Plasma levels of genistein following a single dose of soy extract capsule in Indian women


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Received March 29, 2006

Background & objectives: Soy isoflavones are being used as therapy for menopausal syndrome in many countries. Marketed preparations show variability in bioavailability and there are variations in kinetics due to ethnicity and diet. Inspite of soy isolavone being available in the Indian market there are no studies to show whether the preparation is likely to be effective in women. This study was carried out to determine circulating levels of genistein, a bioactive soy isoflavone, in Indian women after a single dose of soy extract.

Methods: Six healthy vegetarian women volunteers, between 36 and 62 yr and with a mean body mass index (BMI) 25.01±2.02 (kg/m²), were enrolled after an informed consent. Women with antibiotic or Soy food intake within 1 month of study were excluded. A single dose of standardized soy extract capsule containing 64.12 mg of total isoflavones (genistein content equivalent to 31.76 mg) was ingested under supervised fasting condition and multiple blood samples were collected at 0, 1, 2, 4, 6, 8 and 24 h. Genistein levels were measured by high performance liquid chromatography (HPLC) method with a detection level of 2.5 ng/100 µl of injection volume. The intra- and inter-assay coefficients of variation were < 5.32 per cent.

Results: Genistein was detected (10.3 to 16.2 ng/ml) in 3 volunteers in baseline samples. Within one hour genistein levels rose from 42 ng to 215 ng/ml with a maximum concentration of 117 to 380 ng/ml at 4 to 8 h. A secondary peak suggestive of enterohepatic circulation was seen between 4 and 6 h in 2 out of 6 volunteers. The mean $C_{\text{max}}$ was 315.5 ± 57.1 ng/ml. All women had detectable levels from 25.2 to 109.3 ng/ml at 24 h.

Interpretation & conclusion: Our study showed adequate circulating levels of genistein in Indian vegetarian women after a single dose of soy extract. Variability in plasma levels of the soy isoflavones may explain differences in responses to therapy.

Key words Menopause - plasma genistein levels - phytoestrogens - soy isoflavones
With an increment in life expectancy the demand for the treatment of menopausal syndrome (MS) has gained importance in India. An estimated 80 million women exist above the age of 50 yr and of these 20 to 50 per cent may have perimenopausal symptoms. Estrogen deficiency with ageing is the primary cause of MS and estrogen replacement therapy (ERT) alone or with progestins hormone replacement therapy, (HRT) was the mainstay of treatment until recently. However, the hazards of hormonal therapy have been defined after long term follow up and controlled studies on millions of women. There has been a continued search for an effective and safe non hormonal alternative therapy. Amongst these soy isoflavones have been found beneficial and have been studied in several clinical trials. These also have beneficial metabolic and chemopreventive effects. Their kinetics has also been extensively studied in various ethnic groups. Large inter-subject variations are reported with different type of intestinal microflora and type of diet. To our knowledge despite several soy isoflavone products including isoflavone extracts being available in the Indian market there are no published data on the circulating levels of the isoflavones in Indian women. We therefore undertook this study to determine the availability of the bioactive isoflavone, genistein, in healthy Indian vegetarian peri- and post-menopausal women after the ingestion of a standardized soy extract.

Material & Methods

The study was carried out at outpatient clinic and day care facility of Bhavan’s Swami Prakashananda Ayurveda Research Centre between May and July 2002. The study protocol was approved by the ethics committee of the institution.

Subject selection: Six out of a total of fifty nine peri- or post-menopausal healthy vegetarian women between 36 and 62 yr of age, with haemoglobin level > 11g per cent and willing to participate in multiple blood sampling under supervised conditions after a single dose of soy extract capsule were included in the study. Women with unexplained or irregular vaginal bleeding, abnormal Papanicolaou smear, on soy containing diet in the preceding month, history of any chronic treatment such as analgesics or antibiotics within one month of study, uncontrolled hypertension, systemic diseases like diabetes mellitus, hypothyroidism, severe dyslipidaemia, liver, kidney or heart disease, and chronic diarrhoea, Crohn’s disease, liver and gall bladder disease were excluded. Informed written consent was obtained from all.

