porous graphite) positioned serially. The applied voltages for electrodes 1 and 2 were −0.200 V and +0.500 V, respectively. Electrode 1 (E1) was the screening electrode for potential interfering substances; electrode 2 (E2) was the detection electrode. The conditioning cell was set at a potential of +0.100 V and was used as an additional screening electrode for interference. Data handling was carried out using a DataApex (Prague, Czech Republic) Chromatography Station (CSW 32 v. 1.4) software. The analyses were carried out on a Waters (Milford, MA, USA) Atlantis reversed phase C18 column (150 mm × 4.6 mm I.D., 5 μm), kept at room temperature (25 ± 3 °C), using a mobile phase composed of phosphate buffer (pH 3.0; 25 mM)–triethylamine–acetonitrile (74.9:0.1:25.0, v/v/v). The mobile phase was filtered through a Phenomenex (Torrance, CA, USA) membrane filter (47 mm membrane, 0.2 μm, NY) and degassed by an ultrasonic apparatus. The flow rate was 1.4 mL min^{-1}. The samples were injected into the HPLC system by means of a 20 μL loop. A Crison (Barcelona, Spain) MicropH 2000 pHmeter, a Universal 32 R centrifuge from Hettich (Tuttlingen, Germany), a Büchi (Flawill, Switzerland) RE 111 rotary evaporator, an Elma (Berlin, Germany) Transsonic T310 ultrasonic bath and a vortex agitator were also used.

2.5. Solid phase extraction procedure

Solid phase extraction (SPE) for the sample pretreatment was carried out using Oasis Hydrophilic/Lipophilic Balance (HLB, 30 mg, 1 mL) from Waters by means of a Varian (Harbor City, CA, USA) VacElut apparatus.

The cartridges were activated with 2× 1 mL of methanol and conditioned with 2× 1 mL of water. For the analysis of the free fraction of isoflavones, an aliquot of 50 μL of IS standard solution (and analyte standard solution for blank plasma samples) was added to 150 μL of plasma sample. The resulting mixture was diluted with 300 μL of water and loaded onto a previously conditioned Oasis HLB cartridge. For the analysis of the total isoflavones, an aliquot of 150 μL of of plasma was treated with 50 μL of IS standard solution and with 300 μL of the enzyme mixture, prepared as reported in Section 2.2; after an incubation for 18 h at 45 °C the mixture was loaded onto an Oasis cartridge. Then, the cartridges were washed with 1 mL of water twice and then with 1 mL of a mixture of methanol–water (20:80, v/v). Elution was carried out with 1.5 mL of methanol. The eluate was brought to dryness in a rotary evaporator, re-dissolved with 150 μL of mobile phase, and then injected into the HPLC system.

2.6. Method validation

Method validation procedures were carried out according to USP XXXII [18] and Crystal City [19] guidelines.

2.6.1. Extraction yield (absolute recovery) and precision

Aliquots of 50 μL of GNS, DDZ and GLC standard solutions at three different concentrations (in order to obtain analyte plasma concentrations of 0.5, 50 and 100 ng mL^{-1}, respectively), containing the IS at a constant concentration (in order to obtain plasma concentration of 200 ng mL^{-1}), were added to 150 μL of blank plasma. After diluting with 300 μL of water the mixtures were subject to the SPE procedure and injected into the HPLC. The analyte peak area was compared to those obtained injecting standard solutions at the same theoretical concentrations and the absolute recovery was calculated.

The assays described above were repeated six times within the same day to obtain repeatability (*intraday precision*) and six times over six different days to obtain intermediate precision (*interday precision*), both expressed as percentage relative standard deviation values (RSD %).

2.6.2. Calibration curves, limit of quantitation and limit of detection

Aliquots of 50 μL of GNS, DDZ and GLC standard solutions at six different concentrations (in order to obtain plasma concentration of 0.5, 25, 50, 60, 80 and 100 ng mL^{-1}, respectively), containing clozapine as the IS at a constant concentration (in order to obtain plasma concentration of 200 ng mL^{-1}), were added to 150 μL of blank plasma. The resulting mixtures were diluted with 300 μL of water, subjected to the previously described SPE procedure and injected into the HPLC system. This procedure was done in triplicate for each point. The analyte/IS peak area ratios obtained were plotted against the corresponding concentrations of the analytes (expressed as ng mL^{-1}). The calibration curves were constructed by means of the least-square method. One stock solution was used for each replicate; different working solutions were prepared from the stock solutions and added to the blank plasma samples to obtain the different concentrations.

