Pharmacokinetic comparison of soy isoflavone extracts in human plasma

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Abstract

The soy isoflavones daidzein and genistein produce several biological activities related to health benefits. A number of isoflavone extracts are commercially available, but there is little information concerning the specific isoflavone content of these products or differences in their bioavailability and pharmacokinetics. This study describes the development and validation of an analytical method to detect and quantify daidzein, genistein, and equol in human plasma using liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS). The method was applied in a crossover, randomized, bioavailability study. Twelve healthy volunteers were administered the same total isoflavones dose from two isoflavone supplement preparations (Super-Absorbable Soy Isoflavones® (Life Extension, US) and Fitoladius® (Merck, Spain)). The pharmacokinetic parameters ($AUC_{0-24}/Dose$ and $C_{max}/Dose$) of the isoflavones from the two preparations differed significantly. Such differences in bioavailability and kinetics may have relevant effects on the health benefits derived from their intake.

Keywords

Soy isoflavone extracts, daidzein, genistein, equol, bioavailability, pharmacokinetics, clinical trial.

INTRODUCTION

Many isoflavones act as phytoestrogens due to the fact that these compounds present similarities in chemical structure and properties to endogenous steroids (e.g. 17- β -estradiol). The most important dietary sources of isoflavones are soy beans and soy foods. The soy isoflavone aglycones daidzein (4',7-dihydroxyisoflavone), equol (4',7-dihydroxy-3,4-dihydroisoflavone), and genistein (4',5,7-trihydroxyisoflavone) (See **Figure 1**) display a number of biological activities which have been related to lower occurrences of cardiovascular diseases, osteoporosis, hormone-dependent cancers, adverse menopausal manifestations, and age-related cognitive decline.^{1, 2}

In order to evaluate the importance of isoflavones on human health, it is necessary to have a better understanding of the bioavailability and pharmacokinetics of these compounds. Isoflavone disposition follows several deconjugation and conjugation steps. Soy and unfermented soy food isoflavones are mainly found as glycosides (e.g. daidzin, genistin, and glycitin), they display high hydrophilicity and molecular weights, and are the object of a number of metabolic biotransformations. Firstly, gut bacterial β -glucosidases³ and the gastric acidic pH carry out the hydrolysis of the sugar moiety. This facilitates the absorption process^{4, 5} that takes place by passive diffusion in the upper small intestine.⁶ Then, the intestinal⁷ and hepatic⁸ phase II enzymes UDP-glucuronyl transferases and PAPS-sulfotransferases carry out the corresponding conjugation with glucuronic acid and sulphate. The excretion of these conjugates can be both renal and biliary, the later undergoing enterohepatic recirculation, which leads to deconjugation, reabsorption, and further metabolism.^{1, 5} Most circulating isoflavones are in the form of glucuronides⁹ and a small fraction is sulphated or left unconjugated.¹ Although it is generally believed that aglycones are the most active biological compounds in humans, conjugates have also been postulated as displaying biological

activities¹⁰ and some of them, such as sulphates, have been reported to have higher activities than the corresponding aglycones.¹¹

Whereas daidzein and genistein are present in soy foods, equol is a metabolite of daidzein whose production depends upon the individual gut microflora¹² and only 30-50% of the adult Caucasian population is able to produce it.¹ Unlike daidzein or genistein, equol presents a stereogenic centre at C-3, and therefore different enantiomeric forms exist. In humans, (*S*)-equol is the only enantiomer which is produced by intestinal bacterial flora.¹³ This fact is of clinical relevance since both enantiomers have different activities; (*S*)-equol, but no (*R*)-equol, displays high affinity for the oestrogen receptor beta (ER β) and its activity is even higher than that of estradiol.¹³

Because of the beneficial health effects that soy isoflavones may provide to humans, several brands of isoflavone extracts have been commercialized over the last decades. However, there is little information concerning these preparations with respect to their specific isoflavone profile and quantity. Differences in bioavailability and pharmacokinetics may have an impact on isoflavone biological and pharmacological activities.

A number of analytical methods have been developed to detect and quantify isoflavone concentrations in biological fluids (plasma, serum, and urine). The most commonly used include gas chromatography coupled to mass spectrometry (GC/MS),¹⁴ high performance liquid chromatography (HPLC) with electrochemical detection,¹⁵ HPLC with UV detection,¹⁶ and liquid chromatography coupled to mass spectrometry (LC/MS).^{4, 17} At present, LC/MS/MS is the most commonly employed analytical technique due to its high sensitivity and selectivity.

The aim of the present study was to develop and validate an LC/MS/MS method for bioavailability studies of commercial isoflavone preparations which would be subsequently used in clinical research. A randomized, crossover, bioavailability study of two different, commercially available soy isoflavone extracts (Soy Isoflavones® (Life Extension, US) and Fitoladius® (Merck, Spain)) was performed.

