Quantification of Soy Isoflavones, Genistein and Daidzein, and Conjugates in Rat Blood Using LC/ES-MS

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Genistein is the principal soy isoflavone to which the putative beneficial effects of soy consumption have been attributed; however, the possibility of adverse biological effects (e.g., estrogenic, antithyroid) has also been raised. This paper describes development and validation of a simple and sensitive analytical method for the determination of genistein in the blood of rats receiving dietary genistein (<0.5−1250 μg of genistein aglycone/g of chow). The method uses serum/plasma deproteination, liquid−liquid extraction, deuterated genistein and daidzein internal standards, isocratic LC separation, and electrospray mass spectrometric quantification using selected ion monitoring. Extraction efficiency is approximately 85%, the detection limits for genistein and daidzein from 50 μL of rat blood are approximately 5 nM, and the limit of quantification is approximately 15 nM. Interassay precision (relative standard deviation 4.5−4.6%) and intraassay precision (3.3−6.7%) were determined from replicate analysis of a spiked control and an incurred serum sample. The distribution of conjugated and unconjugated forms of genistein in the blood of rats was determined using selective enzyme hydrolysis. The glucuronide was the predominant metabolite (>90%), and only small amounts of the sulfate conjugate and the aglycone were observed at all dose levels. No evidence for additional metabolites was obtained. The 7- and 4′-glucuronide conjugates of genistein were identified using electrospray mass spectrometry and ^{1}H NMR. Total blood genistein ranged from <15 nM in animals fed soy-free control diet to as high as 8.9 μM in male rats fed 1250 μg of genistein/g of chow and encompasses blood isoflavone levels observed in humans consuming a typical Asian diet and nutritional supplements (0.1−1 μM) and infants consuming soy formulas (2−7 μM).

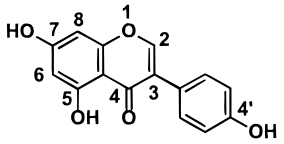
Keywords: *Genistein; soy; isoflavone; electrospray mass spectrometry*

INTRODUCTION

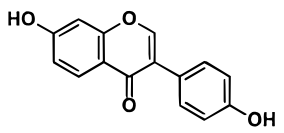
The soy isoflavones, genistein and daidzein (see Figure 1 for structures), have many reported biological activities. Numerous activities have been reported from in vitro studies including inhibition of tyrosine protein kinase, inhibition of topoisomerase II, inhibition of cellular proliferation, stimulation of apoptosis, and interactions with estrogen receptors. The possible significance of such activities has been reviewed by Barnes et al. (1995).

Biological effects in animal and human studies include estrogenic actions in animals and humans (Santell et al., 1997; Cassidy et al., 1994); significant indications of cancer chemoprevention in reproductive tissues of rats, e.g., mammary gland and prostate (Fritz et al., 1998); and goitrogenic actions in rodents (Block et al., 1961), human adults (Ishizuki et al., 1991), and children (Hydovitz, 1960). Human exposure to isoflavones and the bioavailability through consumption of both conjugated and aglycone forms are well-documented. For example, human infants (Setchell et al., 1997) and adults (Xu et al., 1995) show total blood isoflavone levels (aglycone plus conjugated forms) of up to 10 μM following consumption of soy products.

The demonstrated exposure to isoflavones and effects in humans, experimental animals, and in vitro test systems strongly suggest the possibility for both beneficial (e.g., cancer chemoprevention) and adverse (e.g., estrogenic and antithyroid) effects from dietary consumption of soy products. To better understand the dose−response relationships for such biological effects, the National Institute for Environmental Health Sciences/National Toxicology Program and the U.S. Food and Drug Administration have started multigeneration studies to test genistein for possible endocrine toxicity.



Genistein



Daidzein

Figure 1. Isoflavone structures.

Previous workers have used LC−UV (Xu et al., 1995), GC/MS (Adlercreutz et al., 1994; Setchell et al., 1997), and LC-APCI/MS/MS (Coward et al., 1996) for analysis of isoflavones in human blood. This paper describes the development and validation of analytical methods based on LC/ES-MS for use in determining blood isoflavones in rats administered dietary genistein. The sensitivity

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10.1021/jf9902651
Published on Web 08/21/1999

of LC/ES-MS detection in combination with isotopic labeled internal standards serves to add additional confidence over previous LC/MS methods in the accuracy and precision of determinations by directly providing quality control and assurance information (e.g., retention times and recoveries of IS) in every sample throughout large sample sets. In addition, the sample preparation is much less demanding than that required for GC/MS analysis.

EXPERIMENTAL PROCEDURES

Reagents. Genistein was obtained from Toronto Research Chemicals (Ontario, Canada), and daidzein was purchased from Indofine Chemical Co. (Belle Meade, NJ). Hydrolytic enzymes were purchased from Sigma Chemical Co. (St. Louis, MO): the mixture of sulfatase and glucuronidase from *Helix pomatia* (S3009), containing 400−600 units/mg of glucuronidase activity and 15−40 units/mg of sulfatase activity); partially purified sulfatase from *Aerobacter aerogenes* (S1629), containing 2−5 units/mg of protein; and a recombinant bacterial glucuronidase expressed in corn (G2035), containing $>2 \times 10^4$ units/mg of protein. Bovine hepatic microsomal UGT and UDPG were obtained from Sigma Chemical Co. Deuterated daidzein (6,3′,5′-d_3) and genistein (6,8,3′,5′-d_4) were purchased from Cambridge Isotope Laboratories (Andover, MA).