The values of limit of quantitation (LOQ) and limit of detection (LOD) were calculated according to official guidelines as the analyte concentrations which give rise to peaks whose heights are 10 and three times the baseline noise, respectively.

2.6.3. Accuracy

Accuracy was evaluated by means of recovery assays. Aliquots of 50 μL, each one containing analyte standard solutions at three different concentrations (i.e. analyte plasma additions of 2.5, 5.0 and 10.0 ng mL^{-1}; $n = 3$ for each level) and the IS at a constant concentration, were added to 150 μL of plasma from a postmenopausal woman treated with SoymenGN® whose analytes concentrations were previously analysed; then the mixtures were subjected to the SPE procedure described above. Recovery values were calculated according to the following formula: 100 × ([after spiking] − [before spiking])/[added].

3. Results and discussion

3.1. Choice of chromatographic conditions

As isoflavones are lipophilic compounds (log $P = 3.04$ for GNS; 2.51 for DDZ; and 1.97 for GLC), preliminary assays were carried out using a mixture of phosphate buffer (pH 3.0)–acetonitrile (65:35, v/v) as the mobile phase and a pentafluorophenylpropyl (HS F5) reversed phase column. Under these conditions, the chromatographic peaks of DDZ and GLC did not show a good resolution. A concomitant analyte peaks broadening and an increased chromatographic run (>35 min) occurred when the organic phase percentage was decreased from 35 to 25%. Thus, the HS F5 column was substituted with a C18 bonded phase characterized by a lower retention time for analytical compounds than with HS F5. The C18 column provided a good separation of all the analytes and the IS in an acceptable run time (<21.0 min), coupled with a good peak shape. The next step was to find the best electrochemical conditions for improving the sensitivity of the coulometric detector. Some experiments were carried out in a range from +0.100 to +0.650 V for E2, used as the detection electrode; and from −0.300 to +0.050 V for E1, used as the screening electrode. The analytes were monitored

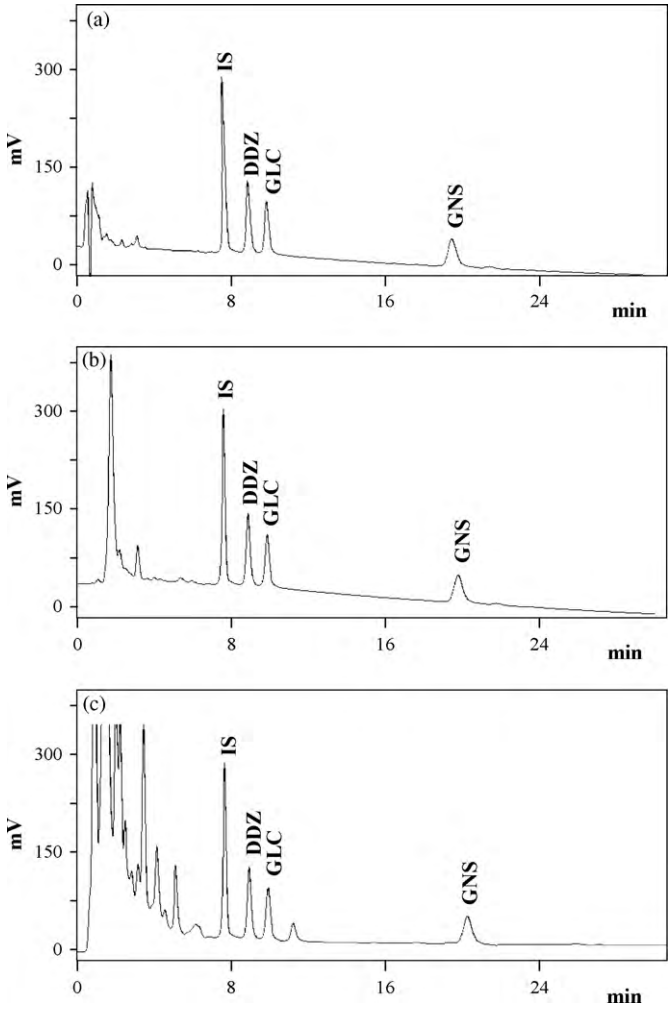

Fig. 2. Chromatograms of (a) a standard solution containing 50 ng mL^{-1} of GNS, DDZ and GLC and 200 ng mL^{-1} of IS; (b) a blank plasma sample spiked with 50 ng mL^{-1} of GNS, DDZ and GLC and 200 ng mL^{-1} of IS, without the hydrolysis step; and (c) the same spiked plasma sample, after the hydrolysis step.