MATERIALS AND METHODS

Chemicals and reagents

Daidzein, *o*-phosphoric acid (85%), β -Glucuronidase from *Helix pomatia* (H-2 and H-1), β -Glucuronidase from *Escherichia coli*, citric acid monohydrate and sodium citrate dihydrate were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Equol and genistin were purchased from LC Laboratories (Woburn, MA, USA). Genistein was purchased from Extrasynthèse (Lyon, France). Daidzein-4-sulphate disodium salt, daidzein-7- β -D-glucuronide potassium salt, daidzein-7- β -D-glucuronide-4-sulphate disodium salt and equol-4-sulphate were custom synthesized by Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Daidzein-d₆ and equol-d₄ were purchased from Medical Isotopes Inc. (Pelham, NH, USA). Methanol, acetonitrile (HPLC gradient grade), ammonium hydroxide and acetic acid were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain). Drug-free plasma was obtained from the Hospital del Mar blood bank (Barcelona, Spain).

Working standards

Standard stock solutions of all analytes and their corresponding internal standards (IS), with the exception of daidzein-7- β -D-glucuronide and daidzein-7- β -D-glucuronide-4-sulphate where water was used were prepared by dissolving 10 mg of

each substance in 10 mL of methanol. Working solutions were prepared by further diluting the starting solutions and were kept in dark flasks at -20 °C.

Instrumentation: LC/MS/MS conditions

Identification and quantification analysis was performed using an Agilent 1200 series HPLC system (Agilent technologies) coupled to a triple quadrupole (6410 Triple Quad LC/MS; Agilent) mass spectrometer with an electrospray interface. Nitrogen was employed as a drying and nebulizing gas. A capillary voltage of 4 kV in negative ionization mode was used and the source temperature was set to 325 °C.

The liquid chromatographic separation of daidzein, equol and genistein was performed at 40 °C on an Acquity UPLC[®] BEH C₁₈ column (100 mm x 3.0 mm i.d., 1.7 µm particle size) (Waters Corporation). The mobile phase was an isocratic solvent system consisting of 0.01% ammonium acetate at pH 5 and methanol (40:60, v/v) at a flow rate of 0.3 mL/min. Injection volume was 10 µL. The total run time was 5 min per sample. All compounds were monitored in negative ionization using the multiple reaction mode. Daidzein and genistein were quantified by comparing their peak area ratios with daidzein-d₆ (IS1) whereas equol was quantified using equol-d₄ as internal standard (IS2). Due to the chemical structure similarities between daidzein and genistein, daidzein-d₆ was used as the internal standard of genistein.

Sample preparation

Firstly, 1 mL aliquot of plasma sample was transferred into a glass tube and spiked with 30 µL of IS1 solution (containing daidzein-d₆ 10 µg/mL) and 10 µL of IS2 (containing 10 µg/mL of equol-d₄). Approximately 3000 units of β-glucuronidase H1 (2 mg/mL, in 0.1 M sodium citrate buffer, pH = 5) were added and sample was incubated at 37 °C for 16 hours in a shaking water bath. Hydrolysis time could be reduced to 1 hour if the temperature of the water bath was increased to 55 °C.

119 After cooling to room temperature, 1 mL of 4% H₃PO₄ was added and all the
120 tubes were centrifuged at 3500 rpm for 5 minutes. They were then submitted to a solid-
121 phase extraction (SPE) procedure using Oasis[®] MAX cartridges (3cc, 60-mg; Waters
122 Corporation, Dublin, Ireland). Cartridges were conditioned with 2 mL of methanol and
123 equilibrated with 2 mL of water. After sample loading, interferences were washed away
124 with 2 mL of 5% NH₄OH and 2 mL of 2% HCOOH containing 10% methanol. The
125 compounds of interest were then eluted with 2 mL of a solution of methanol containing
126 2% HCOOH. After the evaporation of the solvent (<29 °C, <10 psi), analytes were
127 reconstituted in 100 µL of CH₃COONH₄ 0.01%, pH=5/MeOH (50/50). Finally, samples
128 were centrifuged at 12,000 rpm for 10 min at 4 °C and supernatants analyzed by
129 HPLC/MS/MS.

130 **Method validation**

131 *Calibration curves and linearity*

132 Calibration standards of daidzein, equol, and genistein were prepared each
133 analysis day and consisted of two replicates at 6 different concentrations (5, 50, 200,
134 500, 750, and 1000 ng/mL). Calibration curves were prepared by adding the appropriate
135 volume of working solutions, evaporating the solvent under N₂ (25 °C, 10 psi), and
136 adding 1 mL of isoflavone-free plasma. Calibration curves with their corresponding
137 slope (s), intercept and correlation coefficient (r) were calculated by weighting (1/x)
138 least-squares linear regression of the peak area ratio (analyte/IS) versus the
139 concentration of the standards. The method was considered linear if the coefficient of
140 determination was greater than 0.99.