Animal Feeding Procedures. Male and female Sprague−Dawley rats (Crl: COBS CD (SD) BR rat out bred, from the NCTR colony) were maintained on a diet similar to the standard NIH 31 rat chow (Purina 5K96, Purina Mills Inc., St. Louis, MO) except that the soymeal and alfalfa components were replaced by casein (NIH 31C). This feed was determined to be essentially isoflavone-free (below the limit of detection for total genistein of 0.5 ppm in feed) using LC-ES/MS analysis for genistein and daidzein after complete hydrolysis of glucoside conjugates (4 h reflux in 10% aqueous HCl). Soy-depleted chow (NIH 31C) was fortified with genistein aglycone at various levels (0−1250 ppm), and the feed concentrations were determined using LC−UV 260 nm (minimum detectable genistein aglycone, 0.1 ppm in feed).

The rats were treated as part of the dose range-finding study for a multigeneration feeding trial to determine possible endocrine toxicity and carcinogenicity of genistein. Adult females (F0 dams, 70−90 days of age) were switched from normal NIH 31 chow to the isoflavone-depleted NIH 31C chow 7−14 days prior to mating. They were then fed genistein-fortified chow at 7 days after conception and well maintained on this food source through lactation until sacrifice at weaning on PND 21 for a total of 35 days on dosed feed. Male and female pups (F1) obtained from the dams were exposed in utero from 7 days after conception (the time when organogenesis begins) and by consumption of maternal milk through PND 21. After weaning (PND 21), the pups were placed on the same genistein-supplemented chow received originally by the mother. The pups were maintained on this food source until sacrifice at PND 63 for a total of 77 days exposure. At sacrifice, blood was obtained by cardiac puncture, allowed to clot at 4 °C, and centrifuged at 3000*g* for 25 min to remove the clot; and the serum was stored frozen at −60 °C. For the F0 females only, plasma was prepared by collection of blood into EDTA tubes.

Sample Preparation and Extraction. Serum/plasma was thawed at room temperature, vortex mixed, and added to an equal volume of acetonitrile in 1.5-mL Eppendorf tubes (75 μL of serum/plasma per determination), vortex mixed, sonicated for 10 min, and centrifuged at 15 000 rpm for 5 min to pellet precipitated proteins. Aliquots of the supernatant (100 μL, equivalent to 50 μL of plasma) were combined with 1.0 mL of sodium citrate buffer (25 mM, pH 5.0) containing the appropriate enzyme for selective deconjugation. The amount of enzymes added were as follows: sulfatase/glucuronidase mixture, 23 units of sulfatase activity, which was about 5% of the glucuronidase activity; partially purified sulfatase, 0.84 units; and recombinant glucuronidase, 3.24 units.

The time course for hydrolysis by the mixture of enzymes was determined at 37 °C over the interval of 0−240 min. Maximal hydrolysis occurred after about 30 min (not shown), and for this reason, a 30-min incubation period was used for all subsequent analyses. After incubation at 37 °C, the deuterated internal standard mixture (5−150 pmol of genistein and daidzein, depending on the anticipated levels present) 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 (25−150 μL, depending on the anticipated levels present). An equal volume of water was added to make the final solvent (50% aqueous methanol) compatible with the HPLC elution solvent and the samples analyzed by LC-ES/MS. It was determined that two freeze−thaw cycles did not affect the determination of genistein content in rat serum.

Liquid Chromatography. The LC separation was performed using a Dionex GP40 pump (Dionex, Sunnyvale, CA) and either a manual 7125 injector (Rheodyne, Cotati, CA) or a Dionex AS3500 autosampler. Chromatography was performed using a Luna C18 column (2 × 150 mm, 3 μm particle size, Phenomenex, Torrance, CA) with isocratic elution for the aglycone (65% 0.1% formic acid/35% acetonitrile) and gradient elution for the glucuronide conjugates (0.1% formic acid containing 20−50% acetonitrile in 10 min, hold at 50% for 5 min, and then reequilibrate at the initial conditions for 5 min). The flow rate for all analyses was 0.2 mL/min, and the entire effluent was introduced into the ES probe. Quality control samples containing a mixture of labeled and unlabeled isoflavone standards were analyzed throughout each sample set to provide a check of instrument responses and the recovery of internal standards.

