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Quantitative analysis of the principle soy isoflavones genistein, daidzein and glycitein, and their primary conjugated metabolites in human plasma and urine using reversed-phase high-performance liquid chromatography with ultraviolet detection

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Abstract

Soy isoflavones are becoming of increasing interest as nutritional agents which can be used to combat osteoporosis and hyperlipidemia, and are also being considered as potential cancer chemopreventive compounds. However, prior to their formulation and distribution as therapeutic agents, thorough pharmacokinetic and toxicological assessment needs to be completed in men and women in a variety of health conditions in order to ensure their therapeutic efficacy and safety. At this time, studies of purified soy isoflavones are possible, and are being designed to fully evaluate the pharmacological utility of these preparations. In support of these studies, quantitative analysis of soy isoflavones in biological fluids can be accomplished with a wide variety of methods and analytical instrumentation. However, the relatively ubiquitous presence of high-performance liquid chromatography with ultraviolet detection (HPLC–UV) in most analytical laboratories, the relative ease of its operation, and the lesser expense of this instrumentation as compared to more sophisticated techniques such as liquid chromatography–mass spectrometry, offers some distinct advantages for its use in pharmacokinetic studies. In this manuscript, the development and validation of an HPLC–UV method for the quantitation of the principal soy isoflavones, genistein, daidzein, and glycitein, and their primary metabolites, in human plasma and urine is described. This analytical approach allows for pharmacologically relevant concentrations of the analytes and their principle metabolites to be detected, and has been validated in close agreement with the US Food and Drug Administration's guidelines for the validation of methods to be used in support of pharmacokinetic studies.

Keywords: Genistein; Daidzein; Glycitein

1. Introduction

Genistein (GEN), daidzein (DDZ), and glycitein (GCT), the principal soy isoflavones (Fig. 1), are

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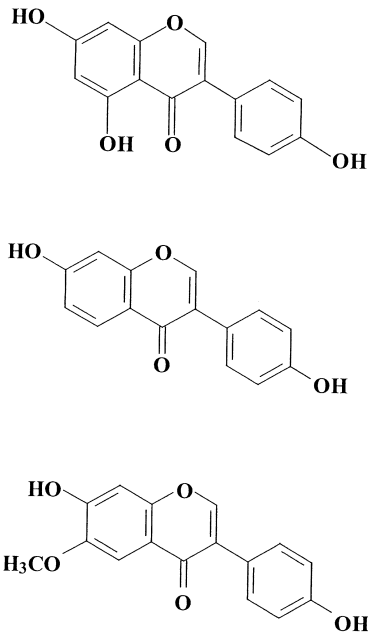

Fig. 1. Structures of the principle soy isoflavones genistein (top), daidzein (middle) and glycitein (bottom).

being considered as potential chemopreventive agents for breast and prostate cancers [1]. In addition to their putative anticarcinogenic effects, soy isoflavones have also been investigated as an antihyperlipidemic agent (reduces the level of low-density lipoprotein [LDL] cholesterol) and as a therapeutic substance to combat osteoporosis. Because of these putative therapeutic and preventative pharmacological properties, consumption of soy or soy isoflavone preparations is increasing, particularly in Western countries where dietary intake of soy-based food is typically 20–50 times less per capita than that of Asians [2,3].

In general, isoflavones are efficiently absorbed from the gut. However, genistein can be glucuronidated in human colon microsomes (UGT 1A10 isoform), suggesting a role for glucuronidation of genistein in the intestine concomitant with absorption [4]. Once absorbed, genistein, daidzein, and glycitein are subject to hepatic glucuronidation at the 7 or 4′-positions, and are also substrates for sulfotransferases. Kidney microsomes are also capable of glucuronidating isoflavones once in circulation [4]. The glucuronides are excreted in the bile where they can be reabsorbed, either before [5] or after cleavage by bacterial gluronidases. The biological activity of the conjugated forms is not well known, but they have been reported to have some therapeutic effects at nutritionally or pharmacologically relevant concentrations, including weak estrogenic effects and activation of natural killer cells [6,7]. Because of their extensive glucuronidation and sulfate conjugation, it is difficult to determine concentrations of free isoflavones; instead, concentrations of total isoflavones or free plus sulfate conjugates in blood and biological matrices are often reported. The low level of free isoflavones, and the formation of biologically active conjugates, has led to experimental difficulty in establishing a relationship between dose and pharmacological effect, identifying the concentration-dependent range of the effect, and identifying doses that produce toxic responses in humans.

A wide variety of analytical techniques have been applied to the quantitation of soy isoflavones in foods and biological fluids, including high-performance liquid chromatography (HPLC) [4,8–25], gas chromatography (GC) [7,26–34], capillary electrophoresis (CE) [35,36], time-resolved fluoroimmunoassay (TR-FIA) [37,38], enzyme-based immunoassays, e.g., enzyme-linked immunosorbent assay (ELISA) [39,40], radioimmunoassay (RIA) [41–43], and other analytical methodologies [44,45]. However, methodological improvements for the quantitative analyses of the free plus conjugated forms of genistein, daidzein, and glycitein in human urine and plasma continue to be needed for clinical trials aimed at defining single and multiple-dose safety, pharmacokinetic, and efficacy profiles. This is particularly important because many of the existing analytical techniques have limited validation data, and therefore their ability to meet the US Food and Drug Administration's (FDA) draft guidelines on the validation of analytical procedures for pharmaceuticals is (in most instances) unknown. In addition, few relatively simple and robust methods have been described for the quantitation of all three of the principle isoflavones in their free as well as conjugated forms, in both plasma and urine. This manuscript reports the development of validated analytical methods using HPLC with UV detection which enable the measurement of (1) the free, non-conjugated molecules, (2) the combined ''free plus the

sulfate-conjugated molecules'', or ''free plus sulfate fraction'', and (3) the total conjugated and free molecules in plasma and urine.

