**ANALYSIS OF SOY ISOFLAVONE CONJUGATION IN VITRO AND IN HUMAN BLOOD USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY**

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**ABSTRACT:**

Soybean products containing isoflavones are widely consumed in Western and Asian diets for putative health benefits, but adverse effects are also possible. The conjugated forms of isoflavones present in a soy nutritional supplement (predominately acetyl glucosides) and in blood from two human volunteers after consuming the supplement (7- and 4'-glucuronides and sulfates) were identified using liquid chromatography coupled with electrospray/tandem mass spectrometry. Circulating conjugates of genistein and daidzein were quantified using selective enzymatic hydrolysis and deuterated internal standards for liquid chromatography-electrospray/mass spectrometry. The levels of isoflavone glucuronides were much greater than the corresponding sulfates or aglycones. The substrate activities of genistein and daidzein were evaluated with recombinant human UDP glucuronosyl transferase (UGT) and sulfotransferase (SULT) by using enzyme kinetics. The SULTs 1A1-2, 1E, and 2A1 catalyzed formation of a single genistein sulfate; however, SULTs 1A2-1 and 1A3 had no observed activity. None of the SULTs showed activity with daidzein. Although several UGTs (1A1, 1A4, 1A6, 1A7, 1A9, and 1A10) catalyzed 7- and 4'-glucuronidation of genistein or daidzein, the UGT 1A10 isofor, which is found in human colon but not liver, was found to be specific for genistein. Glucuronidation of only genistein was observed in human colon microsomes, although nearly equal activity was observed for daidzein in human liver and kidney microsomes. These findings suggest a prominent role for glucuronidation of genistein in the intestine concomitant with absorption, although hepatic glucuronidation of absorbed genistein and daidzein aglycones is also likely.

The soy isoflavones consist of genistein, daidzein, and, to a lesser extent, glycitein, and total isoflavones are in the range of 0.1 to 3 mg/g d.wt. The isoflavones in soybeans were previously characterized chromatographically by using liquid chromatography (LC) \(^1\) with UV and mass spectrometric detection (Barnes et al., 1994; Wang and Murphy, 1994a,b). These procedures have been used to identify several glucoside conjugates, primarily the malonyl esters, in addition to trace amounts of the aglycones (Fig. 1).

The biological effects of soy isoflavones are a topic of considerable current interest. A body of scientific evidence suggests possible anticarcinogenic properties of genistein consistent with its ability to inhibit protein tyrosine phosphorylation, DNA topoisomerase, angiogenesis, and cell growth; to induce apoptosis; and to interact with estrogen receptors (reviewed in Barnes, 1997). Reports of estrogenic activity for soy isoflavones (Barnes et al., 1994; Santell et al., 1997) are currently used as a rationale in the lay press to encourage the use of soy supplements as a "natural" means to ameliorate some symptoms of menopause. However, a study of the effects of soy supplementation on postmenopausal women showed only minimal evidence of any estrogenic responses (Baird et al., 1995).

In addition to putative beneficial effects from soy isoflavone consumption, some evidence exists for potential toxicity. A study showed that a soy diet did affect hormonal status and regulation of the menstrual cycle in premenopausal women (Cassidy et al., 1994). The doses of isoflavones used in this study (0.65–0.85 mg/kg) were approximately 7- to 9-fold lower than the doses consumed by infants receiving soy formula (4.5–8.0 mg/kg; Setchell et al., 1997). It is therefore possible that the estrogenic activity of isoflavones from the doses consumed in soy formula could produce adverse effects in developing infants (Clarkson et al., 1995). Furthermore, we recently described potential hazards from the antithyroid properties of soy isoflavones (Divi et al., 1997). Currently there is insufficient information in the scientific literature to establish isoflavone doses that produce either beneficial or toxic responses in humans.