Mass Spectrometry. A Platform II single quadrupole mass spectrometer (Micromass, Altrincham, U.K.) equipped with an ES interface was used with an ion source temperature of 150 °C. Positive ions were acquired in SIM mode (dwell time = 0.3 s, span = 0.02 Da, and interchannel delay time = 0.03 s). For aglycone analysis, two time functions were utilized: the first time function (0−7 min) monitored the $(M + H)^+$ ions for daidzein (*m*/*z* 255) and daidzein-d_3 (*m*/*z* 258) at a sampling cone−skimmer potential (CV) of 30 V, and a fragment ion was monitored at *m*/*z* 137 (CV = 70V); in the second time function (7−12 min) the $(M + H)^+$ ions for genistein (*m*/*z* 271) and genistein-d_4 (*m*/*z* 275) were monitored at a CV of 30 V, and a fragment ion (*m*/*z* 153) was monitored at CV = 70V. During the genistein glucuronide analysis, the $(M + H)^+$ ion (*m*/*z* 447) and two fragment ions (*m*/*z* 271 and 153) were acquired at 20, 50, and 70V, respectively, in selected ion monitoring mode.

Internal Standard Characterization. Deuterated genistein and daidzein were dissolved in methanol at approximately 1.0 mg/mL, and the concentration of diluted aliquots was determined using LC−UV with 260 nm detection vs a weighed amount of authentic unlabeled isoflavones. Appropriate dilutions of labeled isoflavones were then made into acetonitrile to yield 0.5 pmol/μL of both genistein-d_4 and daidzein-d_3. Additional analysis using LC with 254 nm detection showed no contaminants in the internal standard.

The total isotopic enrichment was determined using full-scan LC-ES/MS (*m*/*z* 100−600 in 1 s) to be 98.1% for daidzein and 94.3% for genistein. The content of unlabeled genistein and daidzein were determined using LC-ES/MS to be less than 0.1%. The distribution of individual isotopic species for genistein was <0.1% d0, <0.1% d1, 2.9% d2, 17.9% d3, and 79.2% d4. For daidzein, the distribution was <0.1% d0, <0.1% d1, 5.1% d2, 87.0% d3, and 7.9% d4. Analysis of the internal standard solution after 6 months storage at −20 °C in methanol showed no change in isotopic purity. The responses for unlabeled and labeled genistein and daidzein were determined from mixtures containing 0.4−40 pmol/20 μL injection of unlabeled and a constant amount of labeled genistein and daidzein (4 pmol/injection). Over this range of concentration ratios (0.1−10), the response ratios were linearly correlated (correlation coefficient >0.999, data not shown). The slopes of the lines were initially

determined to be 1.25 for genistein and 1.15 for daidzein, in accordance with the values determined from full-scan ES-MS (se above). Subsequent small daily fluctuations were compensated by determining the ratio daily by interspacing injections of a mixed labeled and unlabeled standard throughout the sample analyses. This response ratio was used to compute unlabeled/labeled concentration ratios. The possible effect of plasma matrix on the ratio plot was investigated by extracting control rat plasma (50 μL) with ethyl acetate and fortifying with varying amounts of unlabeled genistein and daidzein (5−500 pmol) plus labeled genistein (25 pmol) and labeled daidzein (12.5 pmol). Over these concentration ratio ranges (0.2−20 and 0.4−40, respectively), the linearity and slopes were similar to that observed for the pure standards.

Enzymatic Production and NMR Analysis of Isoflavone Glucuronides. Glucuronides derived from either genistein or daidzein were produced using the following incubation conditions: Isoflavone (100 μM) was incubated with a bovine glucuronosyl transferase (0.1 unit) and $MgCl_2$ (5 mM) in phosphate buffer (0.1 M, pH 8.0) at 37 °C. The reaction was initiated by addition of UDP glucuronic acid (1 mM). Reaction mixtures were analyzed using LC/ES-MS and LC−UV (260 nm detection) after a minimum of 30 min incubation. The product peaks were collected individually from the effluent of the HPLC column, the solvent was removed in vacuo, dissolved in DMSO-d_6, and then analyzed using ^{1}H NMR (Bruker AM500 operating at 500.13 Hz).

RESULTS AND DISCUSSION

General Method Parameters. A reliable method using deuterated internal standards, liquid−liquid extraction, and LC-ES/MS determination was developed and validated for the measurement of isoflavones and metabolites in plasma from rats fed genistein-supplemented chow and control animals fed a soy-depleted chow. This sensitivity of the ES/MS method allowed analysis of small volumes of plasma without derivatization and minimal sample preparation. Initial experiments showed that precipitation of serum/plasma proteins using dilution into acetonitrile was required for reproducible results. Selective enzymatic hydrolysis and subsequent analysis of the respective aglycone were used to determine the forms of isoflavones in plasma: a mixture of glucuronidase plus sulfatase enzymes was used to measure total isoflavones; a purified sulfatase was used to measure sulfate conjugates; a recombinant β-glucuronidase was used to measure glucuronide conjugates; and analysis without enzyme treatment was used to measure aglycone. It was determined using authentic glucuronide conjugates of genistein and daidzein that the glucuronidase preparations catalyzed quantitative hydrolysis under the conditions used and that the sulfatase preparation was devoid of glucuronidase activity (not shown). The ratio of labeled IS to blood level of genistein was chosen to be as close to 1 as possible and within the linear ratio range from 0.1 to 10 that was validated (10−3000 nM added). Figure 2 shows LC-ES/MS responses for the protonated molecules of genistein (*m*/*z* 271), daidzein (*m*/*z* 255), and the deuterated analogues (*m*/*z* 275 and 258, respectively) for a mixture of standards (5 pmol each on-column, left panel), a control serum sample treated with sulfatase and glucuronidase (5 pmol internal standards added, center panel), and an incurred rat plasma sample (1250 ppm F0 female plasma sample treated with sulfatase and glucuronidase; 5 pmol internal standards added, right panel).

