Long-Term Pharmacokinetics of an Extract of Isoflavones from Red Clover (Trifolium pratense)

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ABSTRACT

Objectives: To study the pharmacokinetics of isoflavones from red clover (Trifolium pratense) after long-term administration as a once-daily dietary supplementary.

Design: Fourteen (14) subjects who had been consuming a low-isoflavone diet for 2 weeks were given an oral dose of two isoflavone tablets (approximately 80 mg of total isoflavones) daily for 2 weeks and appeared for a study day at 9:00 AM after an overnight fast on the day that they were to receive the last dose. Plasma samples were collected for a 48-hour period after the last dose. Plasma isoflavones were assayed by high-performance liquid chromatography (HPLC).

Results: Trough plasma levels were significantly higher for daidzein and genistein after long-term dosing than levels taken prior to the commencement of the study and plasma levels of isoflavones after long-term dosing were in the range previously reported in populations that consume an isoflavone-rich diet. The plasma half-lives observed after long-term administration were, in most cases, consistent with once-daily administration.

Conclusions: Isoflavones have pharmacokinetic characteristics that suggest that once-daily administration is adequate when they are administered long-term as dietary supplements.

INTRODUCTION

In recent years there has been increasing interest in the effects of isoflavone phytoestrogens in postmenopausal diseases and symptoms associated with estrogen deficiency. This has arisen because of an apparently lower prevalence of diseases and symptoms associated with estrogen deficiencies in countries where the dietary intake of isoflavones is high (Knight et al., 1996). However, controlled clinical trials of the effects of dietary isoflavone supplementation on menopausal symptoms has produced both positive (Albertazzi et al., 1998; Upmalis et al., 2000) and negative (Baber et al., 1999; St. Germain et al., 2001) results.

There is little published data concerning the pharmacokinetics of isoflavone phytoestrogens in humans and no data on the pharmacokinetics after long-term administration. This study investigated the long-term pharmacokinetics of a commercial tablet preparation of four isoflavones (genistein, daidzein, biochanin, and formononetin). Biochanin and formononetin are metabolized by demethylation in vivo to genistein and daidzein respectively. The recommended dosage for Promensil™ (Novogen, Sydney, Australia) is one to two tablets daily.

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MATERIAL AND METHODS

Study population

Six male and eight female volunteers 22–45 years of age were recruited into a long-term pharmacokinetic study of two tablets of an isoflavone extract tablet derived from red clover (*Trifolium pratense*) consisting of biochanin, 24.5 mg; genistein, 1.5 mg; formononetin, 16 mg; and diadzein, 1.5 mg taken once daily (Promensil). The subjects received two tablets once daily for 2 weeks. The subjects were in good general health, not receiving drug therapy of any kind, did not smoke, or consume more than 40 g of alcohol per day.

The study was approved by the South Eastern Sydney Area Health Service Ethics Committee and written, informed consent was obtained from each subject.

Study procedure

Four weeks prior to the commencement of the pharmacokinetic study the subjects reported for a medical examination and blood sampling for routine biochemistry and haematology. The subjects commenced a diet low in isoflavones for 2 weeks prior to the study (run-in phase) and continued on the diet for the duration of the study. This diet was achieved by providing the subjects with a list of foods to avoid. Compliance with the diet during the week prior to the active treatment period of the study was confirmed by obtaining a 24-hour urine collection for the assay of isoflavones the day before attending for the administration of the first dose. Baseline isoflavone levels were measured at 9:00 AM after an overnight fast the day prior to commencement of isoflavone therapy. Compliance during the active phase of the study was ensured by the maintenance of dietary records and tablet counts of returned study medication bottles.

The subjects fasted overnight and abstained from alcohol for 24 hours and caffeine for 12 hours prior to the administration of the final dose of two Promensil tablets along with 200 mL of distilled water at 9:00 AM.

