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Development of a Simple, Fast, and Accurate Method for the Direct Quantification of Selective Estrogen Receptor Modulators Using Stable Isotope Dilution Mass Spectrometry

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ABSTRACT: A rapid analytical procedure was developed to quantify major selective estrogen receptor modulators (SERMs) simultaneously using stable isotope dilution mass spectrometry (SID-LCMS). Two novel isotopically labeled (SIL) analogues of natural SERMs, genistein and daidzein, were synthesized using a H/D exchange reaction mechanism. Computational chemistry coupled with MS and NMR data confirmed the site and mechanism of deuteration. The SIL analogues, which were mono- and dideutero substituted at the ortho positions, exhibited minimal deuterium isotope effects and were stable under the employed sample preparation protocol and MS analysis. An isotopic overlap correction was successfully employed to improve the accuracy and precision of the analytical method. The developed method, which was found to be sensitive, selective, precise and accurate, could be a valuable tool for research focused on determining the bioavailability of individual SERMs.

KEYWORDS: *isoflavones, selective estrogen receptor modulators, isotope dilution mass spectrometry*

INTRODUCTION

Selective estrogen receptor modulators (SERMs) are non-hormonal compounds that can bind to estrogen receptors and selectively interact with specific coactivators and corepressors depending on the type of tissue. Tamoxifen and raloxifene (Figure 1) are two of the only three synthetic SERMs that are approved by the Food and Drug Administration (FDA) for human use. Naturally occurring isoflavones also exhibit SERM activity.[1−3] Upon ingestion, the metabolic pathway of SERMs and their ensuing bioactivity is dictated by their chemical structure. For instance, equol, which is a metabolite of daidzein, is more estrogenic than daidzein, while the genistein metabolite *p*-ethyl phenol (Figure 1) is not estrogenic.[4]

Liquid chromatographic (LC) techniques coupled with mass spectrometry (MS) are the preferred analytical methods for isoflavone analysis in biological fluids.[5−8] To account for losses during sample preparation, researchers have utilized a variety of internal standards including structural analogues such as apigenin,[9] biochanin A,[10] fluorescein,[11] and dihydroxyflavone.[12] Researchers also used stable isotopically labeled (SIL) analogues, either deuterium (^{2}H) or carbon-13 (^{13}C) labeled, coupled with stable isotope dilution LC−MS (SID−LC−MS) analysis.[13,14] Stable isotopically labeled analogues, which are chemically identical to their respective analytes, have a great advantage over structural analogues because they experience similar chemistry during sample preparation and analysis (chromatography and ionization). One of the major deliverables of a National Institute of Health (NIH) sponsored scientific workshop on soy isoflavone research[15] was the importance of using appropriate SIL analogues to guarantee better quantitation accuracy and traceability.

Because of their high isotopic stability, ^{13}C labeled analogues are preferred over ^{2}H labeled analogues. Nevertheless, ^{2}H labeled analogues are gaining popularity due to their simple synthesis approaches (hydrogen/deuterium (H/D) exchange) compared to the chemical synthesis of ^{13}C labeled analogues. Investigators who had used ^{2}H labeled SIL analogues in their experimental procedures have used trideutero derivatives,[16] tetradeutero derivatives,[17] pentadeutero derivatives,[18] or hexadeutero derivatives of SERMs.[18] However, chromatographic separation of deuterated and proteated isotopologues is commonly observed due to deuterium isotope effects.[19] Wang et al.[20] reported that a chromatographic separation between the analyte and its deuterated analogue can cause up to 25% difference in their ion suppression(s), resulting in an inaccurate analyte-to-internal standard peak ratio.

In addition to the current predicament in the choice of internal standards, available literature lacks an accurate method that can simultaneously quantify all major SERMs. Isoflavones can interact with certain cytochrome class enzymes that take part in the metabolism of tamoxifen, thus altering its physiological activity.[21,22] Although, there is no information on raloxifene−isoflavone interaction on the raloxifene metabolic pathway, a report recommended the use of phytochemicals in tandem with raloxifene to improve its bioavailability.[23] Thus, there is a growing interest to test synthetic SERMs concomitantly with natural isoflavones in an effort to effectively treat various health issues. Therefore, in response to these considerations, the overall objective of this study was to develop and validate an accurate and rapid analytical procedure

