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High-throughput quantification of soy isoflavones in human and rodent blood using liquid chromatography with electrospray mass spectrometry and tandem mass spectrometry detection

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Abstract

Soy-containing foods and dietary supplements are widely consumed for putative health benefits (e.g., cancer chemoprevention, beneficial effects on serum lipids associated with cardiovascular health, reduction of osteoporosis, relief of menopausal symptoms). However, studies of soy isoflavones in experimental animals suggest possible adverse effects as well (e.g., enhancement of reproductive organ cancer, modulation of endocrine function, anti-thyroid effects). This paper describes the development and validation of a sensitive high throughput method for quantifying isoflavones in blood from experimental animal and human studies. Serum samples containing genistein, daidzein, and equol were processed using reverse phase solid-phase extraction in the 96-well format for subsequent LC–ES/MS/MS or LC–ES/MS analysis using isotope dilution in conjunction with labeled internal standards. The method was validated by repetitive analysis of spiked blank serum and the intra-day and inter-day accuracy (88–99%) and precision (relative standard deviations from 3 to 13%) of measurement determined. The lower limit of quantification for all isoflavones was approximately 0.005 μM using MS/MS detection, and 0.03 μM using MS for genistein and daidzein. The degree of method performance, with respect to throughput, sensitivity and selectivity, makes this approach practical for analysis of large sample sets generated from mechanistic animal studies and human clinical trials of soy isoflavones.

Keywords: Isoflavones

1. Introduction

Soy foods and nutritional supplements, which contain isoflavones, are widely promoted and consumed based on a variety of putative beneficial health effects in humans including chemoprevention of breast and prostate cancer [1,2], reductions in serum LDL cholesterol and increases in HDL cholesterol [3]. Consistent with these possible effects in humans, isoflavones have been shown in experimental animals: to accumulate to significant levels in reproductive tissues of adult rats [4]; to be agonists for estrogen receptors α and β which are present in the mammary and prostate glands of adult rodents [5]; to possess antioxidant properties protecting against oxidative damage [6]; to affect during rodent development the pituitary–gonadal axis, reproductive organ development, and sexual behaviors [7].

We have previously reported analytical methods based on LC–ES/MS for the quantification of isoflavones in serum [8,9] and tissues [4] of experimental animals and humans [10]. In these

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studies, various sample preparation procedures were employed. Initially, we used a manual liquid–liquid extraction procedure [8], and later described an automated method for on-line sample preparation using a restricted-access medium trap in a column switching configuration [9]. The latter procedure permitted unattended analysis of large sample sets requiring only a single centrifugation for sample preparation and a total instrument run time of 18 min per sample. To better prepare for the challenges presented by large-scale clinical studies of soy isoflavones involving hundreds of samples, it was necessary to validate a more high throughput analytical method based on parallel processing of serum or plasma samples using solid-phase extraction (SPE) in the 96-well format. As in all our previous work, isotope dilution LC–ES/MS and MS/MS was used because of the high sensitivity and robust quantification of isoflavones provided.

2. Experimental

2.1. Liquid chromatography

The LC separation was performed using a Dionex GP40 pump (Dionex, Sunnyvale, CA) and a Dionex AS3500 autosampler. Chromatography was performed using a Ultracarb ODS column (2×150 mm, 3 μm particle size, Phenomenex, Torrance, CA) with isocratic elution for the aglycones (65% 0.1% formic acid–35% acetonitrile). The flow-rate for all analyses was 0.2 ml/min and the entire effluent was introduced into the ES probe.

2.2. Mass spectrometry

Either a Platform II single quadrupole or a Quattro Ultima triple quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an ES interface was used with an ion source temperature of 150 °C. Positive ions were acquired in selected ion monitoring mode (SIM) for ES/MS (dwell time=0.3 s, span=0.02 Da and interchannel delay time=0.03 s) for the protonated molecule $(M+H)^+$ ions for d0/d4 genistein (*m/z* 271/275), d0/d3 daidzein (*m/z* 255/258) and d0/d3 equol (243/247) which were monitored at a sampling cone-skimmer potential of 30 V. Alternatively, for MS/MS measurements a collision energy of 25 eV was used for the $(M+H)^+$ transitions used to monitor MRM transitions for d0/d4 geinstein (*m/z* 271→215 and 275→219, respectively), d0/d3 daidzein (*m/z* 255→199 and 258→202, respectively),and d0/d3 equol (*m/z* 243→133 and 247→135, respectively) with a dwell time of 0.3 s and a sampling cone-skimmer potentials of 25–60 V and collision energies between 20 and 27 eV. Additional MRM transitions were acquired for genistein (*m/z* 271→243), daidzein (*m/z* 255→137), and equol (*m/z* 243→123) to serve as confirmation in case of coeluting interferences.