141 *Intra-assay accuracy and precision*

142 In order to evaluate the precision and accuracy of the method, quality control
143 (QC) samples were prepared by spiking known amounts of the analytes to isoflavone-

144 free human plasma in order to achieve final concentrations of 5 (lower limit of
145 quantification; LLOQ), 10 (low QC), 100 (medium QC), and 800 (high QC) ng/mL of
146 daidzein, equol, and genistein. Five replicates of each QC were analysed against the
147 calibration curve, and the obtained concentrations were compared to their nominal
148 value.

149 Intra-assay accuracy was expressed as the % relative error of the estimated
150 concentrations. It was evaluated by five replicates of the QC samples that were analysed
151 in one day, at each concentration level. Accuracy was acceptable if the mean
152 concentration was within 15% of the nominal values with the exception of the LLOQ
153 which was acceptable if it was within 20% of the nominal value.

154 Intra-assay precision of the method was calculated as the relative standard
155 deviation (RSD, %) of the estimated concentrations obtained for five replicates of the
156 QC samples that were analysed in one day, at each concentration level. Precision was
157 acceptable if the RSD was within 15% of the nominal values with the exception of the
158 LLOQ which was acceptable if it was within 20% of the nominal value.

159 ***Inter-assay accuracy and precision***

160 Inter-assay accuracy and inter-assay precision were calculated as described for
161 intra-assay accuracy and precision with data obtained during three validation assays that
162 took place during three different days.

163 ***Limit of detection (LOD)***

164 The LOD was calculated using the standard deviations (SD) of the obtained
165 concentrations from five replicates of the LLOQ (5 ng/mL) and the slope (s) of the
166 calibration curve, according to the following formula: $LOD = 3.3 * (SD/s)$.
167 Additionally, at the estimated LOD of the surrogated analytes, it was confirmed that the
168 LOD was at least 3 times the signal to noise ratio of a blank plasma sample.

Lower limit of quantification (LLOQ)

The LLOQ was the lowest concentration calibrator experimentally tested that presented acceptable accuracy (80-120%) and precision ($\pm 20\%$).

Extraction recovery

The extraction process recovery was evaluated by comparing the responses of a mixture of standards with and without extraction. For this purpose, six different sources of isoflavone-free plasma were spiked at a low (5 ng/mL) and high (800 ng/mL) level of concentration. The extraction recovery was calculated as the quotient of response of standard solutions spiked into plasma before extraction (with standard) and the response of standard solutions spiked into plasma after extraction. Results are expressed as a percentage.

Matrix effect

In a similar way, the matrix effect was analysed using six different batches of isoflavone-free plasma at two concentrations (5 and 800 ng/mL). It was evaluated according to the following formula: % Matrix effect = $100 * [(Response\ of\ the\ post\ extracted\ spiked\ sample / Response\ of\ the\ non-extracted\ neat\ solvent\ with\ analytes) - 1]$. A negative % value indicated ion suppression whereas a positive % indicated ion enhancement.

Stability

Stability of working solutions of daidzein, daidzein-d₆, equol, equol-d₄, and genistein in methanol was evaluated to ensure that neither sample preparation nor stored conditions could affect the concentration of the analytes. For this purpose, freeze and thaw stability, short-term stability at room temperature, and long-term stability in the freezer were evaluated.

**Application to real samples: quantification of daidzein, equol and genistein
in human blood samples**

Study design

A randomized, crossover, bioavailability study with a washout period of 3 days was performed. The study was conducted in accordance with the Helsinki Declaration, approved by the local Ethical Committee (CEIC-Parc de Salut Mar), and took place in the Clinical Research Unit of Hospital del Mar Medical Research Institute (IMIM, Barcelona, Spain). Written informed consent was obtained from all the participants prior to any study-related procedure. Participants were financially compensated for any inconvenience caused.

Subjects

A total of 12 healthy, non-smoker volunteers (4 female and 8 male), aged between 20 and 37 years (average 26.1 ± 5.5 years), were selected through a volunteer database. Their mean weight and height were 67.8 ± 10.3 kg and 172.5 ± 12.4 cm, respectively. The body mass index of each subject was within 19.6 and 25.4 kg/m^2 ($22.7 \pm 1.9 \text{ kg/m}^2$). The women had regular menstrual cycles (26-32 days). Subjects underwent a general physical examination, complete chemistry and haematology blood test, urinalysis, and 12-lead ECG. Moreover, during the three days prior to the intervention, they were asked to follow a diet free from daidzin, genistin, daidzein and genistein (including soybeans and soy foods). A list of isoflavone rich food was provided. Alcoholic beverages were not permitted 72 hours prior to each intervention.