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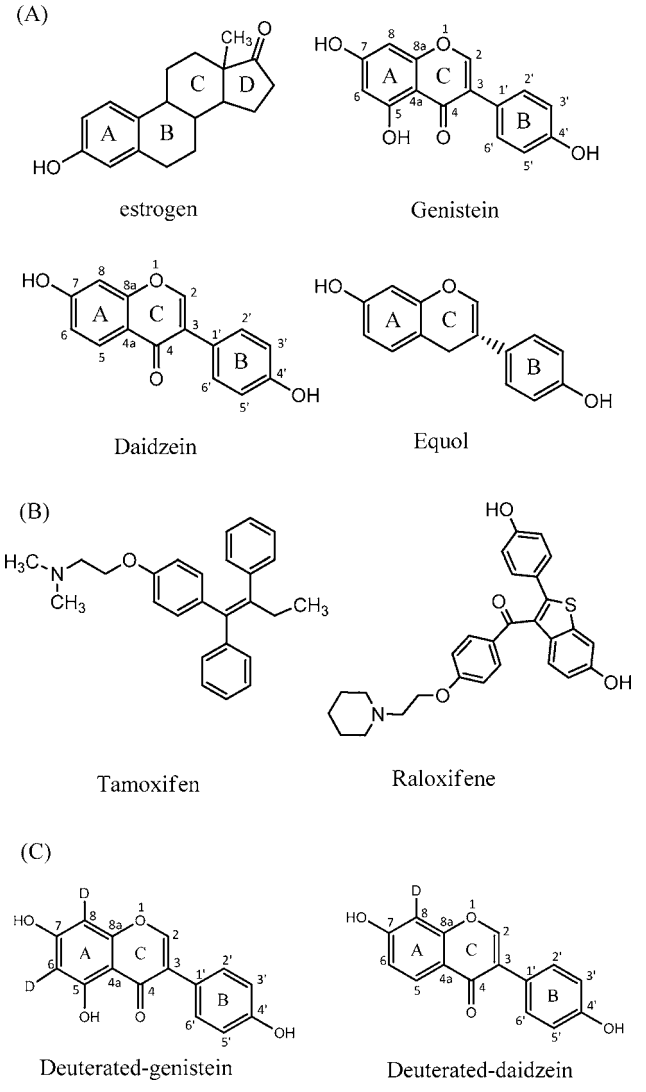

Figure 1. (A) Structures of human estrogen, genistein, daidzein, and equol. (B) Structures of tamoxifen and raloxifene. (C) Structures of deuterated genistein, 6,8-dideutero-5,7-dihydroxy-3-(4-hydroxyphenyl) chromen-4-one, and deuterated daidzein, 8-monodeutero-7-hydroxy-3-(4-hydroxyphenyl) chromen-4-one.

to quantify tamoxifen, raloxifene, genistein, daidzein, and equol simultaneously in plasma using SID-LCMS.

MATERIALS AND METHODS

Materials. High performance liquid chromatography (HPLC) grade acetonitrile and methanol were purchased from Fisher Scientific (Hanover Park, IL, USA). The isoflavones genistein and daidzein were purchased from LC Laboratories (Woburn, MA, USA). Tamoxifen was purchased from MP Biomedicals (Santa Ana, CA, USA). Sulphatase/glucuronidase enzyme (S9626), deuterated methanol (CD_3OD), deuterium oxide (D_2O), raloxifene, phenolphthalein-β-D-glucuronide, *p*-nitrocatechol sulfate, phenolphthalein, and *p*-nitrocatechol were purchased from Sigma Aldrich (St. Louis, MO, USA). Deuterated standards tamoxifen-d_5, raloxifene-d_4, and equol-d_4 were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Rat plasma was generously donated by Professor Daniel Gallaher (University of Minnesota, St. Paul, MN, USA).

Reagents. Sodium citrate buffer (0.01M, pH 5.0): equal volumes of sodium citrate (0.05 M) and citric acid (0.05 M) solutions were mixed and diluted to a final concentration of 0.01M. The pH was adjusted to 5.0 using HCl (0.05 M). Sulphatase/glucuronidase enzyme: the enzyme solution was prepared in sodium citrate buffer (0.01 M, pH 5.0) to a final enzyme activity of 500 U/mL of sulphatase and ~15,000 U/mL of glucuronidase.

Reference Standards. Reference standards of phenolphthalein-β-D-glucuronide, *p*-nitrocatechol sulfate, and *p*-nitrocatechol were prepared in double distilled water (DDW) (500 mg/L). Reference standards of phenolphthalein, genistein, daidzein, raloxifene, tamoxifen, equol, deuterated standards of daidzein and genistein, tamoxifen-d_5, raloxifene-d_4, and equol-d_4 were prepared in 80% aqueous methanol solution (500 mg/L). Reference standards of genistein and daidzein (500 mg/L) were also prepared in deuterated methanol (CD_3OD) for use in the preparation of their respective deuterated standards.

Working Standards. Reference standards were diluted in either DDW or 80% aqueous methanol solution to obtain working standards. Working standards of individual compounds were diluted to 1 μg/L for MS analysis. Working standards for calibration were (1) a cocktail of the analytes (genistein, daidzein, equol, tamoxifen, and raloxifene) at concentrations ranging from 20 μg/L−18 mg/L and (2) a cocktail of all the respective internal standards at a concentration of 6 mg/L prepared in 80% aqueous methanol solution. Working standards of a cocktail of phenolphthalein-β-D-glucuronide, *p*-nitrocatechol sulfate, phenolphthalein, and *p*-nitrocatechol (10 mg/L) were also prepared in DDW after appropriate dilutions using the reference standards. Validation standards were also prepared in 80% aqueous methanol solution at three different levels 200 μg/L, 2 mg/L, and 15 mg/L.