The goals of the present study were to develop analytical methodology to characterize the circulating metabolites of isoflavones in humans and then to characterize the enzymology of such metabolite formation in vitro using expressed recombinant human enzymes. The plasma pharmacokinetics of total genistein and daidzein (aglycones plus conjugates) in humans were previously reported and demonstrated similar bioavailability of these isoflavones when administered as either pure aglycones...
The elimination in urine of daidzein was more prominent than that of genistein (Watanabe et al., 1998). Previous studies in animals isolated and characterized polar isoflavone conjugates (sulfates, glucuronides, and mixed diconjugates) in rat urine and bile (Yasuda et al., 1994, 1996). It was deemed important to characterize the major conjugated metabolites of isoflavones in blood because it is not known whether phase II metabolism would result in altered activity in various endocrine-responsive tissues (i.e., increased, decreased, or equivalent) relative to the aglycones.

In the current study, reversed phase LC and tandem mass spectrometry (MS) were used to characterize the isoflavone glucoside conjugates present in a commercial soy nutritional supplement. Then, LC with electrospray (ES)/tandem MS was used to directly characterize the individual isoflavone glucuronide and sulfate metabolites found in blood from human volunteers who had consumed the commercial soy supplement. In addition, the individual conjugates were quantified in human plasma using selective enzymatic hydrolysis and ES/MS. Finally, the enzymatic basis for formation of isoflavone conjugates was studied in vitro by using recombinant human UDP glucuronosyl transferase (UGT) and sulfotransferase (SULT) isoforms and by using microsomes isolated from several human tissues.

**Materials and Methods**

**Reagents.** Genistin was obtained from Toronto Research Chemicals (Ontario, Canada). Daidzin was obtained from Indofine Chemical Co. (Belle Meade, NJ). Genistin, daidzin, UDP glucuronic acid, bovine hepatic microsomal UGT (1–4 U/g solids), crude glucuronidase/sulfatase from *Helix pomatia* containing 10⁵ U/ml glucuronidase activity plus 5 × 10⁵ U/ml sulfatase activity, and partially purified sulfatase from *Aerobacter aerogenes* containing 2 to 5 U/mg protein were obtained from Sigma Chemical Co. (St. Louis, MO). Deuterated daidzein (6,3',5'-D₃, 95%) and genistein (6,8,3',5'-D₄, 95%) were purchased from Cambridge Isotope Laboratories (Andover, MA). A soy nutritional supplement (Genistin; Source Naturals, Scotts Valley, CA) was obtained from a local health food store. Microsomal recombinant human UGT isoforms, expressed in human lymphoblastoid cells (isoforms 1A1, 1A4, 1A6, and 1A9) were obtained from Gentest (Woburn, MA). Microsomal recombinant human UGT 1A7 and SULT 1A1 expressed in baculovirus-infected Sf9 insect cells were obtained from PanVera Co. (Madison, WI). Filter paper was obtained from Schleicher & Schuell (Keene, NH). The 7- and 4'-glucuronide conjugates of daidzein and genistein were purified from reaction mixtures after incubation of the respective aglycone with bovine UGT as described later and characterized using ES/MS and ¹H-NMR as described previously (Holder et al., 1999).

**Enzymatic Formation of Glucuronides.** Incubations were carried out according to the manufacturers’ recommendations as follows. Reaction mix-
The reactions were initiated by the addition of 0.08 to 3 mM uridine-5'-diphosphate-β-D-glucuronic acid (UDPGA, 0 to 200 μM) and 100 μM MgCl2 in 0.1 M potassium phosphate buffer, pH 8.0, followed by the addition of UDPGA (1 mM). Reactions were linear for at least 2 h. The incubations with SULT 2A1 and 1E were started by the addition of 0.08 to 3 mM uridine-5'-diphosphate-β-D-glucuronic acid (UDPGA, 0 to 200 μM) and 100 μM MgCl2 in 0.1 M potassium phosphate buffer, pH 8.0, followed by the addition of UDPGA (1 mM). Reactions were linear for at least 2 h of incubation (not shown).