2.3. Determination of serum/plasma isoflavones

Samples were processed using specially designed pipetters to pick up fluids from sample vials in 48-well tube racks and deliver them to 96-well plates in which all subsequent operations were performed (Equalizer, Matrix Technologies, Hudson, NH, USA). Depending on the isoflavone concentrations produced in each study, the amount of serum/plasma processed ranged from 5 to 100 μl. The serum/plasma was mixed with an equal volume of acetonitrile to eliminate protein binding, then centrifuged. Further sample cleanup was accomplished using parallel off-line solid-phase extraction (Isolute ENV+, 25 mg, Jones Chromatography, Lakewood, CO, USA) after enzymatic deconjugation for determination of total isoflavones [*H. pomatia* glucuronidase and sulfatase S3009, (Sigma, St. Louis, MO, USA) 100 μg in 0.9 ml of 25 m*M* citrate, pH 5.0] at 37 °C for 30 min. Enzymatic hydrolysis of phenolphthalein glucuronide to phenolphthalein (Sigma) was performed under the same conditions. The isoflavone aglycone content of serum/plasma was determined by omitting enzymatic hydrolysis. Diluted serum/plasma was applied to the SPE cartridge, washed with 1 ml of 30% methanol in water, then eluted with two 0.5 ml aliquots of acetonitrile. The extracts were evaporated to dryness using a heated centrifugal concentrator (SpeedVac, Savant Co., Farmingham, NY, USA), reconstituted in 50% aqueous methanol, then injected onto the LC–ES/MS or MS/MS system for detection using selected ion monitoring (SIM) or multiple reaction monitoring (MRM), respectively.

The deuterated internal standards used in this study were d4-genistein and d3-daidzein, obtained from Cambridge Isotope Laboratories (Andover, MA, USA), and d4-equol, a generous gift from Doctor Kristiina Wahala, University of Helsinki. The individual isoflavones were quantified using isotope dilution by monitoring the respective isotope pairs m/z 275/271 for genistein, 258/255 for daidzein, 247/243 for equol using MS–SIM detection. The analogous MRM transitions were m/z 275→219/271→215, 258→202/255→199, and 247→135/243→133 (see Figs. 2 and 3). In practice, the amount of internal standard added to each sample was selected to lie within an order of magnitude of the endogenous isoflavone content (see validation procedures below), and the amount of serum processed and/or the volume injected on-column was adjusted to give adequate responses (i.e., S/N ratio >10). Quality control procedures included concurrent analysis of isoflavone-fortified rat serum, blank rat serum, and a mixture of labeled and unlabeled standards interspersed throughout each sample set.

3. Results and discussion

3.1. Method validation

3.1.1. Internal standard characterization

Deuterated genistein, daidzein, and equol (see Fig. 1) were characterized using LC–UV and full scan LC–ES/MS. The concentrations of deuterated isoflavones were determined using LC–UV (260 nm detection) using unlabeled compounds as external standards. A calibration curve was constructed from mixtures of each labeled isoflavone at a fixed concentration (4 pmol/injection) along with the respective unlabeled isoflavone present at concentrations from one tenth of the labeled concentration up to ten-fold higher (0.4 to 40 pmol/injection). In all cases, the plot of response ratio versus concentration ratio was highly linear (cc>0.99) and the respective slope was used to determine relative response factors for the predominate deuterated species (i.e., d4-genistein, d3-daidzein, d4-equol). The concentrated stock solutions of deuterated isoflavones were prepared in acetonitrile and stored at −20 °C. Under these conditions, the hydrogen–deuterium exchange rate was very slow. The response factors for each set of labeled/unlabeled isoflavones, which were determined from the slope of response ratio versus concentration ratio plots, were checked daily by analyzing mixtures of labeled and unlabeled standards.