Preparation of Isoflavone Deuterated Standards. Genistein and daidzein were individually dissolved to a final concentration of 25 mg/L in 94.9:5:0.1 (v/v) D_2O, CD_3OD, and deuterated formic acid. Deuteration solvent composition was optimized to obtain rapid and complete deuteration. Genistein solution was incubated for three days, while the daidzein solution was incubated for five days in a heating block maintained at 90 °C. After incubation, deuterated isoflavones were separated from the reaction volume using Sep-Pak C_{18} reverse-phase cartridges (Water's Associates, Milford, MA, USA). Before sample loading, Sep-Pak C_{18} cartridges were primed with 2 mL of methanol, followed by conditioning with the same volume of DDW. The sample was then loaded onto the cartridge, and the deuterated formic acid was subsequently washed away with 3 mL of DDW. The deuterated isoflavones were recovered with 1.2 mL of methanol, which aids in reinstating the protic hydroxyl groups via H/D back exchange. Methanol was subsequently evaporated using a Speed-Vac evaporator (Savant, DNA110), and the residue was either redissolved in 5% (v/v) acetonitrile solution or dimethylsulfoxide (DMSO)-d_6 and immediately analyzed by MS and nuclear magnetic resonance (NMR) to determine the extent of deuteration.

Determination of Deuteration Site. *MS Analysis.* Individual solutions (1.0 μg/L) of isoflavones and deuterated isoflavones were directly infused into the heated electrospray ionization (HESI) interface of a triple stage quadrupole mass spectrometer (5500 QTRAP, AB Sciex, Washington, DC, USA) operating in the positive ion mode. The Q1 and Q3 mass resolutions were set at 0.4 Da (Da) full-width-at-half-maximum (fwhm). Fifteen spectra were collected with a scan time of 1 s. Instrument parameters, namely, sheath gas flow (N_2, 99.99%, flow rate = 5−20 units), vaporization temperature (150 °C), collision cell exit potential (10−17 V), spray voltage (4.0−4.5 kV), entrance potential (5−18 V), declustering potential (38−55 V), and collision energy (15−35 units), were optimized for each isoflavone such that ions of interest were produced in measurable abundance. Tandem MS/MS was employed to determine the fragmentation pathway of the isoflavones and deuterated isoflavones. The precursor ions ($[M+H]^+$) were isolated and analyzed by collision induced dissociation (CID), and product ion spectra were recorded. The collision energy was set to a value (30 units) at which ions of interest were produced in measurable abundance. Spectra were collected in triplicate.

Proton NMR Experiments. NMR experiments were carried out on a Varian 500 MHz Inova spectrometer equipped with a 5 mm triple resonance probe. Proton spectra for isoflavones and deuterated isoflavones were measured in (DMSO)-d_6 at ambient temperature. As

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a nonprotic solvent, (DMSO)-d_6 facilitates the detection of phenol hydroxyl proton resonances due to the absence of proton−deuterium (H/D) exchange, which is commonly observed when protic solvents such as deuterium oxide (D_2O) or CD_3OD are used. Chemical shifts (δ) were referenced to the central solvent signal of (DMSO)-d_6 (δH 2.50 ppm).[24] J values are given in hertz. NMR assignments follow the numbering shown in Figure 1.

Genistein. (500 MHz, DMSO-d_6): H6, 6.21 (d, 2.4 Hz); H8, 6.37 (d, 2 Hz); H3′ and H5′, 6.81 (d, 8.4 Hz); H2′ and H6′, 7.36 (d, 8.4 Hz); H2, 8.31 (s); C4′−OH, 9.57 (s); C7-OH, 10.86 (s); C5-OH, 12.94 (s). Deuterated genistein: H3′ and H5′, 6.81 (d, 8.4 Hz); H2′ and H6′, 7.36 (d, 8.4 Hz); H2, 8.31 (s); C4′−OH, 9.56 (s); C7-OH, 10.85 (s); C5-OH, 12.93 (s). Daidzein: H3′ and H5′, 6.81 (d, 8.4 Hz); H8, 6.85 (s); H6, 6.93 (d, 8.9 Hz); H2′ and H6′, 7.38 (d, 8.4 Hz); H5, 7.96 (d, 8.7 Hz); H2, 8.28 (s); C4′−OH, 9.53 (s); C7-OH, 10.78 (s). Deuterated daidzein: H3′ and H5′, 6.80 (d, 8.4 Hz); H6, 6.93 (d, 8.9 Hz); H2′ and H6′, 7.38 (d, 8.4 Hz); H5, 7.96 (d, 8.7 Hz); H2, 8.28 (s); C4′−OH, 9.53 (s); C7-OH, 10.78 (s).

Quantum Mechanical Modeling of Genistein and Daidzein. Density functional theory (DFT) was employed to perform quantum mechanical modeling of genistein and daidzein. Structures of both genistein and daidzein were optimized to the lowest energy conformations using DFT calculations at the B3LYP level and 6-31G(d,p) basis set using Gaussian 03 software[25] (Gaussian, Inc. Wallingford, CT, USA). The chosen basis set and polarization functions yielded excellent results for structurally similar compounds.[26] Single point energy calculations were performed on the lowest energy conformations of genistein and daidzein at the same level and basis set to plot their electrostatic potential (ESP) maps. Mulliken charges were also determined, which serve as a good indicator for estimating partial atomic charges.