2. Experimental

2.1. Materials

Genistein and daidzein were obtained from Indofine (Somerville, NJ, USA). Glycitein was obtained from Ralston Analytical Labs. (St. Louis, MO, USA) and from Indofine. Formic acid (96%, ACS reagent grade), 4-hydroxybenzophenone (98%), ammonium formate (97%), ammonium acetate, and dimethylformamide (99.8%, ACS reagent grade) were obtained from Aldrich (Milwaukee, WI, USA). Acetic acid (glacial, ACS certified) was obtained from Fisher Scientific (Pittsburgh, PA, USA). Ascorbic acid (crystalline powder, USP-FCC) and 6 *M* hydrochloric acid (reagent grade) were obtained from Baker (Phillipsburg, NJ, USA). β-Glucuronidase/sulfatase from *Helix pomatia* (G-0876) was obtained from Sigma (St. Louis, MO, USA). Solid-phase extraction (SPE) columns (500 mg Bond Elut C_{18} SPE columns) were purchased from Varian (Palo Alto, CA, USA). HPLC-grade solvents (methanol, methyl *tert*.-butyl ether, dichloromethane, dimethylsulfoxide [DMSO]) and UV-grade acetonitrile were purchased from Burdick and Jackson (Muskegon, MI, USA).

Because synthetic or purified conjugates of these isoflavones, either glucuronides or sulfates, are commercially unavailable, all assays relied upon the use of free analytes and optimized experimental methods adopted from the literature. The free plus sulfate fraction for any of the test isoflavones (genistein, daidzein, and glycitein) was defined as free isoflavone after treatment of sample with acid under previously described conditions [7]. Similarly, ''total isoflavone'' (total genistein, total daidzein, and total glycitein) is defined as the total amount of the isoflavone that is in the free form after treatment of the sample with β-glucuronidase and sulfatase enzymes from *Helix pomatia*. For all assays, stock solutions of genistein, daidzein and glycitein were prepared in dimethylsulfoxide. Calibration standards (CalStds) of genistein, daidzein and glycitein were made by adding appropriate amounts of these stock solutions to blank matrix (urine or plasma presumed to be free of the isoflavone analytes) and then serially diluting with additional blank matrix to prepare a range of calibration standards. During the validation studies and the analysis of unknowns, sample matrix controls were similarly prepared, albeit independently of the calibration standards by using independent stock solutions of the isoflavones. During the generation of any isoflavone calibration curve, matrix controls were also analyzed at three concentration levels (low, mid and high range), in either duplicate (for analysis of unknowns) or quintuplicate (for validation of the analytical procedure).

2.2. Free isoflavones in plasma and urine

The internal standard (4-hydroxybenzophenone) was added (200 μl of a 10 μg/ml solution in methyl *tert*.-butyl ether) to 1 ml of plasma in a 10-ml glass centrifuge tube prior to extraction with 6 ml of methyl *tert*.-butyl ether. Using an end-over-end rotating mixer for approximately 30 min facilitated the extraction. The samples were centrifuged at ca. 2000 *g* for ca. 10 min. The methyl *tert*.-butyl ether layer was then transferred to a 12-ml silylated glass culture tube and concentrated to dryness under N_2 at 45–50°C. The residue was dissolved in an appropriate amount (250–4000 μl) of methanol:0.05 *M* ammonium formate, pH 4.0 (20:80, v/v), using a vortex mixer as needed for reconstitution. At least 250 μl of the reconstituted material was then transferred to an appropriate autosampler vial.

2.3. Total isoflavones in plasma and urine

In most instances, calibration standards and controls were made by serial dilutions of a single DMSO stock solution with blank matrix. However, for the preparation of calibration standards and controls in the total urine assay, 5 μl volumes of serially diluted stock solutions of analytes in DMSO were added to each urine. This use of DMSO was thought to help maintain the analytes in solution when added to the urine but was apparently not needed when added to the plasma matrix. Because of the relatively high level of DMSO in each spiked urine sample, the same amount of DMSO was added into subject

samples in order to ensure experimental control and reproducibility. Aliquots (250 μl) of plasma or urine were then transferred to a 10-ml glass disposable centrifuge tube and treated with 0.5 ml of a mixture of β-glucuronidase/sulfatase from *Helix pomatia* to hydrolyze glucuronide and sulfate conjugates of genistein, daidzein, and glycitein. The enzyme mixture was made up freshly and contained 0.15 g ascorbic acid in 10 ml of 0.2 *M* acetate buffer, pH 4.0, and 500 μl of β-glucuronidase/sulfatase from *Helix pomatia* (Sigma G-0876). To allow for complete hydrolysis in the plasma samples, 0.75 ml of 0.2 *M* ammonium acetate buffer was added (this was not required for urine samples), and the tubes were capped and heated overnight (15–18 h) at ca. 37°C. The tubes were removed from the heat and allowed to cool to room temperature. Extraction of the plasma sample was performed with 6 ml of methyl *tert*.-butyl ether after addition of 200 μl of a 50 μg/ml solution of 4-hydroxybenzophenone in methyl *tert*.-butyl ether (internal standard solution) to each tube, as described previously. The residue was dissolved in an appropriate amount (250–4000 μl) of methanol–0.05 *M* ammonium formate, pH 4.0 (20:80, v/v), including mixing on a vortex mixer as needed for reconstitution. At least ca. 250 μl of the reconstituted material was transferred to an appropriate autosampler vial for HPLC analysis.

2.4. Free plus sulfate conjugates of isoflavones in plasma

Initially, 20 μl of a 500 μg/ml solution of internal standard (4-hydroxybenzophenone) was added to 1-ml plasma samples in a glass disposable screw-cap. After mixing on a vortex mixer, the sample was loaded onto a 500-mg Bond Elut C_{18} SPE column that had been pre-wet with 3 ml of HPLC-grade methanol followed by 3 ml of deionized, distilled water. The SPE column was rinsed with 6 ml of deionized, distilled water prior to elution of the analytes with 4 ml methanol–deionized, distilled water (90:10, v/v). The eluent was collected in a 15-ml glass disposable centrifuge tube and concentrated to dryness in a Speed-Vac concentration system (Savant Instruments, Holbrook, NY, USA). To each residue was added, in order listed, 300 μl dimethylformamide, 5 μl 6 *M* HCl and 3 ml dichloromethane, in order to effect solvolysis. The tubes were capped and heated overnight (15–18 h) at ca. 37°C. After incubation, the samples were removed from the heater block and 1.0 ml of deionized, distilled water was added. Extraction of the acid hydrolyzed plasma was done with 6 ml of methyl *tert*.-butyl ether as described previously. The residue was dissolved in an appropriate amount (250–4000 μl) of methanol:0.05 *M* ammonium formate, pH 4.0 (20:80, v/v), including mixing on a vortex mixer as needed for reconstitution. For HPLC analysis, at least ca. 250 μl of the reconstituted material was transferred to an appropriate autosampler vial.