Method Precision and Accuracy. Intraassay and interassay precision for analysis of genistein were determined using a single plasma sample (total genistein in 1250 ppm diet, F0 females) analyzed in quadruplicate on separate days. On day 1, the determined value was 2.63 ± 0.12 μM (mean ± SD; 5% RSD), and on day 2, the same sample gave 2.76 ± 0.09 μM (3% RSD). In another measure of interassay accuracy and precision, the control serum was spiked with 1.0 μM genistein and assayed on two separate days. On day 1, the determined value was 0.88 ± 0.04 μM (4.6% RSD, $n = 3$), and on the second day, the value was 1.04 ± 0.07 μM (6.7% RSD, $n = 3$), and the average accuracy was 96%. In the analysis of plasma from F0 females (four animals per dose group), quadruplicate analyses were performed for the determination of total genistein following enzymatic hydrolysis. The average values for total genistein in rat blood (F0 females) ranged from the limit of quantification, 0.015 μM, to a maximum of 8.48 μM. The RSD values averaged 5.7% with a range of 1.1−15.8% ($n = 112$). The corresponding determinations of aglycone levels ($n = 3$) were made without any enzymatic hydrolysis step. In this case, the values were much lower (0.02−0.61 μM) and the RSD values was greater (average = 8.4%, range 1.3−23.5%, $n = 32$). The precision of isoflavone determinations in blood measured in this study is comparable to that seen in previous studies where isotope dilution GC/MS (Adlercreutz et al., 1994) or LC/MS/MS using structurally related flavonoid internal standards were used (Coward et al., 1996).

Analyte Recovery. The recovery of genistein and daidzein was determined for every sample analyzed based on a comparison of IS signals in samples with those from interspaced standards. The average genistein recovery found in the F0 female study was 80.2 ± 6.7% (RSD = 8.4%, $n = 152$). In the 1 μM spike experiment, recovery of genistein for both days was 85 ± 8% ($n = 6$), and this was typical throughout analysis of ca. 400 samples. Daidzein recoveries were in the same range (not shown). The genistein and daidzein levels in the NIH 31C chow were below the detection limit using LC-ES/MS (<0.5 ppm), and undetectable amounts of daidzein were observed in any blood sample as described below. It was demonstrated that this extraction procedure was 80−100% efficient in recovering isoflavone aglycones and glucuronides (not shown).

Method Limit of Detection/Quantification. The detection limits for genistein and daidzein were estimated by adding known amounts of unlabeled isoflavones (5−20 nM) and a constant amount of IS (50 nM) to control serum. Signal-to-noise levels were evaluated for the series of unlabeled analyte additions. Analysis of the control plasma showed small peaks for genistein and daidzein (see the center panel of Figure 2), and this amount did not appear to change upon treatment of the plasma with the mixture of deconjugating enzymes. The endogenous isoflavone peaks coeluted with authentic unlabeled standards, and the responses were additive with those of the standard addition (see Table 1); however, the endogenous responses were insufficient for structural confirmation of the presence of genistein or daidzein using a response using fragment ions (*m*/*z* 153 or 137, respectively) in addition to the $(M + H)^+$ ions. It is therefore unclear whether these signals result from background isoflavones in blood derived from the low-level contamination in soy-depleted feed or from unrelated serum interferences. These levels in control serum were about 5 nM for genistein and daidzein, and this was defined as the detection limit. These observations