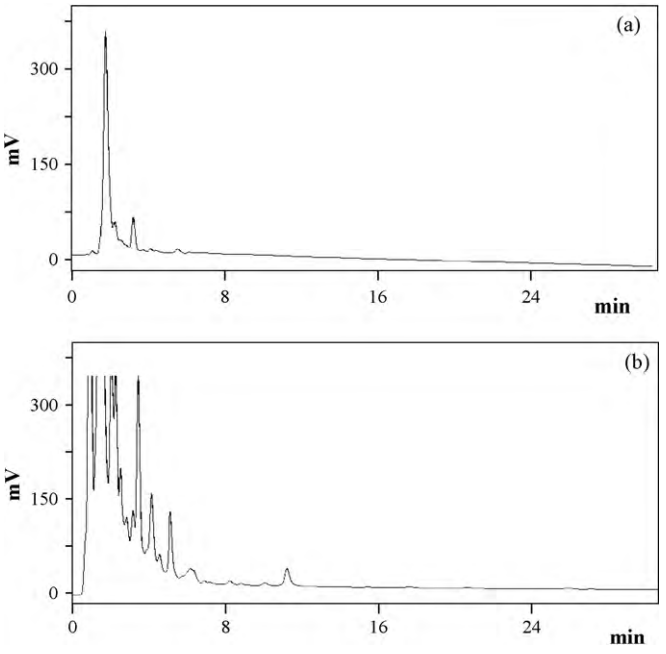

Fig. 3. Chromatograms of (a) a blank plasma sample without the hydrolysis step and (b) the same plasma sample, after the hydrolysis step.

at an oxidation potential of +0.500 V (E2), while a reduction potential of −0.200 V (E1) was chosen obtaining a satisfactory cut-off of biological interference.

Several compounds were tested as possible internal standards; the most suitable was found to be clozapine (log P = 2.24), which showed a relatively short retention time.

The chromatogram of a standard solution containing 50 ng mL^{-1} of GNS, DDZ and GLC and 200 ng mL^{-1} of IS is reported in Fig. 2a. As can be seen, the peaks are neat and well-resolved and the chromatographic separation is within 21.0 min. Retention times (t_R) are: DDZ, t_R = 8.8 min; GLC, t_R = 10.0 min; GNS, t_R = 19.9 min; and IS, t_R = 7.5 min.

3.2. Development of a solid phase extraction procedure (SPE)

The plasma samples needed careful clean-up before proceeding to the step involving the measurement of the soy isoflavones (free and total amounts) levels. In fact, proteins and other biological compounds may result in precocious deterioration of the performance of chromatographic columns and in increased column backpressure. Therefore a SPE procedure was developed using a small amount of plasma sample (150 μL).

Different cartridges that can exhibit polar and weak non-polar interactions were tested: diol, cyanopropyl, C2, C8 and hydrophilic–lipophilic balance sorbents. The lipophilic cartridges (C2 and C8) gave low extraction yields of all the analytes (<60%), while diol and cyanopropyl polar sorbents were inadequate to clean-up the plasma matrix because of interference on DDZ and GLC chromatographic peaks, also giving extraction yields close to 65.0%. Only the HLB cartridges allowed a good purification of the plasma matrix; in particular, the HLB sorbent gave a good clean-up of the plasma samples treated with enzymatic solution for the hydrolysis of isoflavones conjugates (Fig. 3a and b), together with a high extraction yield values for all the analytes (>90.0%). The chromatograms from a blank plasma sample spiked with the analytes and the IS before and after the hydrolysis by means of enzymes from *Helix pomatia* show neat and symmetric peaks and no interference from the matrices (Fig. 2b and c).

3.3. Method validation

Extraction yield and precision assays were carried out at three different concentration levels of GNS, DDZ and GLC, corresponding to the lowest level, highest level and middle point of each calibration curve. The results in terms of extraction yield were satisfactory being always higher than 90.0%. The mean extraction yield of the IS was 92.0%. The precision was also satisfactory with RSD value always lower than 5.5% (Table 1).