Optimization of the Hydrolysis Conditions of Sulphonated and Glucuronidated Isoflavones. In triplicate, an aliquot (20 μL) of rat plasma was mixed with 10 μL of a solution containing both phenolphthalein-β-D-glucuronide and p-nitrocatechol sulfate (10 mg/L), vortexed, and sonicated for 10 min. Sulphatase/glucuronidase enzyme (200 μL) was added, and the samples were incubated at 37 °C and pH 5 for 15, 30, 45, 60, or 360 min. After hydrolysis, the synthetic substrates and products were extracted into ethyl ether (1 mL × 3), vortexed, and centrifuged at 5,000g for 10 min at 15 °C. The supernatant was evaporated under a stream of nitrogen gas, and the residue was dissolved in 200 μL of 80% aqueous methanol. Samples were stored at −80 °C or analyzed immediately by LC-MS. The time required for complete hydrolysis of the synthetic substrates was determined by monitoring their complete disappearance and appearance of their respective deconjugated forms (phenolphthalein and p-nitrocatechol).

Stability of the Synthesized Deuterated Standards. The stability of the synthesized SIL analogues was tested after subjecting them to the optimized enzymatic conditions by monitoring their isotopic profile before and after the enzymatic hydrolysis. An aliquot (10 μL) of SIL analogues of genistein or daidzein (200 μg/L) was added, in triplicate, to rat plasma (20 μL), which was then subjected to the optimized hydrolysis conditions followed by the extraction procedure described above. The final concentration of SIL analogues in the extract was 10 μg/L. Stability was also monitored during MS analysis by varying the vaporization temperature from 100 to 400 °C, with a step size of 100 °C, during SID−LC−MS analysis.

Calibration. An aliquot (10 μL) of each of the six working standards containing all five SERMs (20 μg/L, 1 mg/L, 2 mg/L, 6 mg/L, 12 mg/L, and 18 mg/L) and an aliquot (10 μL) of the cocktail containing the respective SIL analogue of each SERM (6 mg/L) were added, in triplicate, to 20 μL of plasma, which was then subjected to the optimized hydrolysis conditions followed by the extraction procedure described above. The final concentrations of the five SERMS in the standard extracts were 1, 50, 100, 300, 600, and 900 μg/L, and the final concentration of their respective SIL analogues in each standard extract was 300 μg/L. All standards were analyzed following the LC-MS method described below. Calibration curves were obtained by plotting the response ratio of the variable analyte to that of the constant internal standard against the analyte concentration. Analyte response was measured in multiple reaction monitoring (MRM) mode. An additional step was included to correct for the isotopic overlap between genistein/daidzein and their respective SIL analogues. Daidzein and genistein were run separately in the absence of their respective SIL analogues, and the MRM transitions of their natural isotopic peaks, which can interfere with their respective SIL analogues, were monitored. Subsequently, the obtained responses were subtracted from that obtained from the calibration.

LC-MS Analysis. LC-MS analysis was conducted on an ultrahigh pressure LC system (Shimadzu UFLC XR) online with a triple stage quadrupole mass spectrometer (5500 QTRAP, AB Sciex, Washington, DC, USA) equipped with a 50 × 2.1 mm inner diameter, 5 μm, YMC C_{18} column. The column temperature was maintained at 25 °C. An injection volume of 5 μL was chosen. A linear binary gradient at a flow rate of 0.4 mL/min with water and acetonitrile as solvents were used, with each containing 0.1% formic acid. The initial gradient concentration was 20% acetonitrile, which was kept constant for 1 min, linearly increased to 95% in 4.50 min, kept constant for 1 min, and followed by column equilibration steps. The LC column eluate entered the electrospray ionization (ESI) interface of the mass spectrometer operating in the positive ion mode. The MS parameters were sheath gas (N_2 99.99%, flow rate = 20 units); vaporization temperature, 150 °C; collision cell exit potential, 17 V; spray voltage, 4.5 kV; entrance potential, 10 V; declustering potential, 55 V; and collision energy, 28 units. Acquisition was carried out in the MRM mode so as to achieve maximal sensitivity and reliable quantitation over several orders of magnitude of compound abundance.[27,28] The MRM transitions of the analytes of interest are summarized in Table 1. Concentrations of SERMSs were calculated based on peak areas integrated by MultiQuant (version 2.0.2).

Table 1. Multiple Reaction Monitoring (MRM) Transitions of All the Compounds Used in the Present Study

analyte	MRM transitions	
	Q1[a] mass	Q3[b] mass
genistein	271	153
genistein-d_2	273	155
daidzein	255	199
daidzein-d_1	256	200
tamoxifen	372	72
tamoxifen-d_5	377	72
equol	243	105
equol-d_4	247	108
raloxifene	474	112
raloxifene-d_4	478	116
phenolpthalein	319	225
phenolphthalein β-D-glucuronide	495	225
p-nitrocatechol	156	123
p-nitrocatechol sulfate dipotassium salt	234	154

[a]First quadrupole. [b]Third quadrupole.

Validation of the Analytical Procedure. *Linearity.* Calibration curves were constructed by performing least-squares linear regression using Microsoft Excel (2010), and correlation coefficients (R^2) were determined. An R^2 value greater than 0.99 was considered acceptable.