Enzymatic Formation of Sulfate Conjugates. Incubations with SULT 1A1, 1A2, 1A3, were preformed according to the manufacturer’s instructions as follows. The enzyme was diluted to 220 (1A1), 180 (1A2), 400 (1A3), 200 (2A1), or 100 (1E) ng/ml in a prechilled solution containing 5 mM phosphate buffer, pH 6.5, 1.5 mg/ml BSA, and 10 mM dithiothreitol. To start the reaction, 100 μl of diluted SULT was mixed with 50 μl of 25 mM phosphate buffer, pH 6.5, containing 25 mM dithiothreitol, 1.28 μM adenosine-3’-phosphate 5’-phosphosulfate, and varying concentrations of daidzein or genistein (0, 50, 100, 200, or 400 μM) in a final volume of 200 μl. The mixtures were incubated at 37°C for 2 h and then analyzed using LC-UV. The reactions were linear for at least 2 h. The incubations with SULT 2A1 and 1E also contained 0.25 mM MgCl2.

HPLC Analysis. Samples were analyzed by LC with UV 260 nm detection using Prodigy ODS-3 (4.6 × 250-mm column, 5-μm particles; Phenomenex Co., Torrance, CA). The solvent system consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Elution was effected using a mobile phase consisting 95% A and 5% B for 3 min followed by a linear gradient to 50% A and 50% B in 15 min followed by isocratic elution at 50% A and 50% B for 5 min. The flow rate was 1.0 ml/min. The detection limits for glucuronide- and sulfate-conjugated genistein and daidzein were about 1 pmol on-column, and concentrations were quantified using the responses for external standards of genistein and daidzein. It was determined that isoflavone glucoside conjugates had extinction coefficients identical with the aglycone (not shown).

LC-MS. Either a Quattro LC triple-quadrupole mass spectrometer (Micro MASS, Altrincham, UK) equipped with a dual orthogonal atmospheric pressure ionization source (Z-spray) or a Micromass Platform single-quadrupole spectrometer equipped with a conventional atmospheric pressure ionization source was used with an ion source temperature of 150°C. For MS measurements, positive ions were acquired in full scan (m/z 100–400 in 1 s cycle time). For MS/MS measurements, a collision cell gas pressure (Ar) of 2–4 × 10−3 mbar was used. Constant neutral loss (m/z 100–600) and precursor ion scans (m/z 300–600) were used to identify and confirm the presence of isoflavone conjugates. Multiple reaction monitoring (MRM) transitions, used to detect low levels of isoflavone aglycones and conjugates, were optimized by directly infusing isoflavone standards to determine collision energies. Because a similar collision energy was required to dissociate glucoside, glucuronide, and sulfate conjugates, conditions optimized for the corresponding glucoside were used. For aglycone analysis using LC-MS, two time functions were used; the first time function (0–7 min) monitored the (M + H)+ ions for daidzein (m/z 255) and daidzein-d3 at a sampling cone-skimmer potential of 30 V, and the second time function (7–12 min) monitored the (M + H)+ ions for genistein (m/z 271) and genistein-d4 (m/z 275) at 30 V.

Sample Preparation and Analysis. A soy supplement labeled Genistein, purchased from a local health food store, was analyzed in triplicate by extraction with methanol, filtration, dilution, and analysis using LC-MS. The tablets (1.5 g) were pulverized in a mortar and then extracted into acidic methanol (20 ml of 10% concentrated HCl in methanol) with stirring for 2 h at room temperature. The use of room temperature acidic extraction procedures was previously shown not to affect the composition of glucoside conjugates (Barnes et al., 1994). Complete hydrolysis of isoflavone conjugates was accomplished by 6-h reflux in 10 volumes (w/v) of 20% concentrated HCl in methanol (v/v). The acid hydrolysis procedure was shown to completely convert glucosides to aglycones by using authentic genistein and daidzein (not shown).