Blood samples for the assay of plasma isoflavone levels were taken immediately prior to dosing, 10, 15, 30, and 45 minutes and 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12, 24, and 48 hours after dosing. The subjects were allowed an intake of purified water as required from 2 hours after the administration of the test drug and standard light meals with a low-isoflavone content were provided 4 hours and approximately 10 hours after test drug administration. Caffeine-containing beverages were not allowed during the pharmacokinetic study day. The subjects returned home after the 12-hour blood sample and returned the following day for a further blood sample 24 hours after the last dose and again 48 hours after the last dose.

Assay methods

The analytical method was validated by determining the limit of qualification (LOQ), linearity, range, and specificity. Validation samples were prepared by spiking blank plasma, obtained from patients on isoflavone-free diets, at a level of 0, 5, 10, 50, and 100 ng/mL for each of the four isoflavones. The isoflavones were quantitated at a wavelength of 283 nM. The limit of quantitation for each of the four isoflavones was determined as 5 ng/mL (minimum signal to noise ratio of 3:1). The method was found to be linear over the concentration range 5–100 ng/mL for all four isoflavones $R^2 > 0.95$.

The standard analysis of unknown samples consisted of running a system suitability standard prior to each run. The system suitability standard was prepared by dissolving flavone in mobile phase at a concentration of 60 ng/mL. The flavone solution was then injected six times. The system was deemed operating suitably when the root mean square of the standard deviation expressed as a percentage (%)RSD of the retention time and peak area were less than 2% for all six injections. After a system suitability run, a calibration curve was generated from a minimum of four concentration levels of isoflavones prepared in isopropanol:water (1:1). Linear regression was performed on the calibration curve with a minimum acceptable level of $R^2 > 0.95$. A series of 10–15 unknown samples were then analyzed followed by a single injection of standard. This analysis sequence was followed to the end of the run. The entire analysis was only passed when the %RSD of both
peak area and retention time for the standards throughout the entire run was less than 2%.

A series of in study validation samples were run in each analysis. The in-study validation samples were prepared by spiking blank plasma with the four isoflavones at 5 ng/mL, 50 ng/mL, and 100 ng/mL, then performing the normal sample preparation methodology. A series of six in-study validation samples were prepared at each level. The isoflavones were then quantitated based on the standard calibration curve. All plasma samples from an individual were assayed on the same day in the same assay run.

Plasma samples from the pharmacokinetic study were pretreated with glucuronidase to convert glucuronide conjugated to free isoflavones. Two microliters of plasma was incubated with 10 μl of β-glucuronidase (132,500 U/mL) (Sigma, St. Louis, MO) for 17 hours at 23°C. Deconjugation was demonstrated to be complete after 12 hours. Flavone (7.5 μg) was used as an internal standard in each plasma sample, and the plasma was extracted in ether. The organic phase was evaporated and the residue dissolved in 70 μL of 50% isopropanol. Ten μL of the solution was injected onto the column. Recovery of the isoflavones was corrected for using the peak height ratios method. Recoveries were typically 75%–80% and always greater than 55%.

The high-performance liquid chromatography (HPLC) used a gradient system with a mobile phase of 25%–100% acetonitrile with water and 0.5% trifluoroacetic acid. The system used a waters C18 25-cm symmetry column (Waters, Sydney, Australia). Separation between genistein, daidzein, formononetin, and biochanin and other naturally occurring isoflavones and metabolites in plasma was demonstrated. The intra-assay coefficients of variation at 5 ng/mL were 5.65% (daidzein), 13.73% genistein, 14.21% (formononetin), and 3.92% (biochanin A) at 50 ng/mL they were 1.91%, 2.32%, 0.99%, and 1.93% respectively and at 100ng/ml they were 1.10%, 7.54%, 0.54% and 5.63% respectively. This data falls within the acceptable level of 20% at LOQ and 15% at medium and high levels. The assay was modified from previously published HPLC methods (Setchell et al., 1987; Franke et al., 1995).