Accuracy and Precision. Three validation standards of low range (10 μg/L), middle range (200 μg/L), and upper range (750 μg/L) of the calibration curve were prepared as follows. An aliquot (10 μL) of each of three working standards containing all five SERMs (200 μg/L, 4 mg/L and 15 mg/L) and an aliquot (10 μL) of the cocktail containing the respective SIL analogue of each SERM (6 mg/L) were added to 20 μL of plasma, which was then subjected to the optimized hydrolysis conditions followed by the extraction procedure described above. Extracts were analyzed following the LC-MS method described above. Accuracy was determined by comparing the measured

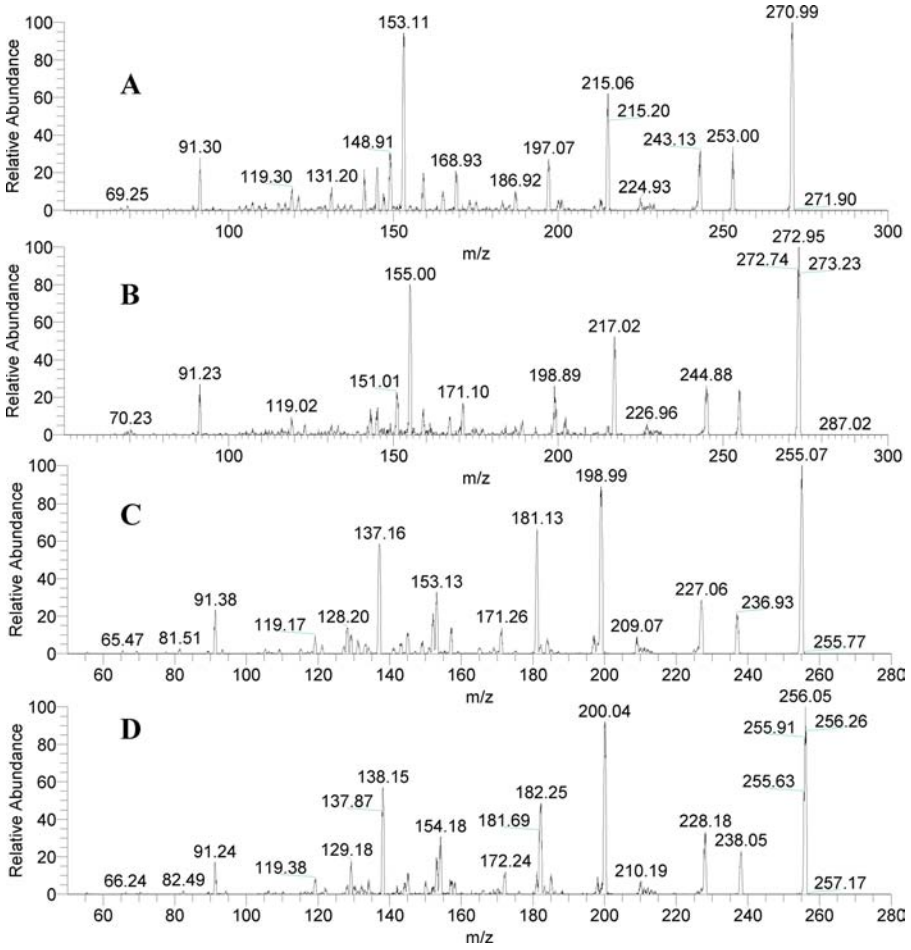

Figure 2. Tandem MS of (A) genistein, (B) deuterated genistein, (C) daidzein, and (D) deuterated daidzein.

concentration of the validation standards to the nominal concentration. Accuracy criterion for the measured concentration was set at nominal concentration ±7% (measured in terms of percentage relative error, % E_{rel}). Precision criteria were set at ≤7% (measured in terms of percent relative standard deviation, % RSD) for both intra-assay precision and instrument precision (reinjection repeatability).

Stability of the Working Standards. Working standards of the analytes (5 μg/L for daidzein and10 μg/L for the rest of the analytes) were analyzed, in triplicate, immediately after preparation and after being held at room temperature (25 °C) for 3 h. The stability of the SIL analogues was tested by monitoring their isotopic profiles before and after being held at 25 °C for 3 h. Stability of the validation standards was also monitored after holding them in the autosampler at 4 °C for 12 h. The analysis time never exceeded 12 h. The same acceptance criterion stated for the determination of accuracy and precision was chosen.

Carryover. A blank plasma extract was analyzed immediately after analyzing the standard with the highest concentration (900 μg/L). Concentration of the analytes should not be more than 5% of the lowest standard concentration (1 μg/L).

Method Application in a Model Rat System. Two male Wistar rats (100−125 g) were housed and cared for in the Research Animal Resources (RAR) facility by trained personnel. The RAR animal facility is guided by the Reagents' policy, USDA Animal Welfare Act, NIH Guide for the Care and Use of Laboratory Animals, AAALAC, and Public Health Service Policy. The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC). The rats were subjected to an adjustment period of 10 days, during which they were fed a casein-based diet following the formulation described by the American Institute of Nutrition (AIN − 93M). After the adjustment period, each rat was gavaged with either genistin or daidzin at a concentration of 100 μmol/kg body weight. The dose is based on an average intake of ∼10 mg/day of isoflavones by humans, which was converted to an equivalent amount based on an energy equivalent intake for rats compared to a human diet. Blood (125 μL) was collected from the saphenous vein of each rat at various time intervals including 0, 2, 4, 6, 8, 10, 12, and 24 h. The collected blood was centrifuged for 3 min at 4 °C, 6000*g*, and plasma was collected and stored at −80 °C until analysis. SIL analogues (300 μg/L) were added to the plasma samples on the day of analysis, and the plasma samples were subjected to the optimized hydrolysis and extraction condition as outlined above. Dilution of the extracts was experimentally determined to fit within the tested linear range. Isoflavone extracts were stored at −80 °C for later analysis. Plasma concentrations of genistein and daidzein and its metabolite equol were monitored following the SID−LC−MS analysis method outlined above.