Analysis of Isoflavones in Serum. Isoflavones present in serum were quantified using LC-MS after selective enzymatic hydrolysis to the respective aglycones and the addition of deuterated internal standards (Holder et al., 1999). One male and one female subject consumed 4 soy tablets/day for at least 5 days. Blood was drawn via venipuncture 4.5 h after the final dose, allowed to clot at room temperature for 30 min, and centrifuged at 15,000 rpm for 10 min to pellet the clot, and aliquots were stored at −80°C. Serum was thawed at room temperature and vortex mixed, and equal volumes of serum (75 μl/100 μl) and acetonitrile were placed in 1.5-ml Eppendorf tubes. The samples were then vortex mixed, sonicated for 10 min, and centrifuged at 15,000 rpm for 5 min to pellet precipitated proteins. Aliquots of 100 μl (equivalent to 50 μl of serum) were combined with 1.0 ml of citrate buffer (0.1 M, pH 5.0), and the appropriate enzyme for selective deconjugation was added. The amounts of enzyme that were added were 23 U sulfatase/glucuronidase, 0.84 U partially purified sulfatase, and 3.24 U recombinant glucuronidase. After a 30-min incubation at 37°C, the deuterated internal standard mixture containing 5 pmol each of d3-daidzein and d4-genistin was added to each sample, the isoflavone aglycones were extracted into ethyl acetate (3 × 1 ml), the solvent was removed in a nitrogen stream, and the residue was reconstituted in methanol in an amount equal to one half of the total final volume (50 μl). The remaining volume was made up with water, and the samples were analyzed by LC/ESI/MS using a selected ion monitoring method to detect (M + H)+ ions for genistein, daidzein, and the deuterated isomers. Average recovery of genistein and daidzein throughout a typical overnight sample analysis run, determined versus authentic standards of comparable concentration, was 86 ± 20 and 69 ± 25%, respectively.

Alternatively, blood was obtained from fingerpricks 2 to 3 h after the consumption of the final soy tablets, and aliquots of 50 μl were spotted onto filter paper and dried under ambient conditions. Isoflavones were extracted from cut-out spots by vortex mixing with 3 × 0.5 ml portions of methanol. The extract was evaporated to dryness and dissolved in 50 μl of methanol and then 100 μl of 0.1% formic acid was added, followed by vortex mixing and centrifugation. Aliquots of 20 μl were injected onto the LC column for positive or negative ion LC-MS/MS analysis.

Results

The isoflavone conjugates present in the commercial soy-based Genistein nutritional supplement were characterized using reversed phase LC coupled with tandem MS in the parent ion scanning mode (Fig. 2). In the parent ion scanning experiment, all components that produce a specified daughter ion after collision-induced dissociation in the collision cell and detection at the third quadrupole are identified by synchronous scanning of the first quadrupole. Figure 2 shows mass spectral responses for parent ions that lead to the formation of isoflavone aglycone fragment ions at m/z 271 for genistein, m/z 285 for glycine, and m/z 255 for daidzein. For example, Fig. 2, left, shows all conjugates that produce daidzein on collision-induced dissociation in the chromatogram. The 12.57-min peak corresponds to daidzein acetyl-glucoside (m/z 459), the 10.92-min corresponds to the peak to the malonyl-glucoside (m/z 503), and the 6.96-min peak corresponds to the glucoside (m/z 417). The total ion chromatogram shows the relative responses for these components. In all three cases, the respective acetyl glucoside of genistein, glycine, and daidzein (m/z 475, 489, and 459, respectively) was the major component observed in the total ion chromatogram, followed in relative intensity by the malonyl glucoside (m/z 519, 533, and 503), and the glucoside (m/z 433, 447, and 417).
The total daidzein and genistein content of the tablets was determined after acid hydrolysis at reflux with LC-UV detection (260 nm) to be 8.9 and 1.4 mg/tablet, respectively. This represents, respectively, 84 and 48% of the isoflavone content given on the label. The lack of an authentic standard for glycitein precluded similar quantitative analysis.