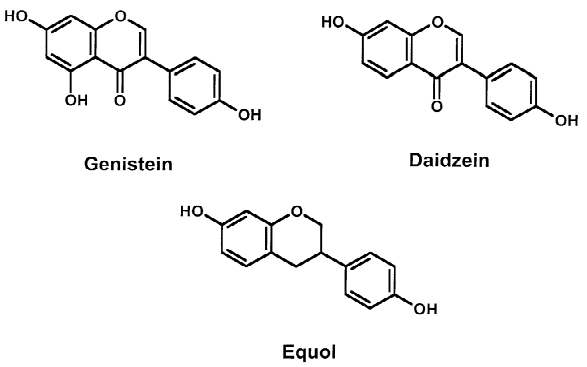

Fig. 1. Structures of isoflavones.

3.1.2. Verification of glucuronidase activity

The time course of isoflavone hydrolysis by commercial hydrolytic enzymes was previously determined to be maximal at 30 min [8]. An independent means to ascertain enzymatic activity was validated using hydrolysis of phenolphthalein glucuronide to phenolphthalein. The same concentration of glucuronidase/sulfatase, SPE conditions, and incubation temperature were used as described above for serum/plasma. The progress of the reaction was monitored by LC–ES/MS in positive ion mode by measuring the loss of phenolphthalein glucuronide (m/z 495) concomitant with the formation of phenolphthalein (m/z 319). Quantitative formation of phenolphthalein occurred at about 2 h, reflecting its lower substrate activity relative to the isoflavones. Each preparation of enzyme was checked using this procedure. Enzymatic activity was stable to at least five freeze–thaw cycles, and stable in the frozen state for at least 2 weeks.

3.1.3. Detection limit

Minimum detection limits were determined by using LC–ES/MS and MS/MS to analyze serum from rats that had been maintained on a soy- and alfalfa-free diet [4,9]. Small responses were observed in the MRM chromatograms of m/z 255→199 and