Commercial isoflavone preparations

The soy isoflavone preparations used in this study were two commercially available brands: Super-Absorbable Soy Isoflavones® (Life Extension, US, hard gelatine capsules) and Fitoladius® (Merck, Spain, soft gelatine capsules), As provided

218 by the manufacturer, each capsule of Super-Absorbable Soy Isoflavones® contains 123
219 mg of soy extract, with 54 mg of isoflavones (44%), 22 mg of which are daidzin and
220 daidzein, 28 mg genistin and genistein, and 6 mg glycitin and glycitein. Each capsule of
221 Fitoladius® contains 60 mg of soybean seed extract (*Glycine max*), with 24 mg (40%)
222 of total isoflavone content, the specific amount of each isoflavone is not, however,
223 described in the information leaflet.

224 ***Drug administration and sample collection***

225 The treatment with Super Absorbable Soy Isoflavones® consisted of a single
226 oral intake of 2 capsules (total dose: 108 mg isoflavones), whereas that of Fitoladius®
227 was 4 capsules (total dose: 96 mg isoflavones). The objective of this approach was to
228 compensate the previously mentioned differences in isoflavone content of the extracts,
229 as provided by the manufacturers (54 mg vs 24 mg in each capsule). On the intervention
230 days, each volunteer received an oral dose of a soy isoflavone extract. In order to avoid
231 batch-to-batch differences in isoflavone composition, all the capsules of each treatment
232 were from the same batch number. Moreover, as explained bellow, the composition of
233 five different capsules corresponding to that batch was analysed.

234 Subjects arrived at the clinical trial unit on the intervention day at 07:45 a.m.
235 after an overnight fast. Spot urine samples were collected for drug testing (opiates,
236 cocaine, amphetamines, and cannabis) by a rapid test device (Instant-View®, Alpha
237 Scientific Designs, Inc, Poway, CA, USA). In addition, a breath ethanol test was also
238 conducted. A positive screen test was considered an exclusion criterion. Intervention
239 days were separated by at least a 3-day washout period. An indwelling intravenous
240 catheter inserted into a subcutaneous vein in the forearm of the non-dominant arm and
241 blood samples were collected at baseline and 1, 3, 5, 6, 7, 8, 9, 12, and 24h after
242 treatment administration. Blood was collected into 10 mL tubes containing EDTA and

centrifuged (3000 rpm, 10 min, 4 °C). The plasma samples were then separated and frozen at -20 °C until analysis.

Quantification of isoflavones in soy extract capsules

Although the treatment dosages (based on the information provided by the manufacturers) were designed to contain similar doses of isoflavones, it was expected that there would be differences in the plasmatic concentrations of specific isoflavones (e.g. genistein and daidzein) as in the Fitoladius preparation their composition was not declared. Consequently, it was necessary to measure the exact content of isoflavones of the capsules. The isoflavone content of the preparations [aglycones (daidzein and genistein) and β -glycoside conjugates (daidzin and genistin)] was determined by LC/MS/MS as follows: a sample stock solution (1 mg/mL) from each was prepared by dissolving 10.0 mg of capsule content in 10 mL of methanol and sonicating it for 5 minutes to achieve complete dissolution. Analysis solutions of 100 μ g/mL and 10 μ g/mL were prepared by further diluting the stock solution. One milliliter of the analysis solution, spiked with 30 μ L of a solution containing daidzein- d_6 10 μ g/mL, was subjected to the previously described solid phase extraction procedure. Ten μ L of the solution were injected in the LC/MS/MS system. Each determination was performed by analysing five different capsules corresponding to the same batch used for the study. The separation of the compounds was carried out using the same conditions described earlier. The aglycones were quantified directly whereas the glycosides were quantified indirectly after hydrolysis. Calculations were based on the response factor of the ratio of the analyte versus internal standard.

Pharmacokinetic analysis

The maximum plasma concentration ($C_{\max}$) and the time to reach $C_{\max}$ ($t_{\max}$) were determined from the curves constructed by plotting the plasma concentrations over

time. The terminal slope (k_e) of the concentration-time curve was determined by log-linear regression using at least the last three points. Elimination half-life ($t_{1/2}$) of the terminal log-linear phase was calculated following the equation $\ln 2/k_e$. The area under the curve (AUC_{0-24h}) was calculated by the trapezoidal method during the 24 hours following the ingestion of the corresponding soy isoflavone extract. All the plasma samples collected from each subject were analyzed in the same batch of analysis. In order to compare the pharmacokinetics of genistein and daidzein in both preparations, the AUC_{0-24h} and C_{max} were adjusted for the dose administered ($AUC_{0-24h}/Dose$ and $C_{max}/Dose$). Taking into account that the isoflavone extracts contained a mixture of glycosides and aglycones, the dose administered was calculated as the sum of the amount of glycoside and aglycone, expressed in mg of aglycone.