Calibration curves were set up for each isoflavone and good linearity (r_c > 0.9990) was found in the 0.5–100 ng mL^{-1} concentration range for all the analytes. The LOQ was 0.5 ng mL^{-1} for GNS and 0.25 ng mL^{-1} for DDZ and GLC, while the LOD was 0.15 ng mL^{-1} for GNS and 0.008 ng mL^{-1} for DDZ and GLC.

3.4. Selectivity

The selectivity of the method into HPLC system was evaluated by injecting standard solutions of some drugs usually co-administered in postmenopausal women, such as antihypertensive, sedative–hypnotic and diuretic agents. The compounds tested for interference are reported in Table 2. As one can see, none of these interfered with the determination of GNS, DDZ and GLC. The analysis of blank plasma samples from six sub-

Table 1
Validation parameters.

Analyte	Amount added (ng mL^{-1})	Extraction yield (%)[a]	Repeatability (RSD %)[a]	Interday precision (RSD %)[a]
GNS	0.5	96.0	5.3	5.4
	50.0	93.4	5.0	5.1
	100.0	92.5	4.7	4.9
DDZ	0.5	97.1	4.8	5.3
	50.0	95.0	4.7	5.0
	100.0	92.9	4.4	4.8
GLC	0.5	96.2	4.9	5.2
	50.0	93.0	4.6	5.1
	100.0	90.1	4.3	4.9
IS	200	92.0	4.2	4.8

[a] Each value is the mean of six independent assays.

jects, who did not receive soy administration, was carried out; no interference at the retention time of the isoflavones and IS was observed.

3.5. Application to postmenopausal women: preliminary pharmacokinetic study

The validated method was applied to the analysis of plasma samples from some postmenopausal women under treatment with SoymenGN® for at least 15 days. The chromatograms of a plasma sample drawn 1.5 h after the last drug administration, before and after the hydrolysis with the enzymes from *Helix pomatia*, are reported in Fig. 4a and b, respectively. As one can see, no interference from the matrix is apparent and analyte peaks are neat and symmetric. The concentrations of the free fractions of soy isoflavones were 7.4 ng mL^{-1} for GNS, 6.2 ng mL^{-1} for DDZ and 4.9 ng mL^{-1} for GLC; while the total amounts of isoflavones were 16.0 ng mL^{-1} for GNS, 12.9 ng mL^{-1} for DDZ and 9.5 ng mL^{-1} for GLC.

Moreover, the plasmatic concentrations of GNS, DDZ and GLC were evaluated in four postmenopausal women treated with SoymenGN®. Blood withdrawals were carried out at 0.5, 1.5, 3.0 and 4.0 h after the evening administration of the dietary supplement. The total plasma concentrations of GNS, DDZ and GLC (mean value of plasma levels found in four women) at different time sampling are reported in Table 3. A large inter-subject variation was observed and this is accounted by several factors like differences in isoflavone metabolizing enzymes, type of diet, intestinal absorption, enterohepatic recirculation and intestinal microflora [20]. A double peak in the plasma concentration–time profiling of GNS, DDZ and GLC is evident; the second one after 4 h resulted

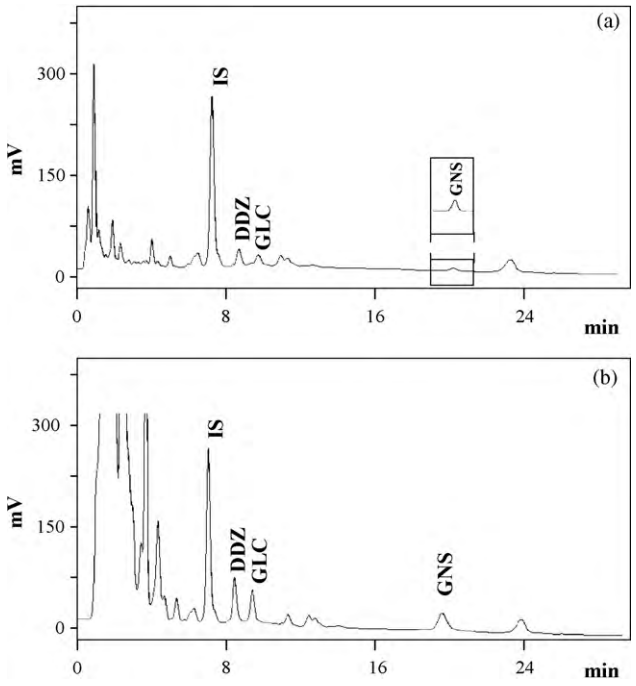

Fig. 4. Chromatograms of (a) a plasma sample from a postmenopausal woman taking SoymenGN® and (b) the same plasma sample, after the hydrolysis step.