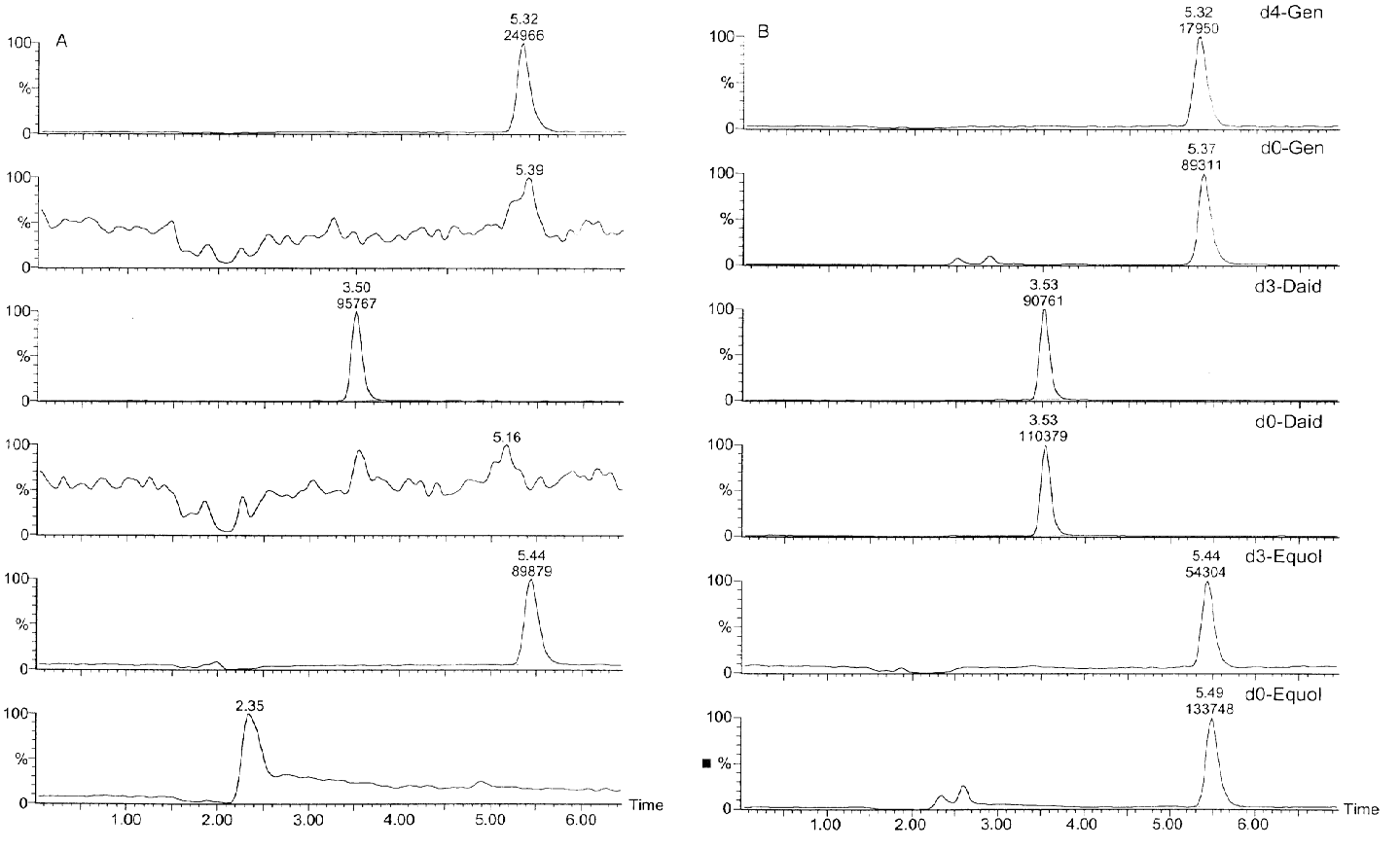

Fig. 2. LC–ES/MS/MS analysis of isoflavones in mouse serum. Serum was analyzed from female BALB/c mice that were fed either an AIN-93G semipurified basal diet (A) or the basal diet fortified with 750 ppm each genistein and daidzein aglycone (B). The MRM transitions monitored were: m/z 275→219 (d4-Gen), 271→215 (d0-Gen); 258→202 (d3-Daid), 255→199 (d0-Daid); and 247→135 (d3-Equol), 243→133 (d0-Equol). The concentrations of genistein, daidzein, and equol were 2.01, 0.95, and 1.90 μM, respectively, in serum from the mouse consuming the isoflavone-fortified diet. Undetectable levels of isoflavones were observed in serum from the mouse consuming a control diet.