Routine biochemistry, prior to selection included complete blood count, peripheral smear, erythrocyte sedimentation rate, liver function tests and renal function tests as well as fasting sugar and serum thyroid stimulating hormone (TSH).

Selected women were given a single dose of standardized soy extract capsule containing total isoflavones 64.12 mg (SoyEstro® Gland Pharma, Hyderabad). The standardized extract contained genistin 38.42 mg, genistein 7.73 mg, daidzin 15.09 mg and daidzein 2.88 mg per capsule. Women had soy free diet for 1 month before and during the study.

Kinetic study: Multiple blood samples were collected at the Center under supervised conditions with standard breakfast, meal and tea. Fasting blood was collected at 0800 h followed by ingestion of SoyEstro® capsule. Standard breakfast (300 cal) was provided after one hour and lunch (450 cal) after four hours. Tea (60 cal) was provided at 7 h. Blood samples (6 ml) were collected using an intravenous canula at 0, 1, 2, 4, 6, 8 and 24 h in EDTA tubes. In 1 volunteer blood was collected at 3 h instead of 2 h after the dose. Plasma was separated and preserved in 500 µl aliquots at -70°C until analysis was carried out.

Assay method: Genistein assay was standardized using pure genistein (Sigma, USA). All reagents used were of analytical grade (Qualigens, Mumbai). The
standard curve was prepared using pure standard as well as spiked plasma. The Jasco HPLC pu 980 pump (Jasco, Japan) fitted with AS – 1555 – 10 auto-sampler was used with an analytical column of C 18 (Cosmosil, Japan), 150 × 4.6 mm, (internal diameter 5µ). The injection volume was 100 µl and a flow rate of 1 ml/min was maintained. The mobile phase was methanol : distilled water (v/v 45:55) at pH 3.5. A UV detector was used at a wavelength of 259 nm with an integrator from Borwin Integrator software, version 1.21.

Six calibrants of concentrations of standard genistein from 2.5 ng/100 µl to 80 ng/100 µl, were used in triplicate. An additional calibrant of 200 ng/100 µl was used for samples with > 100 ng concentration. A non weighted regression analysis showed a coefficient of correlation for standard curve from 0.9974 to 0.9999.

**Precision and accuracy:** Standard curve was plotted in triplicate and 3 times a day for 3 days. The coefficient of variation (CV) was 5.77 per cent for a concentration of 2.5 ng/100 µl, and 0.21 per cent for a higher concentration of 80 ng/100 µl. The accuracy obtained was 103.5 per cent for 2.5 ng/100 µl, and 100.89 per cent for 80 ng/100 µl concentration. Intra-assay CV was < 0.73 per cent and inter-assay CV (11 assays) was < 5.32 per cent for various concentrations. The limits of quantification for 100 µl injection volume were 2.5 to 200 ng.

**Sample hydrolysis and extraction:** 1 ml plasma was mixed with 1 ml of acetonitrile, vortexed for 10 min and centrifuged to precipitate proteins. One ml of supernatant equivalent to 0.5 ml of plasma, was incubated with 6 ml of enzyme sulphatase (Sigma, USA, 23 units) and glucuronidase (Sigma, USA, 3.24 units) in trisodium citrate buffer (25 mM, pH5) at 37°C for 30 min. It was extracted with 6 ml of ethyl acetate three times and centrifuged. The supernatant was evaporated under nitrogen gas. Dried extract was then reconstituted in 0.2 ml of mobile phase;100 µl of the reconstituted sample was injected into the column hence value were corrected for the concentration factor. This helped to measure values as low as 10 ng/ml.

Based on standard HPLC curves with pure genistein standard and genistein peak from a plasma sample (Figs 1 a & b), value of genistein in individual timed plasma samples were calculated.

