Effect of *Lactobacillus sporogenes* on oral isoflavones bioavailability: single dose pharmacokinetic study in menopausal women

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Abstract

*Purpose of the study:* To verify the single dose bioavailability of two oral formulations of soy isoflavones, with and without lactobacilli, in menopausal women in antibiotic therapy.

*Methods:* Twelve menopause women (mean age 54.3 years, BMI 25.0 kg/m²) participated in a controlled cross-over study. Reference and test treatments were: R = tablets containing soy isoflavones 60 mg (genistein 30 mg + daidzein 30 mg) + calcium and vitamin D₃; E = R + 500 million vital spores of *Lactobacillus sporogenes* (E is Estrominal), a food supplement containing soy isoflavones 60 mg, calcium 141 mg and vitamin D₃ 5 μg.

The design included 2 periods of 5 days of amoxicillin + clavulanate treatment with a 2-week wash-out. After each period alternatively a single dose of each formulation was given in randomised sequence. Genistein and daidzein were determined in plasma by HPLC, sampled 10 times within 24 h after dosing.

*Results:* Genistein pharmacokinetics parameters were higher after E than after R administration: peak plasma concentration (Cₘₚ) +2.4 %, area under the concentration curve (AUCₜ₋₂₄) +2.4 % and mean residence time +11.0 %. Daidzein Cₘₚ and AUC showed a larger variability on E, evidenced by higher scatter from the mean on the formulation without lactobacilli.

*Conclusion:* A trend is shown for a greater absorption of genistein from a formulation containing lactobacilli.

Key words

- Food supplement
- Genistein
- Isoflavones
- *Lactobacillus sporogenes*
- Pharmacokinetics
- Phyto-oestrogens

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1. Introduction

Epidemiological observations that a diet rich in soy is associated with a lower incidence of menopausal symptoms, osteoporosis, cardiovascular disorders and uterus, breast and colon cancer in Asian populations increased the interest in phyto-oestrogens in menopausal women [1].

The use of natural non-steroidal substances extracted from soy to control menopausal symptoms is important, as it avoids the adverse effects of hormone replacement therapy thanks to their potency being 1000 to 10,000 times lower than that of oestradiol, whose structure they mimic.

Soy isoflavones are the class of phyto-oestrogens with the greatest effect on menopausal symptoms. They are present in soy and are attached to carbohydrates (glycosides) [2]. They are absorbed at the intestinal level only if hydrolyzed to aglycones (genistein, daidzein and its metabolite, equol) by glycosidases, enzymes produced by the intestinal flora, and in particular by lactobacilli [3]. It follows that certain situations, such as the use of antibiotics or some diseases that reduce the number and activity of the intestinal flora, can adversely influence the absorption and action of isoflavones, which are on the other hand similarly absorbed in pre- and post-menopausal women [4].

These considerations, therefore, formed the basic rationale to develop a food supplement in the form of soy isoflavones enriched with *Lactobacillus sporogenes*, a promoter of intestinal absorption. The aim of this study was to verify the single dose bioavailability of an oral formulation of soy isoflavones, with and without *L. sporogenes*, in menopausal women with gut microflora depletion due to antibiotic therapy.
2. Subjects and methods

2.1 Study subjects

Twelve healthy postmenopausal Caucasian women, aged 45–65 years, who gave informed and signed consent before submission to any study procedure, were recruited. Their mean age was 54.3 years (± 4.0 SD, range 47–62) and BMI was 25.0 kg/m² (± 1.8, range 22.2–27.3).

The pharmacokinetic study was conducted in the Department of Clinical Pharmacology and Therapeutics, UMHAT “Queen Joanna”, Sofia, Bulgaria – Second MBAL, Clinic of Cardiology, Sofia, Bulgaria. It was approved by the local ethics committee at Second MBAL Hospital and the Bulgarian Drug Agency (BDA) and carried out in accordance with the Helsinki Declaration and its revisions and Good Clinical Practice.

The inclusion criteria included a body weight within 10% of the ideal body weight (as defined in the Metropolitan Height and Weight Tables), normal physical examination and laboratory evaluations, history of treatment with penicillin or other β-lactam antibiotics without suffering any allergic reaction.