Tablets were dissolved in methanol and 10-μL of solution was injected directly onto the column.

Data analysis

The maximum and minimum plasma concentrations (C max and C min) were recorded as the actual maximum and minimum values measured (rather than being estimated by pharmacokinetic modeling.) The T max was recorded as the time after dosing that the C max was measured. The area under the plasma concentration versus time curve (AUC) was calculated using the trapezoidal rule using a standard freeware pharmacokinetic computer program (MOMENT) which was supplied by Andrew McLauchlan (Sydney University). The AUC was estimated from the time of dosing to infinity by adding the last measured plasma concentration divided by the terminal elimination constant (β) to the AUC measured up to that point, if the last plasma concentration measured was above the limit of detection of the assay. The terminal elimination rate constant (β) was calculated as the slope of the log of plasma concentrations versus a time plot where the terminal part of the plot appeared linear. The slope was determined by regression analysis from four or more of the final concentrations selected by visual inspection. The value for β was accepted if the correlation coefficient for the regression analysis (r2) was 0.90 or greater. The terminal elimination half-life (T1/2) was calculated as 0.693/β.

Data are expressed as the mean ± standard error.

RESULTS

Demographic data

The mean age of the subjects was 30.0 ± 8.4 years (range, 22–45 years) and their mean weight was 73.6 ± 13.3 kg (range, 52.0–96.5 kg).

Plasma pharmacokinetic data

The pharmacokinetic parameters for biochanin, formononetin, genistein, and daidzein after long-term isoflavone tablet administration are presented in Table 1 and the plasma concentration versus time profiles are displayed in Figure 1.

Biochanin. The mean plasma concentrations of biochanin as a function of time are presented
in Figure 1. The apparent half-life after long-term administration was, on average, 17.5 hours. Two subjects had high AUCs for biochanin with moderately high levels of genistein, suggesting a high bioavailability of biochanin rather than a reduced ability to convert biochanin to genistein.

Formononetin. Plasma concentrations of formononetin as a function of time are presented in Figure 1. The \( c_{\text{max}} \) was 5.6 ng/ml. The plasma half-life appeared relatively long after long-term administration (22.8 hours), but similar to biochanin, plasma levels of formononetin were low compared to those of genistein and daidzein, and sometimes below the sensitivity of the assay. As with biochanin this probably resulted from the rapid conversion of formononetin to daidzein.

Genistein. Genistein levels measured in plasma reflect genistein absorbed from the isoflavone tablet formulation and genistein derived from the metabolism of biochanin. Plasma concentrations of genistein as a function of time are presented in Figure 1. Predose (after long-term administration), \( c_{\text{max}} \) and AUC values for genistein are presented in Table 1. Plasma levels measured after long-term therapy were much higher than those observed for formononetin, which is metabolised to daidzein.

Daidzein. Plasma concentrations of daidzein as a function of time are presented in Figure 1. Plasma values prior to the administration of the last dose, the \( c_{\text{max}}, \text{AUC} \) and \( T_{1/2} \) for daidzein after long-term administration are presented in Table 1. Plasma levels following chronic therapy were substantially higher than those observed for formononetin, which is metabolised to daidzein.

Urinary isoflavone levels

Urinary isoflavone levels (formononetin, biochanin, daidzein, genistein) during the run-in phase when the patients were required to maintain a low-isoflavone diet were below the limit of detection of the assay.

Adverse events

No adverse events were reported by the subjects during the study.

Isoflavone content of tablets

The isoflavone contents of the six tablets assayed from the batch of Promensil used (mean ± SD) were daidzein, 0.44 ± 0.02 mg; genistein, 1.29 ± 0.06 mg; formononetin, 15.7 ± 0.64 mg; and biochanin, 26.20 ± 1.13 mg (total, 43.63 ± 1.48 mg).