The peak at retention time (RT) 16 min was subjected to liquid chromatograph mass spectrometry Fig. 1. HPLC chromatograph of (a) standard genistein with peak at 16.07 min and (b) plasma extract of volunteer with peak at 16.06 min with mobile phase methanol: distilled water (45:55) at pH 3.5, injection volume 100 µl and at UV 259 nm.
and the compound was identified as genistein (M+1 peak=271). Area under concentration – time curve (AUC) was calculated using the software Graph – Pad Prism (GraphPad Software Inc., USA). The Pearson’s correlation coefficient between peak plasma concentrations or AUC of genistein and age, weight or BMI of the volunteers was also calculated.

### Results

All six women were clinically healthy and with normal biochemical parameters. Their age was between 36 and 62 yr, and two women were pre-while four were post-menopausal. The mean body mass index (BMI) was $25.01 \pm 2.02$ kg/m², and mean body weight was $57.00 \pm 7.04$ kg. All subjects were vegetarian. None of the subjects had any side effects with single dose of SoyEstro®.

Within 1 h, genistein levels were detected in plasma of all volunteers. These ranged from 42 ng to 215 ng/ml at 1 h with a maximum concentration of 117 to 380 ng/ml at 4 to 8 h (Table I). A secondary peak suggestive of enterohepatic circulation was seen between 4 and 6 h in 2 out of 6 volunteers after an early primary peak (Figs 2 a-f).

<table>
<thead>
<tr>
<th>Volunteer no.</th>
<th>Age (yr)</th>
<th>$C_{\text{max}}$ (ng/ml)</th>
<th>$T_{\text{max}}$ (h)</th>
<th>$\text{AUC}_{0-24 \text{ h}}$ (ng/ml/h)</th>
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<td>9.74</td>
<td>57.1</td>
<td>1.633</td>
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</table>

**Table I.** Plasma concentrations of genistein in six volunteers

**Table II.** Plasma genistein concentrations ($C_{\text{max}}$, $T_{\text{max}}$, $\text{AUC}_{0-24 \text{ h}}$) in six female volunteers

(LCMS) and the compound was identified as genistein (M+1 peak=271). Area under concentration – time curve (AUC) was calculated using the software Graph – Pad Prism (GraphPad Software Inc., USA). The Pearson’s correlation coefficient between peak plasma concentrations or AUC of genistein and age, weight or BMI of the volunteers was also calculated.
Fig. 2. Plasma levels of genistein in 6 women volunteers up to 24 h after a single capsule of SoyEstro®. (a) Volunteer 1, (b) Volunteer 2, (c) Volunteer 3, (d) Volunteer 4, (e) Volunteer 5 and (f) Volunteer 6.
The mean $C_{\text{max}}$ was $315.5 \pm 57.1$ ng/ml and $T_{\text{max}}$ was at an average of $5.3 \pm 1.6$ h. The AUC from 0 to 24 h was $4015 \pm 1138$ ng.h/ml. (Table II).

Genistein levels were undetectable in three volunteers at baseline. However, in the remaining three volunteers genistein was detected in baseline samples (10.3, 12.8 and 16.2 ng/ml). The variation in maximum concentration was less than 2-folds and the variation in AUC (0-24) h was less than 3-folds. At 24 h all 6 volunteers had plasma genistein levels from 25 to 109 ng/ml. Volunteer No. 3 had high peak level of 379.8 ng/ml and also highest level of 109 ng/ml at 24 h amongst the 6 volunteers as also the maximum AUC. It was of interest to observe that volunteer No. 6 had lowest peak level of 221 ng/ml, lowest AUC and lowest level at 24 h amongst the 6 volunteers (Figs. 2 c, f). Thus there was consistency in baseline, peak and 24 h levels.

Two pre-menopausal volunteers (No. 4 and No. 6) had lower $C_{\text{max}}$ and lower AUC whilst 4 post-menopausal volunteers had higher (max) values.

There was no correlation between peak plasma concentrations or AUC of genistein and age, weight or BMI of the volunteers in this study.