Statistical analysis

A Student's t -test for paired samples was used for the statistical comparison of AUC_{0-24h} , C_{max} , $t_{1/2}$, k_e , $AUC_{0-24h}/Dose$ and $C_{max}/Dose$. A non-parametric method was used (Wilcoxon signed-rank test) for t_{max} . Analyses were performed using SPSS Statistics for Windows (version 18.0; SPSS Inc., Chicago, IL, USA). Significance was defined as $p < 0.05$.

RESULTS AND DISCUSSION

Method development and optimization

Optimization of MS/MS conditions

In order to evaluate ionization sources and multiple reaction monitoring (MRM) transitions, the three most intense fragments of each compound (daidzein, daidzein- d_6 , equol, equol- d_4 and genistein) were selected using pure standards at 10 $\mu\text{g/mL}$ in negative ion mode. To further improve MRM conditions, the effect of gas temperature, and collision and fragmentor energies were investigated over the range of 300-350 $^{\circ}\text{C}$,

293 0-45 eV, and 60-140 V, respectively. The optimal gas temperature was determined at
294 325 °C, and the optimized values for fragmentor and collision energy of each
295 metabolite, as well as the selected MRM transitions, are detailed in **Table 1**.

296 *Optimization of LC conditions*

297 Different chromatographic columns were tested to study their effect on peak
298 shapes and separation efficiency. Depending on the polarity of the stationary phase
299 these included: cyanopropylsilyl- (CN; Acquity UPLC[®] HSS cyano 1.8 µm, 2.1x50
300 mm), *n*-octylsilyl- (C₈; Zorbax SB-C₈ RRHT 1.8 µm, 2.1x100 mm), phenylhexylsilyl-
301 (Acquity UPLC[®] BEH Phenyl 1.7 µm 2.1x100 mm), and *n*-octadecylsilyl- (C₁₈;
302 Acquity UPLC[®] BEH C₁₈ 1.7 µm, 3.0x100 mm) moieties on silica. After having
303 compared the four chromatographic columns, the latter was chosen to continue with the
304 optimization of the methodology because it provided the best separation with good
305 resolution for equol, daidzein, and genistein.

306 Chromatographic separation of the analytes was optimized by evaluating four
307 mobile phases (0.1% HCOOH, 0.01% NH₄CH₃COO, acetonitrile, and methanol). A
308 rapid separation (<5 min) of the three compounds of interest was achieved using an
309 isocratic solvent system consisting of 0.01% ammonium acetate at pH 5 and methanol
310 (40:60, v/v) (**Figure 2**).

311 *Optimization of the solid phase extraction procedure*

312 To optimize the extraction of the analytes of interest from plasma, several solid-
313 phase extraction procedures were evaluated by spiking known amounts of the analytes,
314 and calculating the corresponding recoveries and matrix effect. Five different cartridges
315 were evaluated. These included Oasis[®] MCX, WAX, MAX, WCX, and HLB (Waters
316 corporation). The highest recovery of the analytes was achieved when MAX cartridges
317 were used, although a marked matrix effect was present. In order to avoid the co-eluting

matrix components affecting the ionization efficiency of the analytes, different washes were evaluated. Neither basic washes with 5% NH_4OH , nor additional washing steps using methanol, improved the matrix effect. However, when acidic washes were studied using mixtures of methanol and 2% formic acid, the matrix effect was enhanced. The best results were obtained when 2% HCOOH and methanol (90:10; v/v) were employed.

Optimization of the hydrolysis

The efficacy of different enzymes to hydrolyze the sulphate and glucuronic acid moieties present in the phase II conjugates of equol, genistein, and daidzein was evaluated. Different enzyme preparations (β -Glucuronidases from *Helix pomatia* H-2, purified H-2, H-1, and β -Glucuronidase from *Escherichia coli*), glucuronidase and sulphatase units, incubation times, and temperatures were studied. Several custom synthesized products containing sulphate and/or glucuronide moieties (daidzein-4-sulphate, equol-4-sulphate, daidzein-7- β -D-glucuronide, daidzein-7- β -D-glucuronide-4-sulphate) were used as substrates and the percentage of hydrolysis achieved in each case was evaluated.

The best results were obtained when the samples were treated with 3,000 units of β -Glucuronidase H-1 from *Helix pomatia* at $\text{pH} = 5$ after an incubation at 37 °C for 16 hours. Interestingly, similar results were achieved when hydrolysis time was reduced to 1 hour and the temperature of the water was increased to 55 °C. This glucuronidase has been previously used in the analysis of soy isoflavones¹⁸⁻²¹.

Under the conditions described above, the percentage of hydrolysis of daidzein-4-sulphate, daidzein-7- β -D-glucuronide, and daidzein-7- β -D-glucuronide-4-sulphate was higher than 95%. With greater amounts of β -glucuronidase (5,000 units), the percentage of hydrolysis was constant although the matrix effect increased. Whilst the

343 hydrolysis of most of the metabolites was successful, none of the studied conditions was
344 able to hydrolyze equol-4-sulphate, the yield being below 5%. Studies regarding equol
345 metabolism in humans, monkeys, and rats have reported that sulphation is a minor
346 metabolic pathway of this compound.²² As described below, despite having detected
347 equol in plasma samples from three volunteers, the pharmacokinetics of this metabolite
348 has not been evaluated in this report.