Statistical Analysis. All statistical analyses including the calculation of the mean, standard deviation, coefficient of variation, percentage relative error, and linear regression analyses were performed using Microsoft Excel (2010).

RESULTS AND DISCUSSION

Structural Characterization of Deuterated Genistein and Daidzein. *Mass Spectrometry Analysis.* The employed deuteration conditions produced deuterated isoflavones with high isotopic purity (>98%). The quasi-molecular ion of genistein in the positive ion mode was *m/z* 270.99 $[M+H]^+$ and that of deuterated genistein was 272.95 $[M+H]^+$, indicating an incorporation of two deuteriums on the genistein molecule (Figure 2A,B). The main fragment ion of genistein was *m/z* 153 (Figure 2A), which has the A ring intact and is formed

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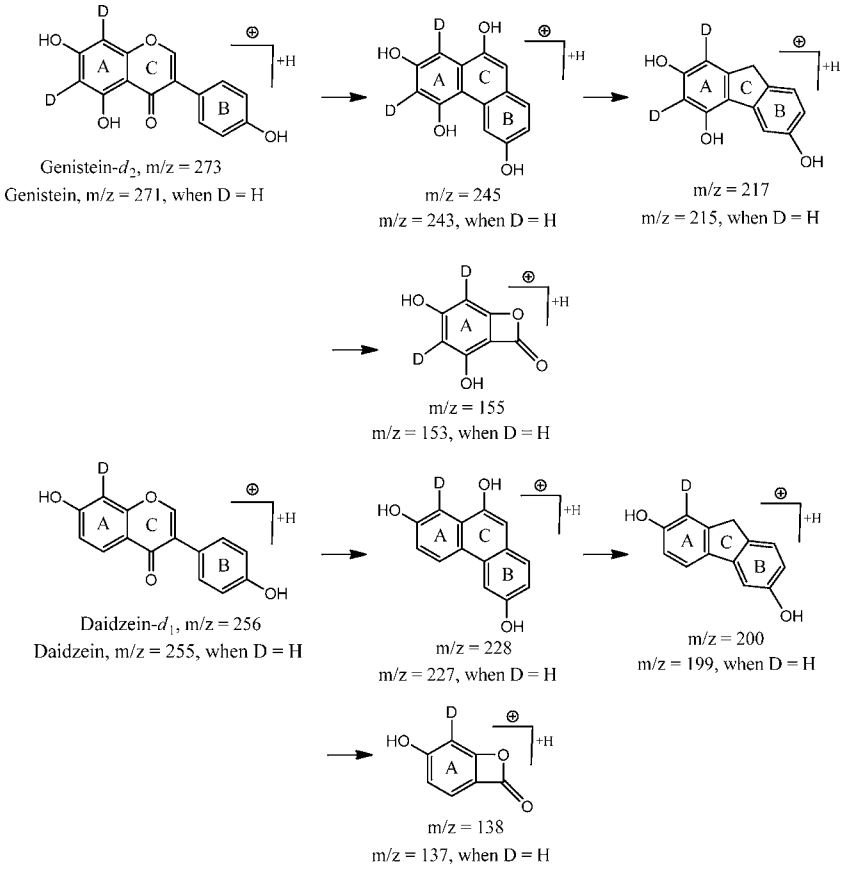

Figure 3. Predicted fragmentation pathway of quasi-molecular ions of genistein, deuterated genistein, daidzein, and deuterated daidzein.

from the parent compound by retro Diels–Alder fragmentation at the C ring (Figure 3). There was a 2 mass unit increase for this ion (m/z 155) in the deuterated genistein spectra (Figure 2B). Therefore, the site of deuteration on the genistein molecule is most likely at the ortho positions of the A ring.

The quasi-molecular ion of daidzein in positive ion mode was m/z 255.07. Unlike what was observed in the case of genistein, we only observed an increase of 1 m/z for deuterated daidzein (m/z = 256.05), indicating an incorporation of 1 deuterium on its structure (Figure 2C,D). The main fragment ion, observed in the fragmentation spectra of daidzein, was m/z 199. This fragment ion, which is composed of three fused benzene rings representing a phenanthrene framework, is formed by retro-Diels–Alder fragmentation upon the loss of 2CO groups at ring C ($[M+H-2CO]^+$) and has both A and B rings intact. The ion with m/z 137 has it is A ring intact (Figure 3). There was 1 mass unit increase for this ion (m/z 138) in the deuterated daidzein fragmentation spectrum (Figure 2D), indicating that the site of deuteration on the daidzein molecule was on the A ring, probably at an ortho position to C7 similar to what was observed for genistein.