2.5. Free plus sulfate conjugates of isoflavones in urine

A 1-ml volume of urine in a 10-ml glass disposable centrifuge tube was frozen and concentrated to dryness by lyophilization. To this residue was added (in the order listed), 300 μl dimethylformamide, 5 μl 6 *M* HCl, and 3 ml dichloromethane. The tubes were tightly capped and heated overnight (15–18 h) at ca. 37°C in order to effect solvolysis. After the incubation, the samples were placed under N_2 at ca. 35°C for 45 min to remove the dichloromethane. A 1-ml volume of deionized, distilled water was then added to the remaining incubation mixture, and the samples were evaporated under N_2 for an additional 30 min at a temperature of 45–50°C to remove the dimethylformamide. After solvolysis, 200 μl of a 50 μg/ml solution of 4-hydroxybenzophenone in methyl *tert*.-butyl ether (internal standard solution) was added to each tube and mixed well with a vortex mixer. An additional 6 ml of methyl *tert*.-butyl ether was then added to each tube and the sample extracted as described previously. The extract was concentrated to dryness under N_2 at 45–50°C. The final residue was dissolved in an appropriate amount (250–4000 μl) of methanol–0.05 *M* ammonium formate, pH 4.0 (20:80, v/v), including mixing on a vortex mixer as needed for reconstitution. At least ca. 250 μl of the reconstituted material was then transferred to an appropriate autosampler vial for HPLC analysis.

2.6. HPLC analysis

A HPLC system consisting of an Eppendorf CH-30 column heater, a Waters (Milford, MA, USA) 717

Plus autosampler, Waters series 600 pumps and pump controller, a Waters 2487 UV detector, and a data collection system running Waters' Millennium 32 software was used for this analysis. The HPLC system and software was validated with respect to the accuracy and reliability of generated data and computed results. Calibration curves for the analytes were constructed relating the concentration of each analyte in the standard to the ratio of their peak's height to the corresponding peak height of 4-hydroxybenzophenone. To determine the concentration of each analyte in matrix controls and in experimental unknowns, the ratios of the same parameters from the analyzed samples were determined and compared with the ratios generated in the calibration. Since matrix components were liberated with glucuronidase and glucuronidase/sulfatase treatments that were not present in the free isoflavone assays, the HPLC methods had to be modified for each assay in order to achieve the greatest sensitivity and specificity.

2.6.1. HPLC analysis of plasma extracts for free and free plus sulfate conjugates of isoflavones

A Luna Phenyl-Hexyl (Phenomenex, Torrance, CA, USA) HPLC column (150×4.6 mm, 5 μm; Waters) with Zorbax Eclipse XDB-Phenyl guard column (12.5×4.6 mm, 5 μm; Mac-Mod, Chadds Ford, PA, USA) was used with the following mobile phase and gradient program to effect separation:

Mobile phase A: 0.05 *M* ammonium formate, pH 4.0. Mobile phase B: HPLC-grade methanol–HPLC-grade acetonitrile (50:50, v/v).

Gradient: isocratic at 10% B for 0.5 min. Linear gradient to 30% B over 0.5 min. Linear gradient to 50% B for 10 min. Linear gradient to 80% B over 1 min. Isocratic at 80% B for 6 min.

The initial HPLC conditions were used to concentrate the analytes on the head of the column. After a brief period under these conditions, a rapid gradient was used to increase the elution strength of the mobile phase. This allowed some matrix components to elute, while retaining the analytes on the column. After this rapid gradient, a slower gradient was used to allow the desired separation to occur. After the analytes eluted, the elution strength was rapidly increased again to elute all remaining matrix components. The flow-rate was maintained at 2 ml/min in order to speed analysis time and column re-equilibration, the column was maintained at 40°C, and the analytes were detected by UV absorption at 259 nm.

2.6.2. HPLC analysis of plasma extracts for total isoflavones

The same HPLC system was used for the analysis of total isoflavones as was used for the analysis of free and free plus sulfate conjugates of isoflavones with the exception of the HPLC gradient. The modified gradient conditions were as follows:

From 100% A, a linear gradient to 40% B over 0.5 min. Isocratic at 40% B for 11 min. Linear gradient to 80% B over 1 min. Isocratic at 80% B for 3 min.

2.6.3. HPLC analysis of urine extracts

For all three assays of isoflavones in urine (i.e., free, free plus sulfate, and total isoflavones) a Zorbax Eclipse XDB-Phenyl HPLC column (75×4.6 mm, 3.5 μm) with Zorbax Eclipse XDB-Phenyl Guard column (12.5×4.6 mm, 5 μm) from Mac-Mod was used. The following mobile phase and gradient conditions were used:

Mobile phase A: 0.05 *M* ammonium formate, pH 4.0. Mobile phase B: HPLC-grade methanol.

Gradient: isocratic at 10% B for 0.5 min. Linear gradient to 45% B over 0.5 min. Isocratic at 45% B for 9.5 min. Linear gradient to 80% B over 1 min. Isocratic at 80% B for 3 min.

The flow rate was maintained at 2 ml/min, the column was maintained at 40°C, and the analytes were detected by UV absorption at 259 nm.

2.7. Validation procedures

2.7.1. Determination of extraction recovery

Extraction recovery was determined using the extraction and analysis of three control levels of analyte, at high, mid, and low levels of the calibration range, for each particular assay. Each control level was prepared with an *n* of 10. After extraction and analysis, the samples were analyzed and the peak heights recorded for the analytes compared to ''recovery standards''. These recovery standards were formulated in HPLC mobile phase to produce a final concentration equal to that produced through the extraction of biological standards, assuming 100% recovery. The average peak heights for biological extracts were compared to the average peak

heights obtained with the recovery standards in order to provide an estimate of the extraction recovery.