349 It is worth noting that a total quantification of isoflavones was chosen due to the
350 large number of isoflavone glucuronide and sulphate metabolites conjugates that have
351 been described to date in humans¹⁶ and animals.⁹ Although some of these conjugates are
352 commercially available, the lack of an appropriate commercially available IS of all of
353 them for their proper quantification was a major limitation for their inclusion in the
354 bioavailability study. For this reason, a total quantification of the isoflavones was
355 performed following sample hydrolysis. To do so, four different representative
356 metabolites presenting glucuronides and/or sulphate moieties were custom synthesized.
357 The hydrolysis procedure was optimized in order to assure that the conditions used were
358 able to release the aglycones. Finally, the quantification of total isoflavones was
359 performed in real plasma samples using the optimized hydrolysis procedure

360 **Method validation**

361 Validation of the analytical method was performed following the European
362 Medicines Agency guidelines on bioanalytical method validation (URL
363 [http://www.ema.europa.eu/docs/en_GB/document_library/](http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf)
364 [Scientific_guideline/2011/08/WC500109686.pdf](http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf) . Accessed 30.04.2014).

365 The linearity of the calibration curve was evaluated from three different,
366 prepared batches as described above. Satisfactory determination coefficients ($r^2 > 0.99$)
367 were obtained for all the analytes in the selected range of 5-1000 ng/mL.

Intra- and inter-day accuracies presented acceptable % relative error for the three compounds, and the intra- and inter-day precision were constantly below 15% RSD (Supplementary Table 1).

The LOD for daidzein, equol, and genistein were 0.6, 0.9, and 1.0 ng/mL, respectively. The LLOQ for these three isoflavones was 5 ng/mL.

The % matrix effect for daidzein, equol, genistein, and their corresponding IS assessed at 5 and 800 ng/mL, indicate that ion suppression was lower than 17.2% and ion enhancement was not higher than 17.8%. The extraction recoveries were between 77.5 and 86% (Table 2).

No significant degradation of daidzein, equol, and genistein in human plasma took place after long-term storage at -20 °C during the analysis period. The low and high QC samples presented acceptable values ($\pm$ 15% the nominal values) during at least 8 months after their preparation.

Quantification of isoflavones in soy preparations

Supplementary Table 2 shows the quantification of daidzein, genistein, daidzin, and genistin present in one capsule of the commercially available soy preparations studied. Both commercial sources presented predominantly β -glycoside conjugates (98%), the free forms representing only a small percentage of the isoflavone content (approximately 2%). In Fitoladius®, daidzin was found to account for 37.4% of the total isoflavone weight, whereas genistin was the most abundant isoflavone, representing 60.3%. On the other hand, Super Absorbable Soy Isoflavones® had almost the same amount of daidzin as genistin (50.0% and 48.1%, respectively). The aglycones were only present in very low amounts, being daidzein at a higher proportion (1.1-1.7%) than genistein (0.6-0.7%). The total isoflavone content of Fitoladius® was found to exactly match the content provided in the information pamphlet (23.8 mg vs 24 mg). In the case

of Soy Isoflavones®, the content of genistin and genistein was 1.2-fold that described by the manufacturer (35.5 mg vs 28 mg). As for daidzein and daidzin concerns, the amount quantified (34.5 mg) was 1.6-fold that given in the specifications (22 mg). This fact is of relevance, since the dosages administered to the volunteers were based on the information provided by the manufacturer. In order to compensate for these differences, the pharmacokinetic parameters were normalised by the dose administered. To do so, each plasmatic concentration value (derived from the calibration curve) was divided by the amount administered of the corresponding isoflavone.

The other constituents present in the soy preparations were excipients. In the case of Fitoladius®, and according to the manufacturer, these are primrose oil, glyceryl monostearate, glycerol, cuprosodic chlorophyll, gelatine, titanium dioxide and purified water. In the case of Super-Absorbable Soy Isoflavones®, these are ascorbyl palmitate and vegetable cellulose. The analysis of these excipients is beyond the scope of this paper.

Quantification of isoflavones in human plasma samples

Equol

Only three of the twelve volunteers were found to be equol producers. In these volunteers, equol concentrations started to increase between 5 and 6 hours after the treatment administration and could still be detected at 24 h. Due to this fact, the pharmacokinetics of this metabolite is not evaluated in this report.