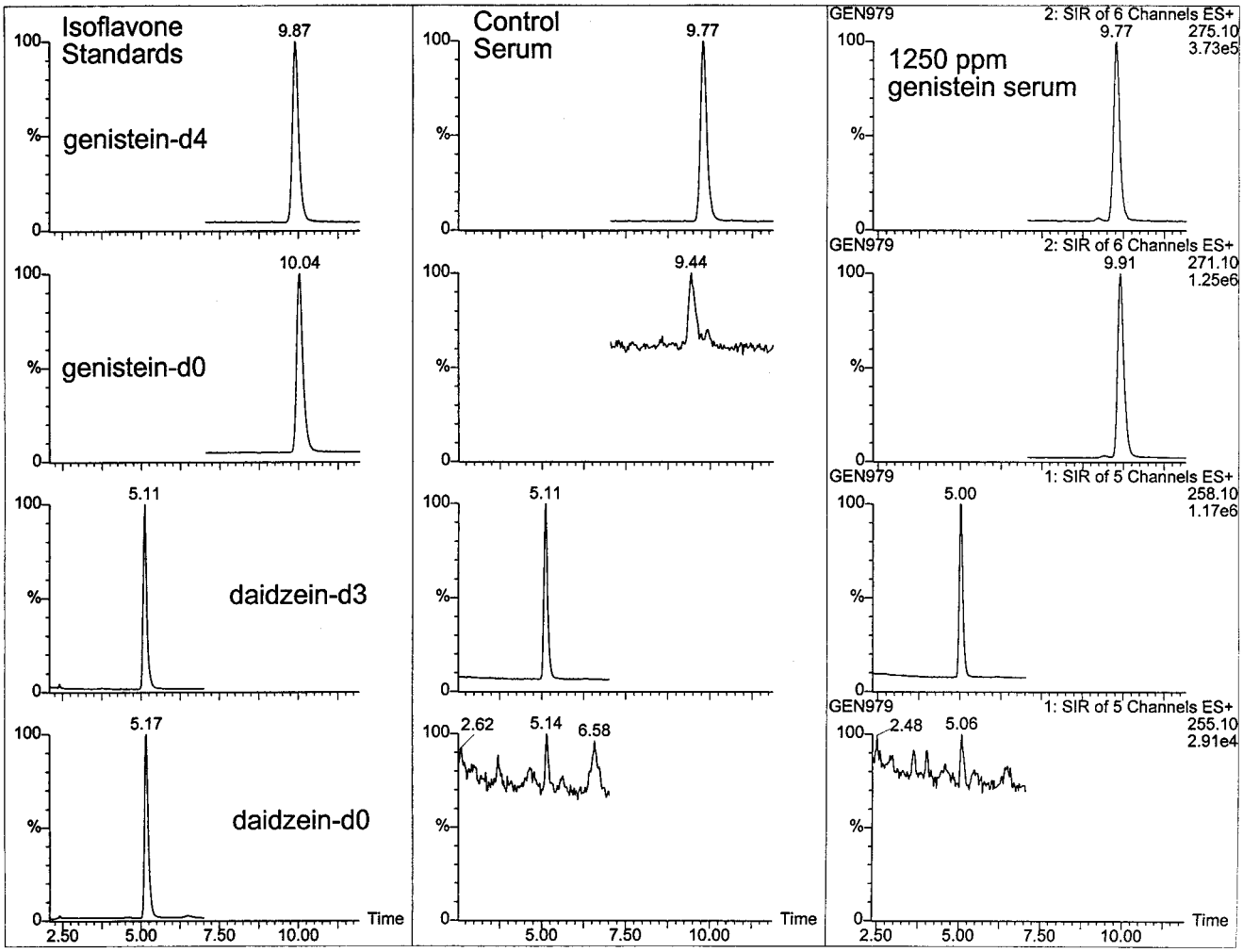

Figure 2. Analysis of isoflavones in rat serum using LC/ES-MS. The ES-MS responses for protonated molecule chromatograms for genistein (*m*/*z* 271), daidzein (*m*/*z* 255), and the deuterated analogues (*m*/*z* 275, 258, respectively) are shown for authentic standards (left panel), serum from a rat consuming the control diet (center panel), and serum from a rat consuming 1250 ppm of genistein in the diet. Note in the center panel that genistein d0 should elute just after the 9.44-min peak shown and that d0-daidzein should elute at approximately 5.14 min.

Table 1. Determination of Extraction Efficiency for Isoflavones near the Detection Limits in Rat Blood[a]

spike level	genistein (μM)	% recovery of added genistein	daidzein (μM)	% recovery of added daidzein
control	6		3	
5 nM	9	60	6	60
10 nM	17	110	13	100
20 nM	26	100	27	110

[a] The different amounts of unlabeled genistein plus daidzein (5−20 nM) and a constant amount of deuterated standards (50 nM) were spiked into control serum and analyzed using LC-ES/MS as described. The isoflavone levels in unspiked and spiked samples are shown.

suggest that the limit of quantitation is about 15 nM for serum isoflavones. Table 1 shows the recovery of unlabeled analytes near the detection limit. The recoveries were in the range of 60−110% and comparable to those seen for the recovery of labeled genistein and daidzein from dosed-rat blood described above. Previous studies had similar detection limits (Adlercreutz et al., 1994; Coward et al., 1996).

Genistein Levels in Rat Blood. The blood levels of total genistein and the aglycone were determined in three groups of animals with and without enzymatic hydrolysis of sulfate and glucuronide conjugates: the F0 dams fed control, 5, 25, 100, 625, or 1250 ppm genistein-fortified feed (n = 4 in each dose group); F1 female and male pups fed control, 25, 250, or 1250 ppm genistein (n = 10 in each dose group). Figure 3 shows the results of analysis for total genistein forms and the aglycone for the F1 females and males at all dose groups. In all treatment groups, blood genistein concentration was linearly related with the dosed feed concentration for total and aglycone forms of genistein for male and female rats (correlation coefficients 0.95−0.999). No evidence for gender differences was observed. The data for F0 females, obtained in the dose range-finding study, showed higher interanimal variability in total blood genistein. This was attributed, in part, to different animal handling practices than those used for the F1 studies. Table 2 shows that the genistein aglycone concentration in blood was less than 3% of the respective total genistein concentration. These findings are consistent with previous findings that conjugated forms of isoflavones predominate in rat and human blood (Adlercreutz et al., 1994; Coward et al., 1996).