Enzymatic synthesis of isoflavone glucuronides was first characterized by using purified enzymes. Incubation of either genistein or daidzein with purified bovine UGT, a highly active commercially available preparation, resulted in the formation of two glucuronide conjugates for each (see Fig. 3, bottom, for a combined chromatogram). These products were characterized by monitoring the constant neutral loss of the glucuronic acid moiety ($m/z$ 176) from positive ions or full-scan MS after LC separation (data not shown). A number of recombinant human UGTs of the 1A class were also tested for the ability to catalyze isoflavone glucuronide formation; two examples are shown in Fig. 3. In Fig. 3, top, UGT 1A6 produced only the 7-glucuronide from both daidzein and genistein. Figure 3, middle, shows that although the 1A1 isoform produced predominantly the 7-glucuronides, a small amount of the 4'-glucuronides was also formed. The daidzein-7-glucuronide produced by the 1A1 isoform showed a slightly different retention time (7.7 versus 7.4 min). The reason for this small but consistent retention time difference is not explicable and is likely due to components of the commercial enzyme preparation; however, the mass spectral properties for both peaks were identical.

Enzyme kinetic data for genistein and the six human recombinant 1A isoforms tested are shown in Table 1. The relative activity, $G_7/G_4$, measured by the ratio of specificity constants ($k_{cat}/K_m$) decreased in the order of $1A10 > 1A9 > 1A1 > 1A6 > 1A7 >> 1A4$. Formation of the 4'-glucuronide, at approximately an order of magnitude lower than the 7-isomer, was observed only for the four most active isoforms. Table 2 shows the analogous results for daidzein. Formation of the 7-glucuronide was also favored by up to an order of magnitude over the 4'-isomer. The activity decreased in the order $1A9 > 1A1 > 1A4 >> 1A10 = 1A6 = 1A7$. For the isoforms with common substrate specificity, 1A9 and 1A1, there was greater substrate activity for daidzein (2- to 3-fold). The $K_m$ values for genistein
The glucuronides, formed by incubation of either genistein or daidzein with either bovine hepatic or recombinant human UGT in the presence of UDPGA, were analyzed after mixing incubations together using LC with constant neutral loss scans of \( m/z \) 176 (loss of glucuronic acid). Alternatively, MRM corresponds to the transitions for \( (M + H)^+ \) to \( (M + H^- \text{glucuronic acid})^- \), respectively. For daidzein, the MRM transition was \( m/z \) 431–255, and for genistein, it was \( m/z \) 447–271. Note the scale expansion by 36-fold to show the trace amount of genistein-4'-glucuronide. TIC, total ion chromatogram.
and daidzein were comparable for the most part and were in the range of 100 to 600 μM. The notable exception was UGT 1A10-catalyzed 7-glucuronidation of genistein ($K_m = 27 \mu M$).

The ability of human tissue microsomes to catalyze the glucuronidation of genistein and daidzein was also measured (Table 3). The activity with genistein decreased in the order kidney > colon > liver; for daidzein, comparable activity was seen in kidney and liver and no activity was observed in colon microsomes. The relative activity for genistein versus daidzein was nearly equal in liver, 2-fold greater in kidney, and much greater in colon microsomes. The specificity constant for 7-glucuronidation versus 4-glucuronidation of genistein or daidzein was of the same order as seen with the recombinant enzymes, except the range was greater for genistein.

A similar experiment was carried out with recombinant human SULTs; these data are shown in Table 4. Data are shown for genistein only because no activity was seen with daidzein under these conditions. The activity decreased in the order 1A1:2 > 2A1 > 1E >> 1A2*1 = 1A3. No evidence indicated the formation of isomeric sulfates as seen for glucuronidation. Unfortunately, insufficient amounts of product were formed to permit further structural characterization using NMR and LC-MS. The predominantly cytosolic nature of human SULTs precluded their study in the microsomal systems.