Daidzein and genistein pharmacokinetics

Time-course plasma concentrations of total genistein and daidzein are shown in **Figure 3**. Some studies have described that daidzein presents higher bioavailability than genistein²³ whereas others have reported the contrary.²⁴ In our study, no differences in bioavailability were observed between daidzein and genistein after the treatment with

418 Super Absorbable Soy Isoflavones® or Fitoladius®. The double peak found in the time-
419 course plasma concentrations suggests an enterohepatic circulation, which is
420 characteristic of these compounds (**Figure 3**).^{6, 25} To a lesser extent, the small
421 percentages of aglycones present in the soy extracts could also contribute to this
422 pharmacokinetic profile, as the free forms are absorbed faster than the glycosides.²⁶

423 The pharmacokinetic parameters studied (AUC_{0-24h} , C_{max} , $AUC_{0-24h}/Dose$,
424 $C_{max}/Dose$, t_{max} , $t_{1/2}$, k_e) are reported in **Table 3**. As shown using the $AUC_{0-24h}/Dose$
425 ratio, the bioavailability of daidzein and genistein was higher after the intake of
426 Fitoladius® ($p<0.05$ and $p<0.001$, respectively), compared to Super Absorbable Soy
427 Isoflavones® (**Figure 3**).

428 $C_{max}/Dose$ values of genistein also differed significantly between treatments
429 ($p<0.001$). After the intake of Fitoladius® these values were higher than the other
430 studied preparation. No differences were observed in t_{max} , $t_{1/2}$, k_e .

431 Several factors can influence the metabolism and bioavailability of isoflavones in
432 humans. These include age, ethnic background, dietary habits, food matrix, intestinal
433 microflora, intestinal transit time, previous exposure to isoflavones, and glycosidase
434 production.^{27, 28} In our case, the differences in bioavailability suggest that the
435 excipients/matrix in which these products are present may play an important role in the
436 absorption process, which could have a direct impact on the bioavailability of soy
437 isoflavones. This fact would explain the different pharmacokinetic profile observed
438 between treatments when the same 12 volunteers received two different soy extracts.

439 Our study has strengths and limitations. One of the strengths is the crossover design,
440 which allowed the same participants to be included in the different treatments, thus,
441 minimising interferences with other confounder variables. Another strength lies in the
442 fact of including both genders among the participants, thus, having a better

443 representation of the overall population. One limitation of our study is that, due to the
444 available commercial sources of the soy extracts, the isoflavone composition of the two
445 tested products differed significantly. Consequently, the participants did not receive
446 exactly the same doses in the two soy isoflavone extracts. In order to minimise these
447 differences, the exact amount of each isoflavone was firstly analysed, and normalisation
448 by the dose administered was performed prior to pharmacokinetic analyses.

449 In conclusion, in the present study we developed a LC/MS/MS method based on an
450 enzymatic hydrolysis with β -glucuronidase H1 followed by SPE procedure using
451 Oasis® MAX cartridges. The analytical method was successfully validated following
452 the European Medicines Agency guidelines on Bioanalytical Method Validation. This
453 methodology presents a good recovery of the analytes, very low matrix effects, a rapid
454 chromatographic separation and high sensitivity. The limits of detection for daidzein,
455 equol, and genistein in 1 mL of plasma sample were 0.6, 0.9 and 1.0 ng/mL,
456 respectively. Once the method was validated, a crossover, randomized, bioavailability
457 study was performed. The method was then applied to the plasma samples of the 12
458 volunteers. The pharmacokinetic profile of daidzein and genistein was studied.

459 After having been normalized, Fitoladius® presented higher bioavailability and
460 achieved higher Cmax values than Super Absorbable Soy Isoflavones® for daidzein and
461 genistein. As the other pharmacokinetic parameters were similar between both
462 treatments, we suggest that these differences in bioavailability come from the
463 matrix/excipients present in the capsules of the isoflavone extracts.

464 The preparations studied are over-the-counter dietetic preparations, they do not
465 require medical prescription, and some of them can be purchased on the internet. Thus,
466 the regulation of these products is not as strict as that applied to prescription drugs.
467 Consequently, a detailed and accurate description of the specific isoflavone content

468 present in the capsules is lacking. Indeed, describing the content of the capsules as “X
469 mg of isoflavones” or “Y mg of genistin and genistein” does not provide clear and
470 accurate information about composition. This is an important fact since there are a
471 number of different isoflavones, which vary in molecular weight, pharmacokinetic
472 profile, and biological activities.²⁹ As a consequence, the exact content of each
473 isoflavone should be provided by the manufacturers in order to have a better knowledge
474 of the content of the capsules and their corresponding beneficial health effects.

475 The purpose of this study was the selection of one isoflavone extract preparation for
476 its use in a clinical trial with the aim of treating drug addiction. In this context we
477 performed the bioavailability study. Results from this report not only show that
478 isoflavones extracts vary in their composition (not unexpected) but also in the amounts
479 reported by manufacturers in each preparation. In a clinical pharmacology context and
480 looking at therapeutic indications with methodological robust designs, results from this
481 study are quite disturbing.