Genistein Conjugates in Blood. The nature of the conjugated forms was further investigated in individual animals using selective enzymatic hydrolysis of the glucuronides, the sulfates, or both. Table 2 shows the results for analysis of several serum/plasma samples (F0 female, 250 ppm dose group; F1 female, 250 and 1250

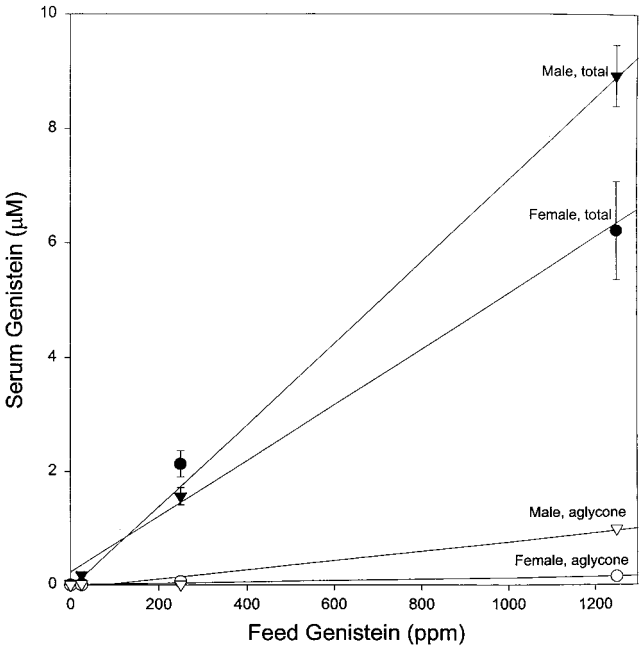

Figure 3. Total blood genistein levels in F1 male and female rats fed different amounts of genistein. The average blood levels of total and aglycone genistein and the associated SEM are shown for each dose group of 7−10 animals. The linear regression lines are shown, and correlation coefficients were 0.97, 0.95, 0.999, and 0.96 from male total, female total, male aglycone, and female aglycone, respectively.

Table 2. Quantification of Genistein Conjugates in Rat Serum/Plasma

dose group	aglycone	sulfate	glucuronides	total
250 ppm (F0)	30	ND	2190	2100
250 ppm (F1)	38	96	1350	1310
1250 ppm (F1)	150	20	5330	5300

[a] Duplicate samples (F0 female, 250 ppm dose group, and F1 females 250 and 1250 ppm dose groups; average values shown) were analyzed for genistein aglycone following selective enzymatic hydrolysis using a recombinant glucuronidase, a purified sulfatase, a mixture of both enzymes, or no enzyme. The amount of genistein present as each form (in nM) is shown. Samples designated ND had genistein levels below 15 nm.

ppm). Similar results were observed for the F1 males (not shown). The amount of sulfate conjugate found in all dose groups was of the same order as the aglycone and was much smaller than the glucuronide conjugates. In all cases, the amount of genistein glucuronide conjugates was essentially equal to the total genistein determination. These results demonstrate that glucuronide conjugates are the predominant circulating form of genistein in rat blood at all dose levels examined. The results in Table 2 show increases in blood levels for each form that mirror the dose dependence observed for the total blood genistein (see Figure 3). This suggests that no saturation of any conjugation pathway occurred over this dose range. The level of aglycone also showed no clear evidence for saturation at these doses (see Figure 3). Another such study with F1 males showed similar dose responses, although the presence of sulfate conjugates was undetectable (not shown).

Other workers characterized the urinary and biliary conjugates of genistein in rats as the 4′-sulfate, the 7-glucuronide, and the 4′-sulfate−7′-glucuronide diconjugate (Yasuda et al., 1996); however, no evidence for diconjugate formation in rat blood was observed in the present study from direct analysis of blood extracts

Table 3. ^{1}H NMR Characterization of Genistein 4′-Glucuronide[a]

chemical shift (ppm)	assignment
6.23 (1H, d)	H6
6.39 (1H, d)	H8
7.07 (2H, d)	H3′,5′
7.49 (2H, d)	H2′,6′
8.38 (1H, s)	H2
10.89 (1H, s)	C7-OH
12.89 (1H, s)	C5-OH
absent	C4′-OH

[a] NMR spectra were obtained in DMSO-d_6 at 500 MHz. Chemical shifts are relative to the DMSO residual proton signal, and integration values with peak multiplicities are listed (s = singlet, d = doublet).

using LC-ES/MS in positive and negative ion modes. Furthermore, no discrepancy was observed between the total genistein determined using a mixture of deconjugating enzymes and the sum of individual treatments (aglycone + glucuronides + sulfates) from the indirect analysis using selective enzyme hydrolysis procedures (see Table 2). This suggests that the diconjugate form of genistein is not present in rat blood.