2.7.2. Determination of linearity, dynamic range, accuracy, precision, and limit of quantitation

Stock solutions were prepared using DMSO to dissolve each analyte. These stock solutions were then serially diluted to form a set of calibration standards covering the desired range of each analytical assay. To determine assay linearity and range, each calibration standard was assayed in duplicate. The standard curves were then fitted by least squares linear regression analysis using a $1/x$ weighting.

Quality control solutions were prepared independently from calibration standards using DMSO to dissolve each analyte. The stock solutions were then diluted to various concentrations within the calibration curve range, including one high concentration, one mid concentration, and one low concentration at the desired limit of quantitation. These samples were prepared in quintuplicate for determination of assay accuracy, precision, and limit of quantitation. Our level of acceptance was set at 20% for both accuracy and precision at all levels of the control samples, including the limit of quantitation. Our definition of the limit of quantitation was therefore the lowest level of each analyte that could be determined with $\pm 20\%$ accuracy and precision when determined with an $n=5$. The method validation of the entire assay required all three levels of quintuplicate controls to meet these $\pm 20\%$ acceptance criteria. During the analysis of a sample set containing subject samples, three levels of controls (at high, medium, and low levels of the calibration curve) were run in duplicate. Acceptance of an analytical run was then dependent upon four of the six controls passing the 20% cut-off (with the additional acceptance criteria that the two failing controls could not be at the same concentration level).

2.7.3. Determination of freeze–thaw stability

To demonstrate freeze–thaw stability, five replicate samples of each of three control concentrations were prepared and then subjected to four freeze–thaw cycles prior to analysis. Since the freeze–thaw samples were typically extracted and analyzed with the accuracy and precision samples, the same calibration curves described in the preceding paragraph were used for their quantitation. If this was not the case, then the system was recalibrated prior to analysis. Acceptance criteria were based on the mean determined value for each concentration set being within 20% of the nominal value and the relative standard deviation (RSD) for each concentration set not exceeding 20%.

2.7.4. Determination of long-term stability

Long-term stability was determined using the quality control samples at high, mid, and low levels of analytes with respect to the calibration range of the particular assay. These control samples were prepared in quintuplicate and stored for over 90 days at -20°C. At the time that the samples were removed from storage for analysis, a new calibration curve was prepared and analyzed along with the stability samples. Acceptance criteria for long-term storage stability of the free analytes, the biological matrices, and the assay procedures in general were dependent upon the quintuplicate control samples (on average) assaying to within $\pm 20\%$ with respect to their accuracy and precision.

2.7.5. Determination of assay specificity

Specificity of the method was assessed by analyzing plasma from six independent sources (obtained from volunteers who had agreed to avoid eating food substances containing genistein, daidzein, and glycitein). Each plasma sample was analyzed in triplicate, with two samples spiked with internal standard for quantitation of any interfering peaks at the retention times of analytes, and one sample that was not spiked with internal standard in order to determine if interfering peaks were present at the retention time of the internal standard. Any interference peaks detected in these samples with the relative retention ratio (as compared to the internal standard) within the range of the relative retention range determined for each analyte during the analysis of calibration curve and control samples was recorded. The ratio of the ''interference'' peak height to the internal standard peak height was calculated and compared to the ratio for the actual analyte in the determination of limit of quantitation (LOQ) samples. In this way the amount of the interference peak could be calculated as a percentage of the assay's LOQ value.

Table 1
Extraction recovery of free isoflavones in plasma and urine

	Low control, % recovery				Mid control, % recovery				High control, % recovery			
	DDZ	GCT	I.S.	GEN	DDZ	GCT	I.S.	GEN	DDZ	GCT	I.S.	GEN
Urine	99 (4)	98 (4)	105 (6)	92 (4)	100 (2)	98 (2)	106 (5)	94 (2)	96 (2)	93 (2)	108 (4)	90 (2)
Plasma	55 (4)	44 (3)	44 (5)	41 (4)	52 (4)	37 (3)	42 (4)	42 (3)	51 (6)	40 (4)	44 (5)	46 (6)

Data reported as mean (standard deviation); $n=10$, with the exception of the high control for free urine, where $n=8$.

3. Results

3.1. Recovery studies

The extraction recovery (Table 1) of isoflavones in urine was quite high, with genistein tending to have the lowest recovery (ranging from 90 to 94%). Daidzein, glycitein, and the internal standard appeared to be completely extracted using this procedure. In contrast to the extraction procedure for urine, plasma recovery of all three analytes and the internal standard were considerably lower, ranging between ca. 40 and 60%. The variability of the extraction recovery was greater in the plasma procedure as well, but was typically below 10% of the mean. The recovery did not appear to be dependent upon analyte concentration. The recovery from plasma might be increased with repeated extractions, but this was not attempted in our experimentation.

3.2. Chromatography

When analyzed by HPLC, both plasma and urine extracts yielded peaks for the analytes that were well separated from each other (Fig. 2). The chromatograms recorded for blank matrix extracts are devoid of interference peaks at the retention times for the analytes. Thus, the analyte peaks are also separated from significant interference from co-extracted components from the matrices. Other buffer systems