482

483 **Supporting Information:**

484 **Supplementary Table 1.** Intra- and inter-day precision and accuracy of quality
485 control samples of daidzein, equol and genistein in human plasma.

486

487 **Supplementary Table 2.** Isoflavone content of one capsule of Fitoladius® and
488 Super Absorbable Soy Isoflavones®. Data are expressed in milligrams as mean $\pm$
489 standard deviation.

490

491 This material is available free of charge via the Internet at <http://pubs.acs.org>

492

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Figure captions

Figure 1. Chemical structures of daidzein, equol and genistein.

Figure 2. Chromatogram of an extracted plasma sample. A blank plasma was spiked with daidzein, equol and genistein (500 ng/mL), and their corresponding internal standards (300 and 100 ng/mL). Following extraction and using an isocratic gradient, analytes were separated within 5 minutes.

Figure 3. Time-course plasma concentrations of genistein and daidzein after a single oral administration of two different isoflavone extracts to human subjects (n=12). For comparison purposes, the concentration values were normalized by the dose administered. **A**, Daidzein time-course plasmatic concentrations after Super Absorbable Soy Isoflavones® and Fitoladius® treatment; **B**, Genistein time-course plasmatic concentrations after treatments. Data are expressed as mean $\pm$ standard error of the mean.

Tables

Table 1. MS/MS Condition Settings

Compound	Retention Time (min)	Collision Energy (eV)	Precursor ion (m/Z ⁺)	Product ion (m/Z ⁺)	Dwell (s)	Fragmentor (V)
Daidzein	2.53	30	253	223, 208, 195	0.03	120
Daidzein-d ₆	5.53	35	259	229, 214, 137	0.03	140
Equol	2.87	10	241	146, 121, 93	0.03	80
Equol-d ₄	2.87	10	245	150, 138, 123	0.03	100
Genistein	3.09	30	269	159, 133, 107	0.03	120

Table 2. Linearity, Limit of Detection, Matrix Effect and Extraction Recovery for the Studied Analytes.

Compound	Determination coefficient (r^2)	Limit of detection (ng/mL)	Matrix effect (%)		Extraction recovery (%)	
			5 ng/mL	800 ng/mL	5 ng/mL	800 ng/mL
Daidzein	0.999	0.6	17.8	2.1	86.2	85.9
Daidzein-d ₆	-	-	-7.1	-11.6	79.2	77.5
Equol	0.999	0.9	-7.6	-4.8	79.0	84.5
Equol-d ₄	-	-	-12.3	-17.2	76.7	80.2
Genistein	0.996	1.0	-1.8	-9.4	79.1	80.5

Table 3. Plasma Pharmacokinetic Parameters of Daidzein and Genistein after a Single Oral Administration of Fitoladius® (4 capsules) and Super Absorbable Soy Isoflavones® (2 capsules). Data Are Expressed as Mean $\pm$ Standard Deviation.

	Daidzein		Genistein	
	Soy Isoflavones®	Fitoladius®	Soy Isoflavones®	Fitoladius®
AUC _{0-24h} ^a (ng·h/mL)	5408 $\pm$ 928**	3376 $\pm$ 839**	5723 $\pm$ 1587	5838 $\pm$ 1275
AUC _{0-24h} /Dose (ng·h/mL·mg)	120.8 $\pm$ 20.7*	144.8 $\pm$ 36.0*	131.5 $\pm$ 36.5**	159.7 $\pm$ 34.9**
C _{max} ^b (ng/mL)	618.6 $\pm$ 116.8**	350.3 $\pm$ 90.8**	630.8 $\pm$ 206.3	620.5 $\pm$ 196.9
C _{max} /Dose (ng/mL·mg)	13.8 $\pm$ 2.6	15.0 $\pm$ 3.9	14.5 $\pm$ 4.7*	17.0 $\pm$ 5.4*
t _{max} ^c (h)	6.3 $\pm$ 2.0	6.8 $\pm$ 0.8	6.3 $\pm$ 2.1	6.9 $\pm$ 1.0
t _{1/2} ^d (h)	4.5 $\pm$ 1.6	5.4 $\pm$ 3.3	5.9 $\pm$ 2.1	5.8 $\pm$ 2.6
k _e ^e (h ⁻¹)	0.18 $\pm$ 0.07	0.16 $\pm$ 0.07	0.13 $\pm$ 0.05	0.14 $\pm$ 0.07

Abbreviations: ^a AUC_{0-24h}: Area under the curve from 0 to 24 h; ^b C_{max}: Peak plasma concentration; ^c t_{max}: Time at peak plasma concentration; ^d t_{1/2}: elimination half-life; ^e k_e: elimination rate constant. **p* < 0.05; ***p* < 0.005

Figure 1.

Figure 2.

Figure 3.

TOC Graphic