Genistein Glucuronide Structure Elucidation. The LC/ES-MS chromatograms in Figure 4 show that two glucuronide isomers of genistein can be formed in vitro and in vivo. The two glucuronides, produced from genistein on a preparative scale (ca. 500 μg) using a bovine hepatic UGT in vitro, were characterized using ^{1}H NMR. The ^{1}H NMR spectra of genistein and the 7-glucuronide were essentially the same as that previously reported derived from rat bile (Coward et al., 1996). The assignments and the associated ^{1}H NMR data for the 4′-glucuronide, which was not observed in rat bile, are shown in Table 3. The assignment of 7- vs 4′-conjugation was made primarily by the loss of the appropriate hydroxyl proton present in the aglycone (C7−OH or C4′−OH) and downfield chemical shift changes of the 3′-/5′- and 2′-/,6′-ring protons relative to aglycone protons.

Analysis of rat blood using LC/ES-MS showed that the 7-glucuronide was the major form and the 4′-isomer was the minor form. The right panels of Figure 4 show the LC-ES/MS positive ion analysis of a rat serum sample processed as described above for aglycone analysis (no hydrolytic enzyme treatment). The left panels show the same analysis for the 7- and 4′-glucuronide conjugates of genistein produced by a bovine hepatic microsomal UDP-glucuronosyl transferase-catalyzed conjugation of genistein as a positive control. Ions corresponding to the protonated conjugate (*m*/*z* 447), the aglycone fragment ion formed by facile loss of the glucuronide moiety through in-source CID, and a further isoflavone ring fragmentation product (*m*/*z* 271 and 153, respectively) were monitored. Not only were the mass spectral properties identical between the rat serum glucuronides and the bovine enzyme products, but the LC retention times were also identical (7.10 and 8.08 min for the 7- and 4′-glucuronides, respectively).

Comparison of Human and Rat Genistein Intake and Blood Levels. Table 4 shows selected measurements or estimates of total soy isoflavone intake (genistein plus daidzein) in several human groups along with the resulting total blood isoflavones. Total isoflavone intake ranges from ca. 1 mg kg^{-1} day^{-1} in adults on a typical Asian diet to 200 mg kg^{-1} day^{-1} in individuals consuming a soy-based cancer supplement