### Table 1. Pharmacokinetic Parameters of Biochanin, Formononetin, Genistein, and Daidzein After Long-Term Administration

<table>
<thead>
<tr>
<th>Isoflavone</th>
<th>Biochanin</th>
<th>Formononetin</th>
<th>Genistein</th>
<th>Daidzein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long-term</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( c_{\text{min}} ) (pre-dose) (ng/mL)</td>
<td>17.3 ± 3.8</td>
<td>&lt;5.0</td>
<td>54.0 ± 14.1</td>
<td>39.9 ± 9.0</td>
</tr>
<tr>
<td>(pre-dose) (ng/mL)</td>
<td>(5.0–43.7)</td>
<td>(&lt;5.0–28.6)</td>
<td>(&lt;5.0–408.2)</td>
<td>(&lt;5.0–116.6)</td>
</tr>
<tr>
<td>( c_{\text{max}} ) (ng/mL)</td>
<td>47.6 ± 5.4</td>
<td>11.2 ± 2.2</td>
<td>114.4 ± 30.0</td>
<td>62.84 ± 9.4</td>
</tr>
<tr>
<td>(18.4–79.6)</td>
<td>(&lt;5.0–35.4)</td>
<td>(42.4–403.6)</td>
<td>(28.8–154.2)</td>
<td></td>
</tr>
<tr>
<td>( T_{\text{max}} ) (h)</td>
<td>3.6 ± 0.8</td>
<td>3.1 ± 0.7</td>
<td>2.8 ± 0.5</td>
<td>4.3 ± 0.9</td>
</tr>
<tr>
<td>(0.5–11.0)</td>
<td>(0.5–9.0)</td>
<td>(0.5–6.0)</td>
<td>(0–11.0)</td>
<td></td>
</tr>
<tr>
<td>( T_{1/2} ) (h)</td>
<td>17.5 ± 3.0</td>
<td>22.9 ± 7.8</td>
<td>12.9 ± 3.1</td>
<td>16.0 ± 3.0</td>
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<tr>
<td>(4.9–37.7)</td>
<td>(4.3–119)</td>
<td>(3.4–39.5)</td>
<td>(6.6–54.2)</td>
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<tr>
<td>AUC (ng.h/mL)</td>
<td>1070 ± 254</td>
<td>240 ± 96</td>
<td>2934 ± 836</td>
<td>1752 ± 682</td>
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<td>(0–infinity)</td>
<td>(232–3112)</td>
<td>(40–1424)</td>
<td>(382–26162)</td>
<td>(492–10,468)</td>
</tr>
</tbody>
</table>

DISCUSSION

This study provides the first detailed description of the long-term pharmacokinetics of isoflavone phytoestrogens in humans. Previous studies have indicated that biochanin is largely converted to genistein and formononetin to
FIG. 1. Plasma levels of biochanin A, formononetin, genistein, and daidzein after long-term administration of a concentrated isoflavone food supplement. The vertical bars represent the standard errors of the mean. ——, chronic biochanin; —, chronic formononetin; ——, chronic daidzein; ——, chronic genistein.
daidzein after oral administration (Knight et al., 1996). This suggests absorption from the upper gastrointestinal tract and rapid demethylation of formononetin and biochanin to daidzein and genistein respectively (Knight et al., 1996; Nilsson, 1961). Rapid and extensive demethylation of biochanin and formononetin is supported by the relatively low plasma levels of these isoflavones after the long-term administration of isoflavone tablets, despite them being the predominant components of the isoflavone tablet. The pharmacokinetic parameters for formononetin derived from the present study should be considered unreliable because of the low plasma values compared to the limit of sensitivity of the assay. However, they have been estimated because we believe that the results would be of interest in illustrating the rapid and extent of metabolism of formononetin to daidzein.