The exclusion criteria were: history of alcohol or drug abuse, smoking more than ten cigarettes/day, history of serious gastrointestinal, renal, hepatic, pulmonary or cardiovascular disease; or history of epilepsy, asthma, diabetes, psychosis or glaucoma, history of allergic response to soy or related substances, participation in a previous clinical trial within the past three months, blood donation of 250 ml or more within the past three months, history of allergic reactions to any penicillin or β-lactam antibiotics in general, previous history of cholestatic jaundice/hepatic dysfunction associated with amoxicillin + clavulanic acid treatment, practising vegetarian, abnormal diets (<1600 or >3500 kcal/day) or substantial changes in eating habits within the past 4 weeks, treatment with any known enzyme inhibiting or inducing agents (barbiturates, phenothiazines, cimetidine, etc.) within the past four weeks, positive to drugs of abuse (qualitative screen in urine), use of any prescription or over-the-counter medication on a regular basis, drinking excessive amounts of tea, cacao, coffee and/or beverages containing caffeine (>5 cups/day) or wine (>0.5/l/day or equivalents), or likely to be non-compliant or non-cooperative during the study. Women on a specific diet (i.e. vegetarian) were not admitted to the study, during which no soy food was allowed. The volunteers were requested not to take any prescription or over-the-counter medication for a period of at least four weeks prior to and during the study (including the washout period), no alcoholic or caffeine-containing beverages from 48 h prior to and until the last blood sampling time (24 h) after each administration and to avoid yogurt and any food containing soy or its derivatives from 48 h prior to the start of antibiotic treatment and until the last blood sampling time after each administration. Moreover, avoidance of fresh grapefruit or grapefruit juice from two weeks prior to and during the entire period of the study (possible hepatic enzyme induction) was requested.

Subjects were hospitalised in the Clinical Centre in the evening of the day before trial substance administration, and remained there under permanent medical and nursing supervision for 36 h after administration in each treatment period. After the last administration, the physical examination, haematological, biochemical and urine analyses were repeated. Vital signs and ECG at 24 h after dose, for the second period, were considered the final evaluation.

2.2 Nutraceutical products

The test product (E) was a food supplement in tablets containing soy isoflavones 60 mg (genistin 30 mg + daidzin 30 mg) + 500 million vital spores of L. sporogenes + calcium 141 mg and vitamin D3, 5 µg (Estromineral®, Rottapharm S.p.A., Monza, Italy, Food Supplements Italian Register XAD 00462-Y, batch no. 507478) and the reference product (R) was a food supplement in tablets with the same composition as E but without L. sporogenes (batch no. ES020/04 prepared by Rottapharm | Madaus Pharmaceutical Development Department).

2.3 Controlled, two-period cross-over study with randomised sequences was planned according to a repeated Latin Squares design.

Each subject underwent two periods of five days of antibiotic treatment with a two-week wash-out at the end of each period. Alternatively, a single dose of one of the two formulations in random sequence was given. E and R were administered in the morning of the 6th day of each study period with 200 ml of water at room temperature under fasting conditions. The antibiotic treatment was represented by amoxicillin trihydrate corresponding to amoxicillin 875 mg + potassium clavulanate corresponding to clavulanic acid 125 mg (Augmentin®, GlaxoSmithKline, Verona, Italy), three times daily for five days. For the morning dosing, subjects were not allowed to drink water from 1 h before until 2 h after administration, except that planned for the drug administration. Four hours after morning drug administration, lunch was served. After the last administration, physical examination, vital signs, ECG, haematological, biochemical and urine analyses were repeated.

Plasma was sampled at time 0 (just before dosing) and 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12 and 24 h after dosing. Blood samples (approximately 10 ml) were collected by an indwelling catheter into heparinised test tubes, mixed by gently inverting them immediately after blood withdrawal and rapidly centrifuged at +4°C (1500 g for 10 min). Resulting plasma was separated, transferred into polypropylene tubes, stopped (airtight) and immediately frozen at ~20°C until assayed.

2.4 Analysis of plasma samples

Genistein, daidzein and equol were analysed in plasma by a validated LC-MS/MS method after enzymatic hydrolysis with β-glycosidase and aryl-sulphates by BT Biotecnica Laboratory, Varese, Italy, in compliance with GLP rules, as requested by the CPMP Guidance on the Investigation of Bioavailability and Bioequivalence of July 2001.

Calibration curves were linear in the range of 5–1000 ng/ml with a coefficient of correlation of r ≥ 0.999 for genistein, daidzein and equol. The lower limit of quantification was 5 ng/ml, the precision was 5.8% for genistein, 5.3% for daidzein and 5.9 for equol, and the accuracy was 115% for genistein, 105% for daidzein and 88% for equol.

Intra-assay precision of quality control samples (expressed as a coefficient of variation) was ± 2.10% for genistein, ± 4.01% for daidzein and 3.34% for equol. Intra-assay accuracy ranged from 95 to 111% for genistein, from 101 to 111% for daidzein and from 98 to 107% for equol.