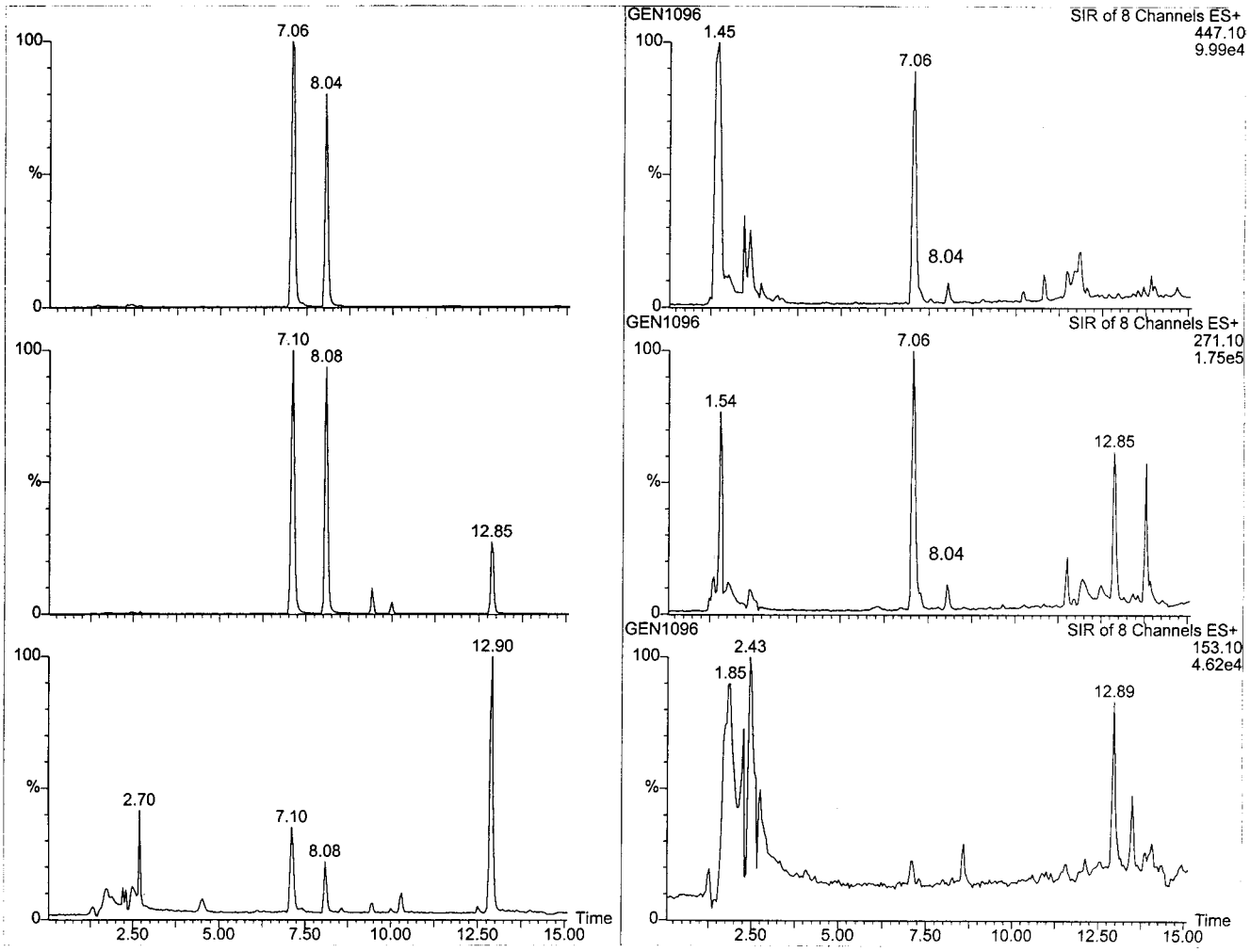

Figure 4. Identification of genistein glucuronides in rat serum using LC/ES-MS. A gradient LC separation and positive ion ES/MS detection (selected ion monitoring of $M + H^+$, *m/z* 447; aglycone fragment ion, *m/z* 271, and a further genistein fragment ion, *m/z* 153) were used to characterize genistein glucuronide conjugates (7- and 4′-isomers elute at 7.1 and 8.0 min, respectively) in an incubation with a bovine liver UGT preparation (left panel) or in the serum of a rat consuming 1250 ppm of genistein (right panel). Note that genistein aglycone elutes at 12.9 min.

Table 4. Isoflavone Intake and Blood Concentrations in Humans

population group, soy form	daily isoflavone intake (mg/day)[a]	total isoflavone dose (mg kg^{-1} day^{-1})[b]	blood isoflavones (μM)
adults, Asian diet	ca. 50	<1[c]	0.1−1.2[d]
infants, soy formula	28−47	6−9[e]	2−7[e]
adults, soy nutritional supplement	50[f]	0.7	0.5−0.9[g]
adults, soy cancer supplement	14000[f]	200	?

[a] Total active isoflavones = genistein + daidzein. [b] 70 kg body weight. [c] Soy intake on typical Asian diet estimated at 50 g/day containing 1 mg/g isoflavones. [d] From six Japanese men (Adlercreutz et al., 1994). [e] From seven infants (Setchell et al., 1997). [f] As per label instructions. [g] Doerge et al., unpublished.

at the label-recommended level. Figure 3 shows the total genistein levels measured in rat blood after continuous dietary genistein intake of <0.04, 2, 20, and 100 mg kg^{-1} day^{-1} for the control, 25, 250, and 1250 ppm dose groups, respectively. The blood genistein levels found in rats in these studies encompass the range observed in previous studies from humans consuming soy products. For example, the 25 ppm dose yielded blood genistein levels in rats similar to those determined in humans on a typical Asian diet (Adlercreutz et al., 1994), and the 250−1250 ppm doses yield rat blood genistein levels similar to the range observed in infants on soy formulas (Setchell et al., 1997). This suggests that any biological effects, either beneficial or adverse, observed in rats receiving these doses may be relevant to humans because of similar blood concentrations encountered.

Potential Impact of Soy-Based Diets on Rodent Bioassays. The total content of genistein and daidzein in NIH 31, the rodent chow used in carcinogenicity bioassays conducted at this institution, is approximately 30 and 20 ppm, respectively, resulting primarily from the 5% soy content. For this reason, a specially formulated chow, NIH 31C in which casein was substituted for soy meal, was used in the present study to study genistein effects. This chow contains no detectable isoflavones. Other chows widely used for rodent carcinogenicity studies contain up to 20% soy and concomitant increases in the isoflavone content (Thigpen et al., 1992, 1999). The results shown in Figure 3 predict that

animals consuming typical soy-based chows will have amounts of genistein and daidzein in blood on the order of 0.1−1 μM. Thigpen and co-workers have measured uterotrophic effects in animals consuming these feeds (Thigpen et al., 1992), and other biological effects, both beneficial and toxic, are possible. For example, a recent study demonstrated that perinatal exposure to dietary genistein was sufficient to significantly reduce chemically induced mammary carcinogenesis in adult female Sprague−Dawley rats (Fritz et al., 1998). The possible impact of these isoflavone activities (e.g., estrogenic, antiestrogenic, anticarcinogenic, or antithyroid) on carcinogenicity and toxicity studies remains to be determined.

ABBREVIATIONS USED

APCI, atmospheric pressure chemical ionization; CV, sampling cone-skimmer potential; ES, electrospray; IS, deuterated internal standard; $(M + H)^+$, protonated molecule; PND, postnatal day; RSD, relative standard deviation; SD, standard deviation; SEM, standard error of the means; SIM, selected ion monitoring; SIR, selected ion recording; UDP, uridine 5′-diphosphate; UGT, UDP glucuronosyl transferase.

ACKNOWLEDGMENT

The assistance of Ms. Connie Weis, Ms. Betty Blaydes, and Dr. Barry Delclos in obtaining blood samples is gratefully acknowledged, as is the assistance of Ms. Joanna Deck in obtaining NMR spectra.

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Received for review March 8, 1999. Revised manuscript received July 6, 1999. Accepted July 12, 1999. This research was supported in part by Interagency Agreement 224-93-0001 between NCTR/FDA and the National Institute for Environmental Health Sciences/National Toxicology Program.

JF9902651