The plasma half-lives of genistein and daidzein after long-term administration were relatively long, in the order of 13–16 hours, although there was considerable variability between subjects. Two earlier studies of the short-term pharmacokinetics of isoflavones (King and Bursill, 1998; Watanabe et al., 1998) included only six and seven men, respectively, and used methods of estimating the plasma half-life that may have led to the reporting of lower values (5–8 hours) than for the terminal elimination half-life calculated by our methods. Specifically, these studies appeared to use all time points after $T_{\text{max}}$ to estimate the half-life. We used the final plasma concentration time points where the log (plasma concentration versus time) satisfied prespecified criteria (a linearity). The former method may yield a composite half-life for distribution and different phases of elimination, while the latter method provides an estimate that is closer to the terminal elimination half-life. However, a recent study (Satchell et al., 2001), which estimated the terminal elimination half-lives of daidzein and genistein after short-term administration of a soy extract, found half-lives of 9.3 and 6.8 hours, respectively. Differences between the results of this study and ours may have reflected differences between short-term and long-term administration or differences between the subjects studied. The relatively long half-lives of these isoflavones when given long-term were supported by the relatively high predose levels prior to administration of the last dose.

The levels of daidzein and genistein during long-term administration were within the range of plasma levels previously reported in patients who traditionally consume a high-isoflavone diet (Adlercreutz et al., 1993; Morton et al., 1997) approximately 30 mg/mL for daidzein and 70 mg/mL or genistein and in subjects undergoing short-term dietary supplementation with soy (Goodyear et al., 1996; Xu et al., 1995). These data support once-daily administration of isoflavone supplements in most people.

It should be emphasized that in a number of cases, particularly for formononetin, the mean plasma concentrations calculated and displayed graphically for some time points were actually lower than the limit of detection of the assay. This was because values less than the limit of detection of the assay were assigned a value of zero. It should also be emphasized that in instances where most plasma concentrations were near the limit of detection of the assay, the estimation of pharmacokinetic parameters would be less accurate than when most plasma concentrations were relatively high.

The plasma levels of the isoflavones (particularly daidzein and genistein, where levels were generally in the range where the assay was reliable) showed significant variability after long-term administration. However, much of the variability in isoflavone levels was contributed to by one subject who had high plasma levels of genistein and a prolonged elimination half-life of genistein after short-term and long-term administration. This may represent genetic polymorphism in the demethylation process of formononetin and biochanin.

Previous studies of the short-term pharmacokinetics of isoflavones after the administration of soy preparation or meals have reported longer times to reach $T_{\text{max}}$ of (6–10 hours) (King and Bursill, 1998; Watanabe et al., 1998). This possibly reflects slower gastrointestinal absorption of conjugated isoflavones present in soy compared to the aglycone form present in
red clover (Setchell et al., 2001). In addition, the administration of genistein and diadzein as a meal rather than as a concentrated extract tablet administered to fasting subjects may influence the rate of absorption.

The observation of clinical importance from this and previous studies is that it is more appropriate to estimate the dosing interval required during long-term therapy from half-lives determined after long-term administration than after short-term administration. Previous studies have also demonstrated accumulation of isoflavones during long-term administration (Izumi et al., 2000; Simons et al., 2000).

We studied the pharmacokinetics of isoflavones in young to middle-aged adult males and females. It is possible that the pharmacokinetics of these compounds vary with age and gender. Our study was not large enough to examine this issue. Further studies are required to examine potential differences between males and females and between young and old persons. Furthermore, it must be emphasized that important pharmacokinetic questions relating to the use of isoflavone tablets as once daily dietary supplements remain unanswered such as the effects of coadministration with food, the pharmacokinetics of higher and lower doses, and possible interactions with drugs such as antibiotics.

In conclusion, this study indicates that the isoflavones genistein and daidzein have sufficiently long plasma half-lives in humans during long-term administration to allow once-daily administration of isoflavone supplements containing biochanin, formononetin, genistein, and daidzein, and that biochanin and formononetin are rapidly and extensively metabolised to genistein and daidzein in humans. The data also suggest that the administration of two 40-mg tablets daily of the isoflavone supplement studied produces plasma levels similar to those found in populations consuming high-isoflavone diets.

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REFERENCES


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