Serum was analyzed from human volunteers who had consumed a commercial Genistein supplement. Two volunteers consumed two 1000-mg tablets twice daily for about 1 week according to the label instructions, and a control volunteer consumed no tablets. The isoflavones actually consumed were 5.6 mg/day total genistein and 35.6 mg/day total daidzein. Figure 4 shows the positive ion LC-MRM transitions monitored from a blood sample. Responses consistent with two glucuronides each from daidzein, genistein, and glycitein were observed. The retention times for daidzein and daidzein glucuronides matched those observed for authentic 7- and 4'-conjugates (see Fig. 3). Although authentic glycine conjugates were not available for comparison, it is likely that the two compounds with retention times of 7.88 and 10.5 min in the m/z 461→285 transition correspond to the 7- and 4'-glucuronide isomers, respectively. These responses suggest that the glucuronides of genistein are present in lower amounts, although lower sensitivity detection could also explain the results. Similar results were obtained from the analysis of blood from other volunteers, and these results were reproduced in a separate soy supplement dosing experiment with a male and a female volunteer except that weak signals for the aglycone of daidzein were also observed (not shown). A blood sample from a control subject was analyzed similarly (Fig. 4, left). In this case, no responses were observed for any of the glucuronides.

Negative ion MRM transitions showed responses consistent with a single isomeric sulfate conjugate from daidzein, genistein, and glycitein, along with the aglycone for daidzein (Fig. 5). No negative ion signals corresponding to genistein or glycitein aglycones or any isoflavone glucuronide were observed. Similar weak signals for sulfate conjugates were seen in the blood of two other volunteers (not shown). Weak signals consistent with genistein sulfate and daidzein sulfate were observed in a subsequent experiment using LC-MS with selected ion monitoring of the (M − H)$^-$ ion (not shown). The corresponding control blood sample showed none of these responses (Fig. 5, left).

Table 5 shows the serum concentrations in two volunteers of isoflavones present as the aglycones, the sulfates, the glucuronides, and the totals. These values were obtained by using either no enzyme, a partially purified sulfatase, a recombinant glucuronidase, or a mixture of sulfatase and glucuronidase, respectively, for deconjugation. The method validation and performance specifications were previously reported in a study of isoflavones in rat blood (Holder et al., 1999). Their study also showed that the recombinant glucuronidase catalyzed quantitative hydrolysis of authentic glucuronide standards under the conditions cited above. In addition, the purified sulfatase preparation was devoid of glucuronidase activity. The same volunteers consumed the soy supplement on another date, at which time the blood metabolite profiles and determined values were comparable (not shown). In accord with the greater daidzein than genistein content in the soy supplement (~6-fold), the total daidzein in blood exceeded...
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TABLE 3
Human tissue microsome-catalyzed glucuronidation of isoflavones

Microsomes from human tissues were incubated with varying concentrations of genistein or daidzein, and the formation of genistein- and daidzein-7-glucuronides was determined using LC-MS and 1H-NMR to identify genistein-7-glucuronide in bile from human tissues. The detection limits were 0.03 to 0.08 pmol min⁻¹ mg⁻¹ for genistein and daidzein. Measurement of relative activity is based on kcat/Km ratios.

<table>
<thead>
<tr>
<th>Tissue/Isomer</th>
<th>kcat</th>
<th>Km</th>
<th>kcat/Km</th>
<th>G7/D7</th>
<th>7 vs. 4' Glucuronidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney: G7</td>
<td>25</td>
<td>100</td>
<td>0.25</td>
<td>1.8</td>
<td>38</td>
</tr>
<tr>
<td>Liver: G7</td>
<td>13</td>
<td>140</td>
<td>0.093</td>
<td>0.85</td>
<td>2.3</td>
</tr>
<tr>
<td>Colon: G7</td>
<td>56</td>
<td>400</td>
<td>0.14</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td>Kidney: D7</td>
<td>14</td>
<td>100</td>
<td>0.14</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Liver: D7</td>
<td>12</td>
<td>110</td>
<td>0.11</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Colon: D7</td>
<td>&lt;0.08</td>
<td></td>
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</table>

TABLE 4
Human recombinant SULT-catalyzed sulfation of genistein

Various concentrations of genistein and daidzein were incubated with recombinant microsomal SULTs as described in the text, and the kinetics of product formation were determined using LC-UV. The detection limits were 0.03 to 0.08 pmol min⁻¹ mg⁻¹ for genistein and daidzein. Measurement of relative activity is based on kcat/Km ratios.