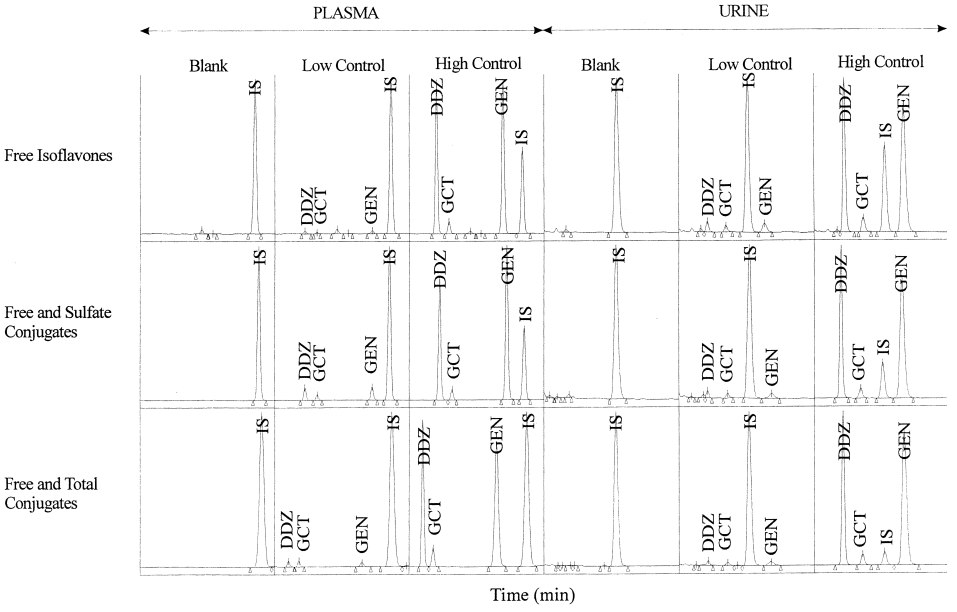

Fig. 2. Analysis of plasma (left panels) and urine (right panels) samples for free (top), free plus sulfate conjugates (middle) and free plus total conjugates (bottom). For each form of the isoflavone, samples were analyzed with no addition of analytes, and after addition of two concnetrations of analytes, one at a low level and one at a relatively high level of each analyte. The peaks for the analytes (when detected) are labeled in each chromatogram.

were tested, including ammonium acetate; however, ammonium formate appeared to afford the best peak shape and resolution. A pH 4.0 buffer also appeared to be optimal for peak shape and resolution. By comparing the chromatograms in Fig. 2, one can conclude that although the extraction procedures for the different forms of the analytes and the biological matrices varied, producing different extracts for HPLC analysis, the chromatographs typically revealed good resolution and peak shape. It is important to note that all of the chromatograms were produced from biological matrices that were spiked with free isoflavones, and then treated according to the various procedures, some of which included solvolysis or enzyme hydrolysis. Thus, only the generation of endogenous substances that are liberated from the matrix upon solvolysis, or treatment with β-glucuronidase and sulfatase enzymes, during our experimental procedures was controlled for. Additional studies were performed with plasma obtained from subjects ingesting soy isoflavones, in order to ensure that the enzyme hydrolysis procedures would go to completion under the conditions used (data not shown).

3.3. Free isoflavones in plasma

Calibration curves for free genistein, daidzein, and glycitein were generated by analysis of fortified plasma extracts. The standards ranged in concentration from ca. 2 to 2000 ng/ml for genistein and daidzein (11 standards in duplicate), and from 2 to 220 ng/ml for glycitein (seven standards in duplicate). The r^2 values of the calibration curves for genistein, daidzein, and glycitein were 0.995, 0.996 and 0.998, respectively. The accuracy and precision data for this analysis are provided in Table 2. The mean determined value for each analyte's set of control plasma samples was within 20% of the nominal value and the RSD around this mean value did not exceed 20%, thereby meeting the acceptance criteria for both precision and accuracy. Analysis of the LOQ data resulted in an LOQ of 2.11±0.22 ng/ml (RSD=4.4%) for genistein, with an accuracy

Table 2
Plasma assay validation

	Determined (ng/ml)								
	Low control			Mid control			High control		
	DDZ	GCT	GEN	DDZ	GCT	GEN	DDZ	GCT	GEN
Data accuracy and precision – free isoflavones in plasma									
Mean	1.76	2.55	2.11	129	92.0	118	1321	179	1252
SD	0.069	0.095	0.093	11.3	5.04	8.58	48.4	4.04	76.1
Precision (%)	3.9	3.7	4.4	8.8	5.5	7.3	3.7	2.3	6.1
Concentration (ng/ml)	2.18	2.48	2.14	131	89.5	129	1306	179	1283
Accuracy (%)	81	103	99	99	103	91	101	100	98
Free isoflavones in plasma after four freeze–thaw cycles									
Mean	15.0	11.7	14.0	148	107.6	135	1411	213	1296
SD	0.552	2.38	0.187	2.74	13.6	2.59	88.3	37.1	64.0
Precision (%)	3.7	20	1.3	1.9	13	1.9	6.3	17	4.9
Concentration (ng/ml)	13.1	9.95	12.9	131	89.5	129	1306	179	1283
Accuracy (%)	115	118	109	113	120	104	108	119	101
Free isoflavones in plasma after storage for 188 days									
Mean	13.6	9.6	12.5	139	74.0	122	1364	143	1248
SD	0.503	0.343	0.331	1.14	0.868	2.55	28.6	3.36	22.7
Precision (%)	3.7	3.6	2.7	0.8	1.2	2.1	2.1	2.3	1.8
Concentration (ng/ml)	12.9	10.9	12.7	129	87.5	127	1292	175	1272
Accuracy (%)	105	88	98	101	85	96	106	82	98

of 99%. The LOQ for daidzein was determined to be 1.76±0.07 ng/ml (RSD=3.9%), with an accuracy of 81%. Finally, the LOQ for glycitein was determined to be 2.55±0.10 ng/ml (RSD=3.7%), with an accuracy of 103%.

Acceptance specifications for both accuracy and precision were also met in samples that had undergone four freeze–thaw cycles (see Table 2). During the analysis of both the accuracy and precision samples and the freeze–thaw samples, matrix control samples (blank or unspiked plasma samples) were also analyzed. In these samples no interference peaks were detected with retention times of any of the analytes that were calculated to be higher than the LOQ for each analyte.

Long-term storage stability of the analytes in this assay was determined in a separate study where five replicate samples of each of three concentrations were prepared and then stored at ca. −20°C for 188 days prior to analysis. As shown in Table 2, each analyte was found to be within 20% of the nominal concentration after the storage period. Furthermore, the precision of the assay was within 20% at each control level. Finally, a matrix control sample (blank or unspiked plasma sample) was also analyzed. In this sample no interference peaks were detected with retention times of any of the analytes. All of these control experiments demonstrate that storage at −20°C for 188 days does not compromise the stability of the analytes or the performance of the analytical methodology.

Specificity of the method was ensured by the observation that no interference peaks for genistein, daidzein, or glycitein were detected during the analysis of the samples (data not shown), indicating that the specificity of the method was acceptable.