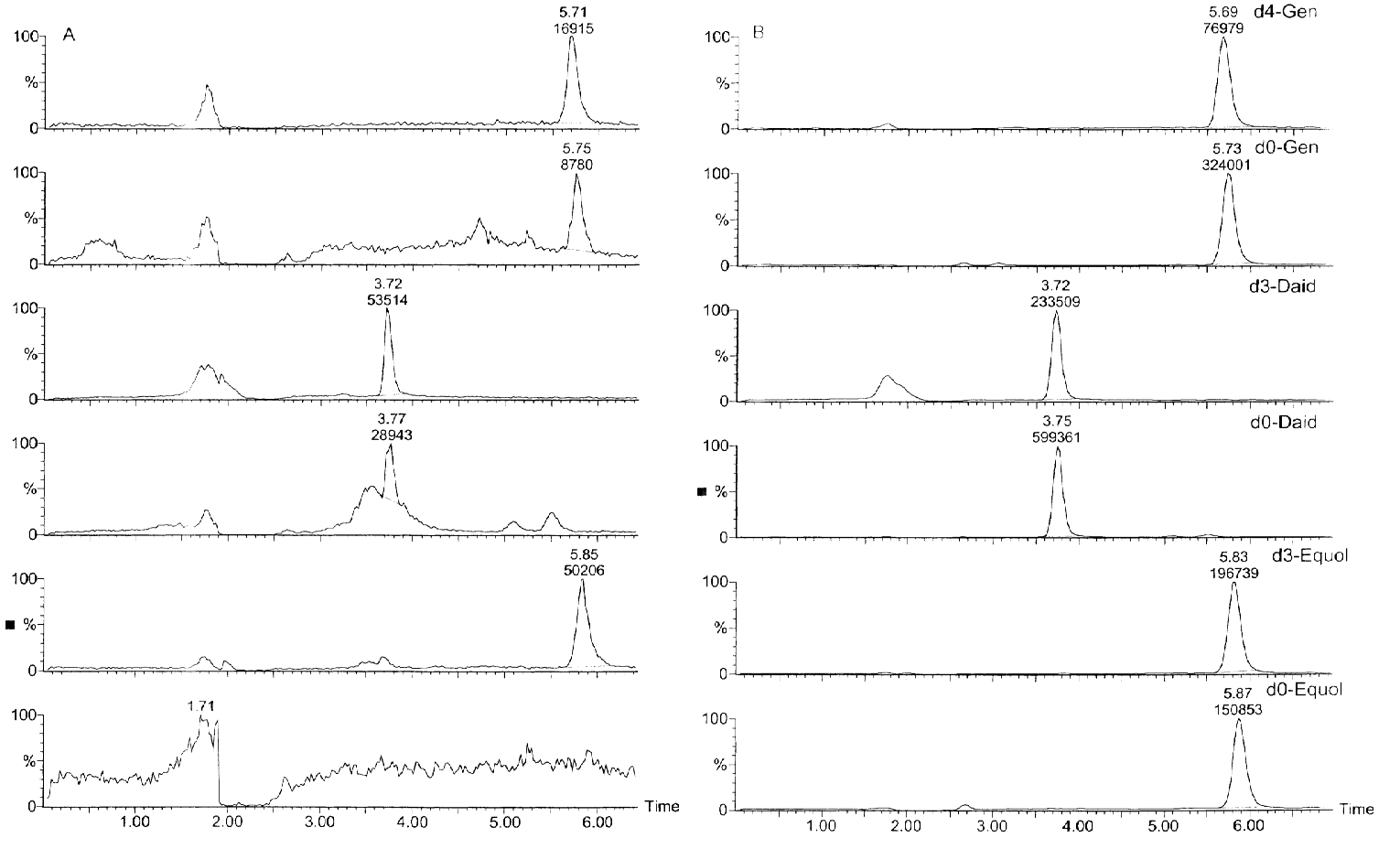

Fig. 3. LC–ES/MS/MS analysis of isoflavones in human plasma. Serum was analyzed from a male volunteer collected before consumption of the dietary supplement (A) and after consumption of a commercial soy dietary supplement containing 100 mg total isoflavones at a ratio of 1.1:1:0.2 for genistein:daidzein:glycitein each day for 3 months (B). The MRM transitions monitored were: m/z 275→219 (d4-Gen), 271→215 (d0-Gen); 258→202 (d3-Daid), 255→199 (d0-Daid); and 247→135 (d3-Equol), 243→133 (d0-Equol). The concentrations of genistein, daidzein, and equol were 0.28, 0.16, and 0.045 μ*M*, respectively, in plasma from the isoflavone-supplemented volunteer. In blank pooled human plasma, the concentrations of daidzein and equol were below the detection limit and the concentration of genistein was 0.007 μ*M*.

271→215 in serum from a control mouse that corresponded to approximately 0.001 μ*M* daidzein and genistein, respectively (see Fig. 2). No equol was observed. The method detection limit (*S*/*N* 3) was estimated to be 0.010 μ*M* for MS–SIM detection of genistein and daidzein, and 0.001 μ*M* for MS/MS–MRM analysis of all three isoflavones using 100 μl of serum/plasma. The detection limit for equol using MS–SIM was unacceptably high and therefore, measurements of equol required use of MS/MS–MRM and the detection limit for equol in serum was similar to genistein. The limit of quantification (LOQ) in serum was set at 0.030 μ*M* for genistein and daidzein using MS–SIM, and 0.005 μ*M* for genistein, daidzein, and equol using MS/MS–MRM detection. The pooled blank human serum sample shown in Fig. 3 shows responses near the LOQ for genistein (7 n*M*) and daidzein (ca. 4 n*M*), although equol was undetectable.

3.1.4. Intra- and inter-assay precision

For the initial validation study, rat serum was fortified with genistein, daidzein, and equol at concentrations predicted to be in mouse blood for an impending analysis (0.02, 0.5, 10 μ*M*). Serum was analyzed in triplicate on two separate days using MS/MS–MRM. The results are summarized in Table 1. The precision of measurements (relative SD, RSD) were in the range of 3–13% across the concentration range and the accuracies were 88–99% of the concentration added. In addition, an incurred serum sample from a mouse maintained on a diet fortified with genistein and daidzein was analyzed in quadruplicate using MS/MS–MRM on 2 days (see Table 2). On day 1, the total genistein concentration was 2.03±0.057 μ*M* (3%), daidzein was 0.98±0.066 μ*M* (7%), and equol was 1.66±0.034 μ*M* (2%); on day 2, genistein concentration was 2.01 μ*M*, daidzein was 0.95 μ*M*, and equol was 1.90 μ*M*. These validation results are comparable to those reported previously using MS–SIM [4,9] and demonstrate that the modifications needed for increased throughput do not adversely affect sensitivity, accuracy, and precision. Representative chromatograms are presented for incurred mouse serum (Fig. 2) and human plasma (Fig. 3) in conjuction with the respective control samples.