<table>
<thead>
<tr>
<th>SULT Isoform: Substrate Specificity</th>
<th>kcat</th>
<th>Km</th>
<th>kcat/Km</th>
<th>Relative Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A1+2: PNP thermostable I</td>
<td>10.6</td>
<td>300</td>
<td>0.035</td>
<td>1</td>
</tr>
<tr>
<td>1E: estrone</td>
<td>20</td>
<td>1000</td>
<td>0.020</td>
<td>0.6</td>
</tr>
<tr>
<td>2A1: DHEA</td>
<td>3.8</td>
<td>500</td>
<td>0.0076</td>
<td>0.2</td>
</tr>
<tr>
<td>1A2+1: PNP thermostable II</td>
<td>&lt;0.05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1A3: dopamine thermolabile</td>
<td>&lt;0.05</td>
<td></td>
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</tr>
</tbody>
</table>

total genistein by 1.8- to 3-fold (see Table 5). The glucuronide was the predominant circulating form for both genistein (69–98%) and daidzein (40–62%), with smaller amounts of the aglycone and sulfate detected.

Discussion

Class-specific conjugate analysis of the nutritional supplement by tandem MS (Fig. 2) showed that acetyl glucosides were the predominant form present. This differs significantly with the principal conjugate found in soybeans, the malonyl glucoside derivatives (Barnes et al., 1994; Wang and Murphy, 1994a,b; also see Fig. 1). This is likely the result of heat processing during tablet manufacture because acetyl glucosides arise from decarboxylation of the malonyl esters during heating (Barnes et al., 1994). Analysis of the total genistein and daidzein as aglycones after complete acid hydrolysis showed significantly lower amounts than that described on the label (48–84%). This finding underscores the potential for incorrect dosing based on label-defined composition of what is essentially an unregulated product.

The results shown in Figs. 4 and 5 provide direct evidence for the presence of isoflavone glucuronide and sulfate conjugates in human blood at a time point near steady state after consumption of the soy-based nutritional supplement. Two isomeric glucuronides and a single putative sulfate conjugate were observed for genistein, glycitein, and daidzein. The same pattern of glucuronidation was also observed in rat blood (Holder et al., 1999) and the recombinant UGTs (Fig. 3). Our previous study identified the sites of conjugation using 1H-NMR to be the 7- or 4'-positions (Holder et al., 1999). Other workers used LC-MS and 1H-NMR to identify genistein-7-glucuronide in bile from rats infused with genistein into the duodenum (Sfakianos et al., 1997) and synthetic genistein-4- and 7-sulfate conjugates (Coward et al., 1996). Although the data in Figs. 4 and 5 are qualitative in nature, the relative responses suggest that genistein glucuronides were present in lower amounts, relative to daidzein and glycitein. This conclusion is consistent with the higher amounts of daidzein and glycitein present in the nutritional supplement.

Quantitative information about the isoflavone conjugates in these blood samples was obtained using selective enzymatic hydrolysis and quantification of the respective aglycones using LC-ES/MS (Table 5). In previous analyses of human blood isoflavones, a mixture of deconjugation enzymes (glucuronidase plus sulfatase) was typically used to measure total isoflavones (Coward et al., 1996; Setchell et al., 1997). This procedure can give high sensitivity for total isoflavones but does not permit a distinction between individual conjugates. In the quantitative procedure used in the current study, aliquots of human serum were treated with either no enzyme, purified sulfatase, recombinant glucuronidase, or a mixture of the enzymes. This liberated the corresponding aglycones that were quantified using LC-ES/MS in the presence of deuterated genistein and daidzein.

The total isoflavones in blood, produced by a defined composition soy-based nutritional supplement, are consistent with those observed in previous human studies using other defined soy dosing forms (Adlercreutz et al., 1994; Xu et al., 1994; Coward et al., 1996; Setchell et al., 1997). As previously suggested, isoflavone glucuronides were the predominant conjugates observed in human blood (Adlercreutz et al., 1994; Coward et al., 1996). Similar blood concentrations of total genistein were observed in rats fed genistein aglycone at 25 to 1250 µg/g in the diet, and sulfate conjugates were found to be a minor component (Holder et al., 1999). Indirect evidence for the formation of isoflavone sulfates in humans came from in vitro studies of genistein metabolism in a human MCF-7 transformed breast cell line (Peterson et al., 1996).