3.4. Free isoflavones in urine

Calibration curves for genistein, daidzein, and glycitein were generated by analysis of extracts of spiked urines. The standards ranged in concentration from ca. 10 to 2000 ng/ml for genistein and daidzein (eight standards in duplicate), and from ca. 10 to 200 ng/ml for glycitein. The r^2 values of the calibration curves for genistein, daidzein, and glycitein were each 0.999. Precision and accuracy of the assay was determined with five replicate urine control samples that were analyzed at each of three concentration levels of genistein, daidzein, and glycitein (at high, mid, and LOQ levels of the calibration curve). The accuracy and precision data for this assay are provided in Table 3. The mean determined value for each analyte's set of control urine samples was within 20% of the nominal value and the RSD around this mean value did not exceed 20%, thereby meeting the acceptance criteria for both precision and accuracy. Analysis of the LOQ samples resulted in an LOQ of 22.3±0.71 ng/ml (RSD=3.1%) for genistein, with an accuracy of 99%. The LOQ for daidzein was determined to be 22.7±0.67 ng/ml (RSD=3.0%), with an accuracy of 101%. The LOQ for glycitein was determined to be 22.5±0.37 ng/ml (RSD=1.6%), with an accuracy of 104%.

Acceptance specifications for both accuracy and precision were met in samples that had undergone four freeze–thaw cycles (Table 3). During the analysis of both the accuracy and precision samples and the freeze–thaw samples, matrix control samples (blank or unspiked urine samples) were also analyzed. In these samples no interference peaks were detected with retention times of any of the analytes. Long-term stability of the analytes in the free urine assay was determined using five replicate samples of each of three concentrations that were prepared and then stored at ca. −20°C for 253 days prior to analysis. As shown in Table 3, each analyte was quantitated to within 20% of the nominal concentration after the storage period. Furthermore, the RSD at each control level was also within 20%. Finally, a matrix control sample (blank or unspiked urine sample) was also analyzed. In this sample no interference peaks were detected with retention times of any of the analytes. These control studies demonstrate that storage at −20°C for 253 days does not compromise the stability of the analytes or the performance of the analytical methodology.

In the specificity study for this particular assay, some interference peaks were detected at the retention times of daidzein and glycitein (data not shown). The interference peak at the retention time of glycitein did not exceed the LOQ value, and therefore, the system's specificity criteria were met

Table 3
Urine assay validation data

	Determined (ng/ml)								
	Low control			Mid control			High control		
	DDZ	GCT	GEN	DDZ	GCT	GEN	DDZ	GCT	GEN
Accuracy and precision – free isoflavones in urine									
Mean	22.7	22.5	22.3	118	87.6	125	1183	174	1276
SD	0.672	0.361	0.712	0.548	1.07	3.05	16.4	2.17	33.6
Precision (%)	3.0	1.6	3.1	0.5	1.2	2.4	1.4	1.2	2.6
Concentration (ng/ml)	22.6	21.6	23.0	122	82.5	124	1218	165	1242
Accuracy (%)	101	104	99	97	106	100	97	106	103
Free isoflavones in urine after four freeze–thaw cycles									
Mean	48.1	42.1	52.3	119	88.2	127	1186	172	1302
SD	1.04	1.15	0.987	1.30	0.856	4.47	9.78	4.76	53.3
Precision (%)	2.2	2.7	1.9	1.1	1.0	3.5	0.8	2.8	4.1
Concentration (ng/ml)	50.5	41.2	51.5	122	82.5	124	1218	165	1242
Accuracy (%)	95	102	102	98	107	102	97	104	105
Free isoflavones in urine after storage for 253 days									
Mean	41.2	38.6	49.3	119	82.1	116	1208	169	1158
SD	132	0.652	1.91	2.19	1.26	265	18.8	1.64	9.12
Precision (%)	2.8	1.7	3.8	1.8	1.5	2.3	1.6	1.0	0.8
Concentration (ng/ml)	50.0	41.1	49.3	120	82.0	119	1199	165	1184
Accuracy (%)	94	94	101	100	100	97	101	102	98

for genistein and glycitein. Unfortunately two of the specificity samples had peak height ratios (analyte to internal standard) for interferences with retention time ratios very close to (although not within) the retention time ratio range (analyte retention time to internal standard retention time) obtained for samples actually fortified with daidzein. The large peak heights of these interferences exceeded the peak height ratio obtained for samples fortified with the analytes at LOQ concentrations by 300% in one sample, and by 600% in another specificity sample.

In order to overcome the problem with assay specificity for free daidzein in urine, an alternative HPLC system was developed which was intended to be used if any levels of daidzein were detected that exceeded the LOQ. Because of the need for a modification of the HPLC system, the entire validation needed to be repeated for this analyte. Using this modified method, we found that our acceptance criteria for linearity, precision and accuracy, freeze–thaw stability, and storage stability could all be met (data not shown). In five of the six specificity samples, no interference was detected at the retention time of daidzein. However, one of the six specificity samples still had an interference peak that exceeded the LOQ value (229% of LOQ), indicating that the levels of daidzein in subjects' pre-dose void needs to be monitored closely.

3.5. Conjugates of isoflavones in plasma and urine

Rather than provide all of the validation data for each of these assays, we have summarized the results for all of the assays in Table 4. The accuracy and precision criteria of 20% (which is the recommended acceptance level for the limit of quantitation given in the US FDA's 1998 Draft Guidance to Industry: Bioanalytical Methods Validation for Human Studies) was met in most instances. The specific exceptions encountered were: a 21% level of precision for the LOQ in the free+sulfate glycitein in urine assay, a 22% level of precision and 77% accuracy for the high control in the 147 day stability study for free+