NMR Analysis. NMR data obtained for genistein and daidzein were consistent with previously published data.[29,30] The signals corresponding to the H6 and H8 protons (δH 6.21 and δH 6.37 ppm, respectively) in the genistein spectra were absent in the deuterated genistein spectra. This indicates that the two protons present in the ortho positions to C7 in genistein took part in the H–D exchange, thus complementing the MS data. In the case of daidzein, only the H8 proton (δH 6.85) was replaced with deuterium. Thus, the deuterated standards of genistein and daidzein are 6,8-dideutero-5,7-dihydroxy-3-(4-hydroxyphenyl) chromen-4-one and 8-mono-deutero-7-hydroxy-3-(4-hydroxyphenyl) chromen-4-one, respectively (Figure 1C).

Quantum Mechanical Modeling. In spite of using an extended basis set and polarization functions, no differences in electron densities at the sites of deuteration were detected based on ESP maps (data not shown). To overcome this predicament, Mulliken charges on each atom were determined. Mulliken charges represent partial atomic charges, making it possible to probe the electron population in a given region of the molecule (e.g., sites of deuteration). Partial atomic charges were used to predict the reactivity preference of the abundant deuterium ion (D^+) in the deuteration of isoflavones. Although partial atomic charges are not quantum mechanical observables, the charge scheme employed could represent all of the properties that can be obtained from the quantum mechanical wave functions. Calculation of the Mulliken charges revealed that the C6 of the genistein molecule is more electron dense than C8. Thus, deuteration of genistein would be favored at C6 followed by C8. However, in the case of daidzein, C8 is more electron dense than C6. Thus, for daidzein, deuteration at C8 would be favored over C6. The difference in the preference of deuteration between genistein and daidzein is due to the presence of an additional hydroxyl group at C5 in genistein. Being an electron withdrawing group, the hydroxyl group reduces the electron density at the C5 position, which invokes a pronounced asymmetric distribution of electrons between C5 and C6, resulting in a higher electron density at C6. Further, a comparison of the overall partial atomic charges between genistein and daidzein at the deuteration sites reveals that the nucleophilic nature of genistein is greater than that of daidzein.

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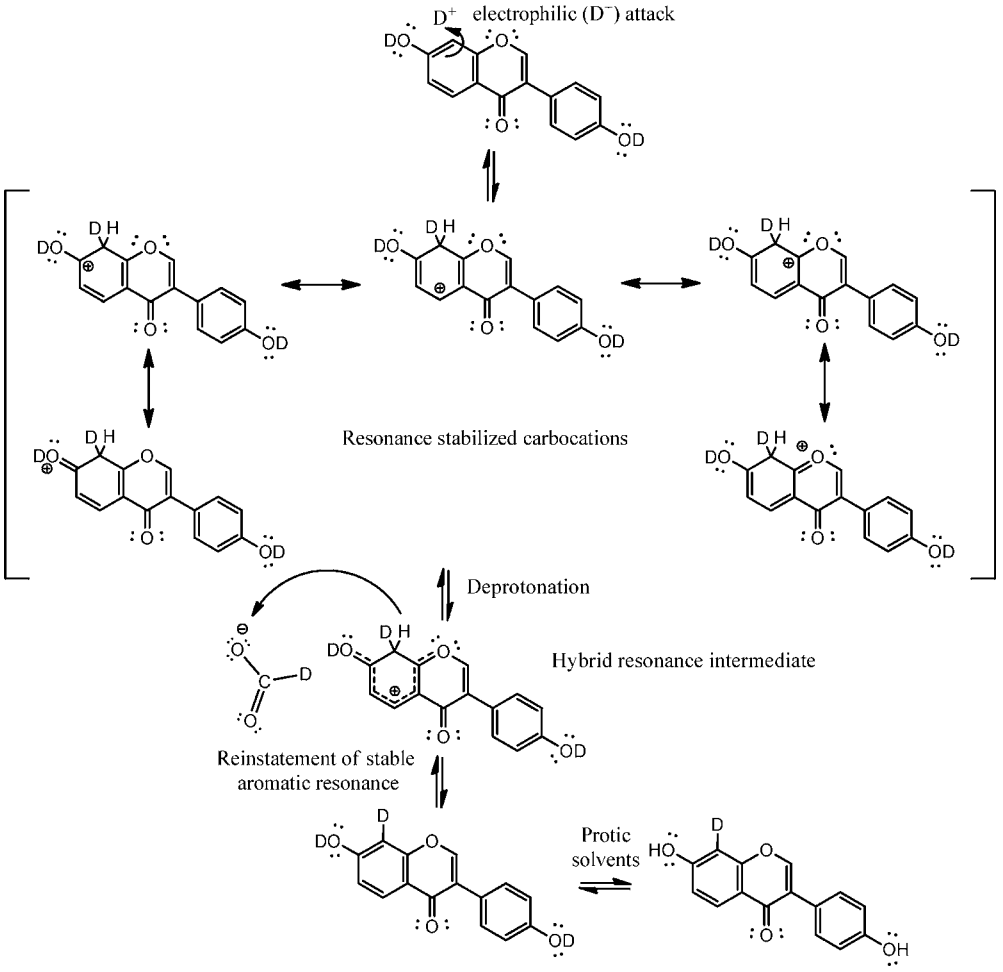

Figure 4. Five intermediate cyclohexadienyl cations involved in the electrophilic aromatic substitution of daidzein with the subsequent formation of stable deuterated daidzein.