Propyl-4-hydroxybenzoate (Sigma-Aldrich, Milan, Italy) was used as internal standard.

Aliquots of plasma extract (50 µl) were subjected to LC-MS/MS on a Shimadzu system 10 AD class-VP, consisting of a binary pump, autosampler at 4°C, column at 25°C, detector UV/
VIS set at 214 nm, analytical column Hypersil BDS C18 3 μm, 150 × 4.6 mm, and a mobile phase of 0.02 M phosphate buffer/acetonitrile (72:28, v/v) eluted at a flow rate of 1 ml/min.

2.5 Pharmacokinetic analysis
The individual time courses of plasma concentrations were analysed according to a non-compartmental approach using the Kinetica® software (Innaphase). The pharmacokinetic parameters assessed were: \( C_{\text{max}} \), \( t_{\text{max}} \), AUC\(_{0\rightarrow24} \), \( t_{1/2} \), MRT. Maximum plasma concentration (\( C_{\text{max}} \)) and the time of maximum plasma concentration (\( t_{\text{max}} \)) were derived from the plasma concentration-time curve. The area under the plasma concentration-time curve, from the time zero to the last measurable concentration (AUC\(_{0\rightarrow\infty} \)) was calculated by the trapezoidal rule, setting to zero the values below the limit of quantification. The area under the plasma concentration-time curve from the time zero to infinity (AUC\(_{0\rightarrow\infty} \)) was estimated by extrapolating to infinity (AUC\(_{0\rightarrow\infty} \)). The apparent terminal rate constant (\( \lambda_{2} \)) was obtained as the slope In-linear regression analysis of plasma concentration-time curves in the terminal phase. The apparent terminal half-life (\( T_{1/2} \)) was calculated as: \( T_{1/2} = \ln(2)/\lambda_{2} \). The mean residence time (MRT) was calculated as the ratio between AUMC and AUC, where AUMC is the area under the plasma concentration × time vs. time curve. The pharmacokinetic bioequivalence of the formulations was assessed by comparing \( C_{\text{max}} \) and AUC.

2.6 Statistical analysis
The statistical analysis was performed with the Kinetica software (InnaPhase) by analysis of variance (ANOVA) on \( C_{\text{max}} \) and AUC logarithmically transformed data, and on non-transformed MRT data. \( t_{\text{max}} \) was analysed by the non-parametric Friedman test. In particular, the bioequivalence calculation was based on the 90% symmetrical confidence interval (CI) for the log-transformed data of AUC\(_{0\rightarrow24} \) and \( C_{\text{max}} \) within the range of 80–125% for the ratio E/R. The two one sided t-test was used.

3. Results
The mean plasma concentrations of genistein and daidzein after oral administration of the two isoflavone formulations are shown in Fig. 1 and 2. The values of equal were below the limit of quantification.

The main pharmacokinetic parameters are reported in Tables 1 and 2.

Genistein pharmacokinetic parameters were higher after E than after R administration and the differences between formulations were more than 20% for \( C_{\text{max}} \) (+24.3%), and for AUC\(_{0\rightarrow24} \) (+24.4%) and less than 20% for MRT (+11.0%). The 90% confidence interval for the ratio E/R was outside the bioequivalence acceptance limits (0.80–1.70 for \( C_{\text{max}} \) and 0.84–1.70 for AUC\(_{0\rightarrow24} \)).

Daidzein pharmacokinetic parameters \( C_{\text{max}} \) and AUC showed a wider variability on R, evidenced by higher scattering from the mean on the formulation without \textit{L. sporogenes}: AUC standard error of the mean (SEM) was 14% for E and 27% for R.

No significant alterations of physiological functions were observed versus baseline and also between formulations. No adverse events were noted during the study and no abnormal laboratory values were found after either treatment regimen with the two isoflavone formulations.

4. Discussion
The mean plasma curve profiles of genistein and the derived pharmacokinetic parameters obtained in this kinetic study after oral administration of isoflavones with and without \textit{L. sporogenes} showed that bioequivalence between E and R formulations can not be stated as the 90% confidence intervals for the AUC and \( C_{\text{max}} \) were more than 20% different from each other.

![Fig. 1: Genistein: mean plasma levels after oral administration of isoflavones with \textit{L. sporogenes} (E) and without \textit{L. sporogenes} (R) (mean and SEM).](image-url)
The lower AUC values of daidzein with E suggest that *L. sporogenes* promoted degradation of daidzein at the intestinal level in favour of other metabolites (e.g. equol), whose levels on the other hand could not achieve the quantification limit due to the single dose design. A steady-state study may clarify this. On average, the formulation containing *L. sporogenes* showed a more constant absorption than R as confirmed by the highly inferior SEM values of E treatment AUC compared with R. MRT and $t_{1/2}$ values of genistein and daidzein justify the recommendation of the once a day regimen for E.