Table 4
Summary of method performance characterizations

	LOQ (μmol/l)			Precision (RSD, % of LOQ)			LOQ accuracy (% of nominal)			Range (μmol/l)						Average precision (RSD, %)[a]			Average accuracy (% of nominal)[a]			Stability tests (days)
	GEN	DDZ	GCT	GEN	DDZ	GCT	GEN	DDZ	GCT	GEN		DDZ		GCT		GEN	DDZ	GCT	GEN	DDZ	GCT	
Plasma																						
Free	0.008	0.007	0.009	4.4	3.9	3.7	99	81	103	0.008	7.40	0.007	7.87	0.009	0.774	3.9	4.1	8.4	99	102	101	188
F+S	0.374	0.429	0.387	16.7	3.1	5.1	83	91	105	0.374	37.0	0.429	36.6	0.387	3.52	7.4	3.3	3.8	93	96	98	311
Total	0.426	0.448	0.760	3.2	10	2.8	91	90	98	0.426	77.7	0.448	86.6	0.760	7.04	2.3	3.5	2.5	98	100	98	185
Urine																						
Free	0.084	0.089	0.079	3.1	3	1.6	99	101	104	0.085	7.40	0.090	7.87	0.081	0.739	2.7	1.7	1.6	101	98	103	253
F+S	0.418	0.417	0.524	10	3.1	21	102	91	109	0.418	71.8	0.417	77.1	0.524	7.04	3.3	2.5	2.8	107	102	106	285
Total	0.585	0.641	0.344	1.2	1.9	0.9	83	87	98	0.585	718	0.641	744	0.345	70.4	6.8	7.1	5.9	98	99	104	147

[a] Average of all values (≥9) for low, mid and high control levels determined during method validation testing procedures (precision and accuracy, freeze–thaw, storage stability).

total conjugates of daidzein in urine, and a 78% accuracy in the 147 day stability study for free+total conjugates of genistein in urine. Table 4 shows that the average accuracy was within 10% or less of the nominal value. The average precision of all assays also did not exceed 10%.

4. Discussion

The relatively high extraction recovery of the analytes in urine is consistent with the results obtained by other investigators using a similar method [22] and is comparable to that obtained with alternative procedures including column chromatography [17,46,47]. However, our extraction recovery from plasma was considerably lower than that reported by Supko and Phillips [22]. Our average plasma extraction recovery was approximately 50%, whereas Supko and Phillips [22] reported over 90% recovery using a similar liquid–liquid extraction technique. The primary difference between our methods is the larger volumes of plasma we are using to obtain lower limits of quantitation. We used 1 ml of plasma and 6 ml of *tert*.-butyl methyl ether (TBME), whereas Supko and Phillips [22] used 50 μl of plasma and 3 ml of *tert*.-butyl methyl ether. The 10-fold difference in plasma to solvent ratio is an obvious difference in the procedures and might result in our lower extraction recovery. Despite the twofold lower extraction recovery, our procedures were reproducible and reliable and afforded limits of quantitation of approximately 2 ng/ml for all three analytes as compared to the 10-fold higher cut-off of 20 ng/ml reported for genistein with the method of Supko and Phillips [22]. An important difference in the analytical assays described here and many of the other available quantitative methods for isoflavones is the inclusion of complete validation testing for all three analytes in both urine and plasma. Thus, the number of analytes and the extent of the characterization of these methods represents a significant addition to the available analytical literature for soy isoflavones. In most of our assays the linearity, accuracy, precision, and stability of the analytes in the biological matrices were readily validated. Our acceptance criteria of ±20% for accuracy and precision is considerably greater than the acceptable precision levels in other types of analytical methods (such as formulation analyses, etc.), which reflects the increased complexity of biological matrices. However, this level of acceptance is recommended at the level of the LOQ by the US FDA's 1998 Draft Guidance to Industry: Bioanalytical Methods Validation for Human Studies. Above the LOQ, the FDA suggests that acceptance criteria be narrowed further to ±15%. This is similar to method validation criteria that have been proposed previously [48]. However, when endogenous substances or dietary substances such as soy isoflavones are the analytes of interest, method validation can become even more problematic due to the variability in individuals' physiology and food intake. Furthermore, our acceptance criteria involved 18 separate method validations (three analytes, each in three circulating and excretable forms, and in two biological matrices), adding considerably to the demands of method validation. Thus, we set our acceptance criteria at ±20% and found this level to be obtainable in most instances. The few exceptions occurred where either the accuracy or precision exceeded 20% but did not exceed 25%.

In general, lignans and isoflavonoids are much more stable than many estrogens [4], and our experience has been that these compounds are quite stable at −20°C and do not appear to decompose rapidly even when left at room temperature over a short period of time. Perhaps the most problematic aspect of the validation of isoflavone assays according to the FDA recommended guidelines is the assay specificity requirements. It only takes one of six biological samples containing levels of any one of three analytes exceeding the LOQ for the assay to fail the criteria for specificity. Because of this, the issue of specificity in an analytical assay of a dietary component needs careful consideration, particularly given the ubiquitous nature of soy isoflavones in our diets (e.g., fast foods and prepared food items). Specifically, control subjects must have a restricted diet or must be relied upon to be extremely careful in their intake of food items prior to providing biological fluids for analyses.

Another difficulty in validation studies with isoflavonoids involves the lack of a readily available supply of glucuronide and sulfate conjugates of the analytes to use as standards, resulting in our having

to use free substances. The significance of the validation data for conjugated materials when free isoflavone are used includes demonstrating that the analytes of interest are recoverable after the two treatments. In addition, these studies serve to establish that no endogenous materials are released by the gluronidase/sulfatase enzymes or by the solvolysis procedures that interfere with assay linearity, accuracy, precision, stability, or specificity. Other laboratories have attempted to utilize standards synthesized with biological enzyme systems [4] or have purified them from bile [20]. While this approach has been used in non-validated assays (with regard to FDA guidelines), in our case, complete validation would have required standards for the glucuronide, sulfate, and sulfoglucuronide conjugates for each of the three analytes, and in the various positional isomers that can occur with each particular analyte. The completeness of the hydrolysis and solvolysis procedures could have then been assessed, controlled for and validated. Because of the considerable effort that this entails, we elected to use previously described and carefully evaluated methods for the hydrolysis and solvolysis [49]. In fact, most of the analytical methodologies described here are modifications of previously described and applied methods (for free isoflavones, see Ref. [22]; for solvolysis of sulfate, sulfoglucuronide, and disulfate conjugates, see Ref. [49]). Thus, the methods we describe here, and have applied to studies in male and female humans (manuscripts submitted for publication), both extend our knowledge of isoflavone metabolism and pharmacokinetics and also allow for direct comparison with preexisting findings.