A significant discrepancy was observed between the total amount of daidzein generated by enzymatic hydrolysis using a mixture of glucuronidase and sulfatase enzymes and the sum of the two enzyme treatments when used separately (see Table 5). No clear discrepancy was observed for genistein. The most likely explanation comes from the reports of Yasuda et al. (1994, 1996), who isolated and characterized the glucuronide-sulfate diconjugates of daidzein and genistein in rat urine. We tentatively conclude that a diconjugated form of daidzein, observed only after complete enzymatic hydrolysis, accounts for 38 to 56% of the total. Unfortunately, insufficient amounts...
of the putative diconjugate could be isolated from human blood or from incubations of isoflavone glucuronides with sulfotransferases to enable detection using LC-MS/MS in positive or negative ion modes. It is likely that low sensitivity for detection of such negative ions hindered detection. It should be noted that no evidence for such diconjugation was observed in an extensive study of rat blood (Holder et al., 1999).

These findings in vivo are consistent with the survey of kinetic characteristics for commercially available human UGTs and SULTs shown in Tables 1 to 3. The UGT substrate activity for genistein and daidzein was generally comparable, although there were notable differences (see later). The finding that UGT 1A10 is specific for and highly active in glucuronidation of genistein suggests a possible role in metabolic processing in the gut before transport via blood to the liver. A previous study showed that UGT 1A10 is expressed in colon, gastric, and biliary epithelium but not liver (Strassburg et al., 1998). This study also showed that colon contained many UGTs also present in the liver (1A1, 1A3, 1A4, 1A6, and 1A9). The lack of 1A10 activity toward daidzein and the greater activity of 1A1 and 1A9 suggest that daidzein glucuronidation is more likely to occur in the liver rather than the colon. This hypothesis was supported by the finding of much higher genistein glucuronidation activity in the colon versus hepatic microsomes and the reverse pattern for daidzein glucuronidation (see Table 3). These findings are consistent with a previous study that showed genistein infused into rat duodenum was rapidly converted to a glucuronide in the intestine (Sfakianos et al., 1997).

The SULT enzymes tested showed similar $K_m$ values for genistein but greater catalytic activity than seen with the recombinant UGTs, but further quantitative comparisons may not be warranted because of differences between commercial products. However, no detectable activity of any SULT toward daidzein was observed. The participation of other SULT isoforms in vivo is suggested by the evidence of daidzein sulfate (Fig. 5 and Table 5) and possibly a sulfate-glucuronide diconjugate in human blood (see earlier). A recent study showed that daidzein sulfate inhibited sterol sulfatase and steroid SULT activity in hamster liver (Wong and Keung, 1997). This study also pointed out a possible interaction between exogenous isoflavones and the metabolic processing of endogenous hormones or other pharmacologically active compounds.

These results demonstrate the use of tandem MS for the identifi-
Isolavone aglycones and sulfate conjugates were extracted from blood of a human volunteer who had consumed the soy nutritional supplement (right) or from a control subject (left). Extracts were analyzed using LC-MRM by monitoring the following negative ion transitions: daidzein, m/z 253→133; daidzein sulfate, m/z 333→253; glycitein sulfate, m/z 363→283; and genistein sulfate, 349→269.

**TABLE 5**

Quantification of soy isoflavones in human blood

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Aglycone</td>
</tr>
<tr>
<td>Genistein</td>
<td>138±13</td>
<td>N.D.</td>
</tr>
<tr>
<td>Daidzein</td>
<td>671±46</td>
<td>N.D.</td>
</tr>
<tr>
<td>Genistein</td>
<td>383±16</td>
<td>N.D.</td>
</tr>
<tr>
<td>Daidzein</td>
<td>558±14</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

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References