Discussion

Pharmacokinetics of genistein has been reported in women by various groups and has been reviewed extensively\textsuperscript{10-13,19,20}. Peak levels up to 800 ng/ml have been reported after a 50 mg dose of isoflavone aglycone genistein. In the present study, mean peak level of $315\pm57.1$ ng/ml were observed after ingestion of soy extract equivalent to 31.76 mg of genistein indicating good absorption. The AUC (0-24) hours was also proportionally comparable and in all women genistein was detected at levels above 25 ng/ml at 24 h after the single dose. Recently a maximum concentration of $516$ ng/ml and mean plasma level of $261\pm110$ ng and mean AUC (0-32 h) of $2957\pm1372$ ng h/ml for genistein has been reported after a single dose of 2 soy extract capsules (22.57 mg genistin)\textsuperscript{13}. Plasma levels of genistein were similar after 1 standard dose and at the end of 30 days of daily intake of 100 mg of soy isoflavones in a recent study\textsuperscript{21}. Thus multiple dosing did not seem to increase plasma levels.

Very low levels of plasma genistein (<16 ng/ml) were detected in baseline samples in 3 volunteers. Since volunteers on soy foods were not included in this study these could have been generated from other common food items which may have very low concentrations of genistein such as peas and urad. However, all volunteers were instructed not to take soy products 1 month prior to the study and received soy free meals and breakfast on the day of study.

The isoflavones are hydrolysed in intestinal enterocytes by glycosidases before absorption. In the liver, in the presence of glucuronic and sulphuric transferases, the conjugated glucuronides and sulphates are formed. These, being more polar circulate in blood to reach various target organs and are excreted in kidney and in the bile. The conjugated isoflavones in the biliary excretion reach the lower intestines. Here these are acted upon by the intestinal microflora and are reabsorbed. This leads to the secondary peak at 6 to 10 h\textsuperscript{14,19,20}. The structure of soy isoflavones is similar to the natural synthetic female sex hormone 17$\beta$-estradiol and ethinyl estradiol which also undergoes enterohepatic recirculation\textsuperscript{22}. We have earlier reported the secondary plasma peak of both ethinyl estradiol and of norethisterone, in Indian women with low dose oral contraceptives\textsuperscript{23,24}. The extent to which this occurs with soy isoflavones is likely to be variable. Such variations are also probably dependent on the life long dietary habits rather than short term changes as some authors have reported that there was no effect of short term dietary change on pharmacokinetics of isoflavones\textsuperscript{25}. 
Large intersubject variations have been reported for the bioavailability of genistein and daidzein\textsuperscript{14,15,19,20}. This is accounted by several factors like variations in drug metabolizing enzymes, type of diet, intestinal absorption, enterohepatic recirculation and intestinal microflora. Variation in gut transit time can also affect bioavailability of genistein in women\textsuperscript{26}. Apart from enterocytes of the gut, the hydrolysis of flavonide glucosides in the oral cavity could be another causative variable factor\textsuperscript{27}. Another possible factor which may alter the bioavailability of exogenous agents is plasma protein binding. However, unlike natural estrogens, genistein and daidzein are poorly bound to plasma proteins (<3\%) and this is not likely to be a major variable\textsuperscript{19,20}.

In the present study we observed minimum bioavailability in the two premenopausal volunteers. Because of the small sample size it is not possible to say whether this is due to the high levels of endogenous estrogens. In another study no difference was observed in the kinetics of pre- and post-menopausal women indicating that absorption and disposition of isoflavones is independent of age and menopausal status\textsuperscript{28}. Takimoto and coworkers\textsuperscript{29} have studied kinetics and dynamics of genistein in cancer patients and did not observe any change.

In conclusion, the bioavailability of genistein in Indian vegetarian women was similar to that reported in some ethnic groups in literature. The study has demonstrated adequate circulating levels of genistein in Indian vegetarian women after a single dose of soy isoflavone product in the Indian market. Since bioavailability of soy-isoflavones is variable, measurement of plasma levels could assist dosage-adjustment, in those women who fail to respond.

Acknowledgment

Authors acknowledge the financial support received from the Indian Council of Medical Research, New Delhi and Gland Pharma India to provide standardized soy extract capsules. Authors thank Prof. Akhil Vaidya for gifting the enzyme glucuronidase.

References


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