Support for the validity of these assays is also apparent when one examines our pharmacokinetic results. A complete description of our results will be provided elsewhere, but in general we found that levels of free isoflavones in plasma and urine were very low. The average ($n=3$) maximum free plasma levels of genistein over a 24-h time course were 34.0, 41.9, 36.2, and 97.4 ng/ml with increasing doses of genistein of 2, 4, 8, and 16 mg/kg. The average ($n=3$) maximum free plus sulfate conjugate levels of genistein over a 24-h period were also quite low and increased with increasing dose (135.9, 262.1, 291.4, and 610.3 ng/ml at doses of genistein of 2, 4, 8, and 16 mg/kg). In comparison, the average ($n=3$) maximum free plus total conjugate levels of genistein were considerably higher (1523.3, 2343.3, 4116.5, and 6866.5 ng/ml at doses of genistein of 2, 4, 8, and 16 mg/kg). With this particular dosage form, with each dose of genistein the subjects also received 1, 2.1, 4.2, and 8.4 mg/kg of daidzein and 0.085, 0.17, 0.3, and 0.68 mg/kg of glycitein. Since the dose levels of daidzein in this formulation were lower than that of genistein, the free, free plus sulfate conjugates, and the free plus total conjugate levels tended to be lower as well. The average ($n=3$) maximum free plasma levels of daidzein over a 24-h time course were 17.6, 18.7, 25.4, and 44.8 ng/ml, the free plus sulfate conjugates were 39.7, 175.1, 200.2, and 372.1 ng/ml, and the free plus total conjugates were 640.9, 915.0, 1649.3, and 2280.8 ng/ml, at increasing dose levels of daidzein of 1, 2.1, 4.2, and 8.4 mg/kg, respectively. The average ($n=3$) maximum plasma levels of non-conjugated glycitein over a 24-h time course were 3.8, 6.0, 8.7, and 14.4 ng/ml at increasing dose levels of glycitein (see above). However, no free plus sulfate nor free plus total conjugates of glycitein exceeded our LOQ (LOQ free plus sulfate glycitein of 110 ng/ml, LOQ free plus total glycitein of 208 ng/ml). The amount of free plus sulfate conjugates of genistein we describe above is approximately 9% of the free plus total genistein, and the amount of free plus sulfate conjugates of daidzein is approximately 13.4% of the free plus total daidzein (when averaged across the dose range). These values are consistent with the levels of these isoflavones in Japanese men, where the mean free plus sulfate genistein and daidzein were determined to be 3 and 12% of the total [50]. In omnivorous Finnish women, the mean relative amounts of the free plus sulfate fraction of genistein and daidzein were reported to be 14.3 and 16.6%, respectively, whereas in Vegetarian Finnish women, the mean relative amounts were 7.6 and 17.3%, respectively [32]. In this particular study, levels of unconjugated isoflavonoids could not be measured in either omnivores or vegetarians with the GC–mass spectrometry (MS) assay that was used, also consistent with the low levels of free isoflavonoids we could detect. It is interesting to note that while we could detect low ng/ml levels of free glycitein after dosing with this formulation, no levels of free plus sulfate or free plus total conjugates

exceeded our LOQs. However, because of the higher amount of interference in these assays, our LOQs were over 40-fold higher for free plus sulfate conjugates, and over 80-fold higher in the free plus total conjugate assay.

The development of these validated HPLC–UV assays for genistein, daidzein, and glycitein in the free, sulfate conjugate, and total conjugate forms, and in both plasma and urine, provide novel analytical methods with which the pharmacokinetics and pharmacodynamics of the principle active forms of soy isoflavones can be studied. Although in many instances not as sensitive as electrochemical, LC–MS or LC–MS–MS techniques, UV detection is generally available to a greater population of scientists than are these other techniques, and is perhaps the most rugged technique. In addition, these compounds present several problems for LC–MS techniques. For example, in preliminary experiments, we found that the free compounds, particularly glycitein, appear to be somewhat labile under the high temperatures required for atmospheric pressure chemical ionization (APCI) conditions. Additionally, these phenolic phytoestrogens are not ionized in our mobile phase at the acidic pH required for the chromatographic conditions used for their separation, thereby making their analysis by electrospray techniques also problematic (e.g., necessitating post-column modification of mobile phase pH). It does appear that under some electrospray conditions one can get positive ions formed through adduct formation. However, we found that production of daughter ions from genistein and daidzein appears to be insufficient for multiple-reaction monitoring, a finding in agreement with the results of [21]. There has been one report of a successful use of HPLC–MS as a quantitative tool for free genistein and daidzein [20]. However, the calibration curves in this report covered a modest fivefold dynamic range (from 27 ng/ml to 135 ng/ml) and consisted of only three concentrations per curve, and the authors did not fully report the linearity, accuracy, or precision of this assay (although at levels above 2 ng/ml, the RSD of variation was reported to range between 7 and 10%).

In conclusion, while the sensitivity of UV detection might be surpassed by other available techniques, the sensitivity and range of the described techniques has proved adequate for most meaningful measurements to be made. Arguably, the very low levels of free isoflavones in plasma and urine require even lower levels of detection to be available for adequate data to be obtained for reliable pharmacokinetic analyses. However, it may be that isoflavones are not circulated in plasma or eliminated in urine in this form, and therefore the measurement and pharmacokinetic analysis is not as relevant as the measurement of the conjugated (yet pharmacologically active) forms of these substances. Specifically, it has been suggested that since sulfate conjugates of estrogen are a source of biologically active material due to the abundance of phenol sulfatases in tissues [50], it is plausible to consider the sulfate conjugates of isoflavonoids to also be a source of pharmacologically active circulating material. Similarly, the lignans enterolactone and enterodiol are also found in plasma as sulfate conjugates, and in high concentrations (free plus sulfate conjugates of approximately 20%). Furthermore, enterolactone is rapidly sulfated by both MCF-7 human breast cancer cells and HepG2 human liver cancer cells and the monosulfate is the predominant metabolite in these cell cultures [51,52]. With the recent evidence that even the glucuronides of genistein and daidzein are weakly estrogenic and activate natural killer cells at relevant dietarily achieved concentrations, one might conclude that consideration of these forms of the isoflavonoids is important in the complete characterization of the pharmacology and toxicology of these compounds. The methods we describe here can therefore serve to facilitate our understanding of these important dietary components and potential therapeutic agents.

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