Table 2
Compounds tested for interference.

Compound	Retention time (min)
Antihypertensives	
Telmisartan	n.d.
Carvedilol	n.d.
Fosinopril	n.d.
Ramipril	n.d.
Diuretics	
Furosemide	n.d.
Amiloride	n.d.
Torasemide	n.d.
Sedative-hypnotics	
Clonazepam	n.d.
Flurazepam	n.d.
Lorazepam	n.d.
Alprazolam	n.d.
Other drugs	
Simvastatin	n.d.
Melatonin	6.3
Acetylsalicylic acid	n.d.
Analytes	
Genistein	19.9
Daidzein	8.8
Glycitein	10.0

n.d., not detected within 40 min.

Table 3
Mean plasma GNS, DDZ and GLC concentrations found in four postmenopausal women and obtained at different sampling time.

Time sampling (h)		0.5	1.5	3.0	4.0
	Plasma concentrations of total isoflavones (ng mL^{-1})				
GNS	Mean	22.0	17.2	17.0	25.1
	SD	9.0	5.5	1.7	1.0
DDZ	Mean	10.8	8.2	6.3	13.8
	SD	4.7	3.5	4.0	8.0
GLC	Mean	8.0	11.0	7.0	12.8
	SD	3.7	6.5	4.0	6.0

SD, standard deviation of the plasma concentrations found in four women.

from enterohepatic recirculation of the conjugates of isoflavones excreted in bile, as reported in the literature [21]. Moreover, GNS levels were higher than DDZ ones after the consumption of an equal amount of GNS and DDZ contained in SoymenGN®. In accordance with some papers [22], this difference may be partly explained by the higher bioavailability of GNS than DDZ, due to the food matrix in which the isoflavones were incorporated or whether they were given as pure isoflavones, as soy protein isolates or as enriched isoflavone extracts.

3.6. Accuracy

Method accuracy was evaluated at three different concentration levels by adding known amounts of standard solutions of the isoflavones and the IS to real plasma samples taken from postmenopausal woman, whose soy isoflavones content had been already determined. Results were satisfactory: recovery values were always higher than 89.0%.

4. Conclusion

An analytical method based on the use of an HPLC system with coulometric detection and a SPE procedure with Oasis HLB cartridges for the determination of GNS, DDZ and GLC (free fraction and total) in plasma samples from postmenopausal women has been developed.

Good results in terms of extraction yield (>90%), precision (RSD < 5.4%), sensitivity (LOQ = 0.5 ng mL^{-1} for GNS and 0.25 ng mL^{-1} for DDZ and GLC) and accuracy (recoveries always higher than 89.0%) were obtained.

If compared with other analytical methods reported in the literature for the simultaneous analysis of GNS, DDZ and GLC, this method has many advantages. In fact, the coulometric detector is more sensitive and selective than UV or DAD detector [9–12], cheaper and more feasible than a mass spectrometer [15–17]. Moreover, in comparison with the reported chromatographic methods based on the use of electrochemical detectors such as amperometric [11,12] and CoulArray [14] detectors, the proposed method resulted to be more sensitive, rapid and precise. The developed procedure for the solid phase extraction with Oasis HLB cartridges used an aliquot of plasma (150 μL) smaller than the ones reported in the previous methods (0.5–1.0 mL) [11,14]; this is particularly useful for pharmacokinetics studies which require numerous blood withdrawals. Furthermore, the SPE procedure was found to be faster and less polluting than other techniques based on the liquid–liquid extraction [11,16,17].

In conclusion, the proposed method is suitable for the quantitative determination of the free fraction and total amount of soy isoflavones in plasma of postmenopausal women treated with the SoymenGN® capsules and for its application to pharmacokinetics studies. Since the bioavailability of soy isoflavones is variable, measurement of plasma levels could help dosage adjustment, above all in those women who fail to respond.

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