The distribution of genistein in the biologically active aglycone form, as opposed to estrogenically inactive conjugates [11], was measured in serum

Table 2
Precision and repeatability of total isoflavone measurements from incurred mouse serum

Total serum concentration (μ*M*)	Day 1	Day 2
Genistein	2.03±0.057 (3%)	2.01
Daidzein	0.98±0.066 (7%)	0.95
Equol	1.66±0.034 (2%)	1.90

Serum from a mouse maintained on a diet containing genistein and daidzein was analyzed on 2 days. The day 1 means±SD with RSD are shown (*n*=4).

Table 1
Method validation data for genistein, daidzein and equol aglycones fortified into control rat serum

Fortification level	Day 1 mean±SD (RSD)	Day 2 mean±SD (RSD)	Accuracy (% added)
Genistein 10 μ*M*	9.64±0.080 (8%)	9.48±0.35 (4%)	96
Genistein 0.5 μ*M*	0.47±0.016 (3%)	0.46±0.032 (7%)	93
Genistein 0.02 μ*M*	0.017±0.001 (6%)	0.018±0.001 (8%)	88
Daidzein 10 μ*M*	9.15±1.03 (11%)	8.86±0.33 (4%)	90
Daidzein 0.5 μ*M*	0.46±0.024 (5%)	0.46±0.0 (3%)	93
Daidzein 0.02 μ*M*	0.17±0.001 (3%)	0.018±0.001 (5%)	88
Equol 10 μ*M*	10.22±1.33 (13%)	9.61±0.037 (4%)	99
Equol 0.5 μ*M*	0.49±0.023 (5%)	0.48±0.016 (3%)	98
Equol 0.02 μ*M*	0.016±0.001 (4%)	0.017±0.001 (5%)	97

Control rat serum containing undetectable amounts of isoflavones was fortified with aglycones at three levels and processed on two separate days as described in the Experimental section and analyzed using LC–ES/MS/MS. The mean isoflavone concentrations (*n*=4) are shown with the respective SD and RSD (%), and the accuracy compares all measurements with the concentrations added.

Table 3
Determination of total and aglycone genistein in mouse serum

Dietary genistein (μg/g)	Serum total genistein (μM)	Serum aglycone genistein (μM)	% Aglycone
0	<LOQ	<LOQ	–
250	1.79±0.74	0.11±0.03	8.3±1.8
500	4.06±1.50	0.14±0.09	4.5±1.0
1000	10.70±7.26	0.28±0.12	6.6±1.7

Serum was analyzed using LC–ES/MS/MS for genistein with and without enzymatic deconjugation from groups of 6–7 female SCID mice fed a semi-purified AIN diet containing different amounts of genistein. The values shown are means±SEM (n=6–7).

from mice that had received several genistein-fortified diets. The percent present aglycone ranged from 2 to 15%, with a mean for all animals of 6.5% (n=20). Table 3 shows the results for each dose group and no dose-related trend was observed for the aglycone percentage. This fraction is significantly higher than observed previously in Sprague–Dawley rats (1–2%, [8]). We have observed similar aglycone distribution in another study of mice receiving dietary genistein (unpublished). It will be of interest to determine whether a corresponding increase occurs in the accumulation of genistein aglycone in mouse tissues relative to rats [4].

These results show that MS–SIM and MS/MS–MRM yield similar performance for the analysis of human and rodent serum/plasma, recognizing that the higher sensitivity of MS/MS–MRM detection permits measurement of isoflavones at lower serum concentrations or in smaller sample volumes. Advantages in sample throughput beyond our previous studies were realized from decreasing chromatographic analysis time to 7 min by eliminating time-consuming column switching and gradient reequilibration steps, and by employing parallel SPE processing of up to 96 samples simultaneously. These method modifications are required for the anticipated increases in analytical measurements that are presaged by the increasing prevalence of clinical trials to investigate biological effects of isoflavones in humans.

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