The limitations of this study are the relatively small sample size, even though such a design is frequently adopted for this kind of products [5, 6].

The human gut microflora influences the bioavailability of isoflavones, as their absorption occurs in the small intestine where local microbes can hydrolyse soy isoflavone conjugates, transforming them into the corresponding bioactive aglycones for absorption or metabolism [7]. An important metabolite of daidzein is equol, exclusively produced by intestinal bacterial metabolism, and more potent than its precursor daidzein [8]. The rationale to add probiotics to isoflavones is to integrate intestinal microflora in order to improve the hydrolysis of the isoflavone glycosides and the bioavailability of isoflavones, especially when the gut microflora is depleted due to antibiotic treatment. This was validated by the results of the present clinical pharmacokinetic study.

Recent *in vitro* data clearly indicate that the conversion of isoflavones into active aglyconic forms is promoted by *L. sporogenes*: incubation of soy isoflavones with *L. sporogenes* resulted in 98.2% conversion of daidzein to daidzein and in 87.5% conversion of genistin to genistein. In the absence of *L. sporogenes* very poor conversion was observed: 2.4% and 2.0%, respectively (data presented by Arcoraci et al. at the 81st Congress of the Italian Society of Gynecology and Obstetrics, 20–24 September 2005, Bologna, Italy). On the basis of these *in vitro* data, one would also expect a beneficial role of *L. sporogenes* on the conversion of isoflavones to their active forms *in vivo*, especially when gut microflora is depleted due to antibiotic treatment.

This study was performed to comply with the recommendation to improve quality assurance and standardisation of food supplements to support the claims on content and bioavailability [9].

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**Fig. 2: Daidzein: mean plasma levels after oral administration of isoflavones with *L. sporogenes* (E) and without *L. sporogenes* (R) (mean and SEM).**

**Table 1: Genistein: pharmacokinetic parameters after oral administration of isoflavones with *L. sporogenes* (E) and without (R) *L. sporogenes* (R). Mean and SEM.**

<table>
<thead>
<tr>
<th>Genistein</th>
<th>R</th>
<th>E</th>
<th>Reference Cl E/R (0.80–1.25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ (ng/ml)</td>
<td>200.6 ± 31.1</td>
<td>249.3 ± 44.6</td>
<td>No bioequivalence 0.80–1.70</td>
</tr>
<tr>
<td>$\text{AUC}_{0-24}$ (ng/ml·h)</td>
<td>3132 ± 461</td>
<td>3895 ± 590</td>
<td>No bioequivalence 0.84–1.70</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>8.4 ± 1.7</td>
<td>9.0 ± 1.8</td>
<td>NS</td>
</tr>
<tr>
<td>$T_{1/2}$ (h)</td>
<td>11.4 ± 3.0</td>
<td>16.5 ± 6.9</td>
<td>NS</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>18.2 ± 2.9</td>
<td>20.2 ± 5.0</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Table 2: Daidzein: pharmacokinetic parameters after oral administration of isoflavones with *L. sporogenes* (E) and without *L. sporogenes* (R). Mean and SEM.**

<table>
<thead>
<tr>
<th>Daidzein</th>
<th>R</th>
<th>E</th>
<th>Reference Cl E/R (0.80–1.25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ (ng/ml)</td>
<td>234.9 ± 23.8</td>
<td>245.5 ± 30.4</td>
<td>No bioequivalence 0.80–1.31</td>
</tr>
<tr>
<td>$\text{AUC}_{0-24}$ (ng/ml·h)</td>
<td>5968 ± 1638</td>
<td>4224 ± 586</td>
<td>bioequivalence 0.86–1.18</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>5.3 ± 1.9</td>
<td>5.1 ± 1.2</td>
<td>NS</td>
</tr>
<tr>
<td>$T_{1/2}$ (h)</td>
<td>20.2 ± 6.5</td>
<td>12.2 ± 1.3</td>
<td>NS</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>31.3 ± 9.4</td>
<td>19.3 ± 1.9</td>
<td>NS</td>
</tr>
</tbody>
</table>
Moreover, these pharmacokinetic data support the clinical evidence that the combination of isoflavones + \textit{L. sporogenes} (E) is effective in the treatment of menopausal symptoms in various open and controlled clinical studies, even during prolonged treatment for 6–12 months [10–13].

**Conflict of Interest**
C. Benvenuti, MD, ScD and I. Setnikar, MD are consultants for Rottapharm|Madaus.

**References**