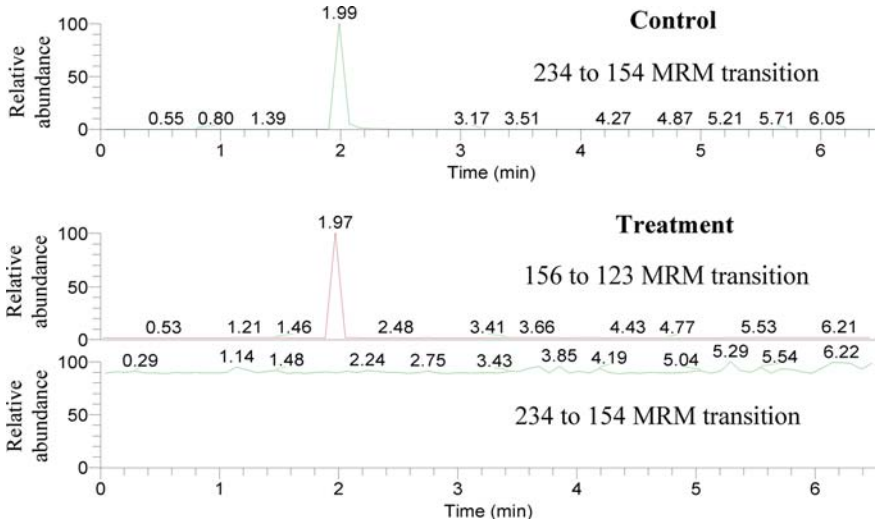

Figure 5. MRM of m/z = 234 to 154 transition for *p*-nitrocatechol sulfate and m/z = 156 to 123 transition for *p*-nitrocatechol, before and after incubation at 37 °C and pH 5 for 60 min.

This explained the necessity to incubate daidzein for an extended period of time (5 days) to achieve deuteration as compared to genistein (3 days).

A reaction in which the hydrogen attached to an aromatic system is replaced by an electrophile (D^+) is an example of the classical electrophilic aromatic substitution reaction.[31] The reaction is initiated with the addition of deuterium(s) to the π complex resulting in the formation of a resonance stabilized cyclohexadienyl cation intermediate (Figure 4). The presence of electronegative hydroxyl groups in the structures of

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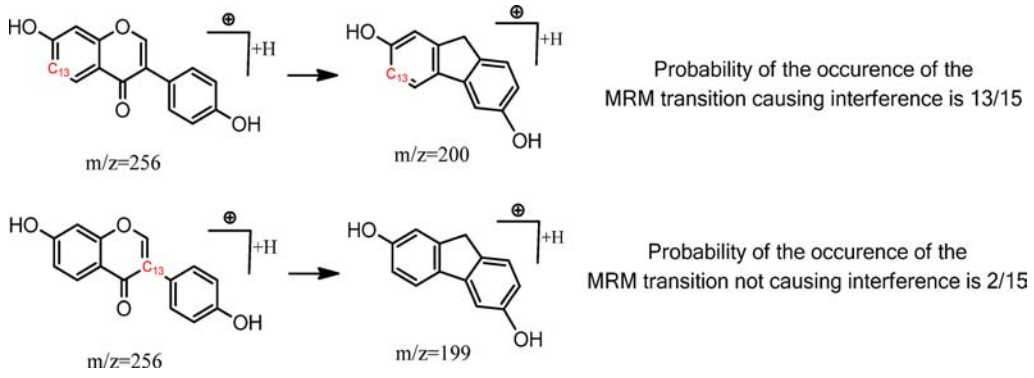

Figure 6. Probability of the occurrence of the higher isotope (^{13}C) in the daughter ion (m/z = 200) of daidzein monitored in the MRM mode.

isoflavones aids in the additional stabilization of the cyclohexadienyl cation intermediate. The hybrid resonance intermediate formed during the electrophilic aromatic substitution reaction allows delocalization of the electrons over a greater volume of the isoflavone molecule (five resonance forms) resulting in its enhanced stability (Figure 4). The final deuterated compound is formed by restoration of the aromatic sextet upon the loss of the original hydrogen bound at the site of the electrophilic attack.[32] Hydroxyl groups in the isoflavone structures also significantly affect the regioselectivity of deuterium substitution, predominantly favoring ortho or para substitution.[32] This is in agreement with the experimental results where substitution for both genistein and daidzein occurred at positions ortho to C7-OH and to C5-OH in the case of genistein.

It is reported that deuteration at the ortho positions has far less effect on isotopic fractionation when compared to that at meta or para positions.[19] Hence, we predict that the SIL analogues reported in this work will exhibit less deuterium isotope effects as compared to the tetra/tri/hexa deuterated isotopes of isoflavones.

Determination of Optimum Hydrolysis Time. Complete hydrolysis of phenolphthalein-β-D-glucuronide and *p*-nitrocatechol sulfate by sulphatase/glucuronidase was achieved after 60 min of incubation at 37 °C and pH 5 (Figure 5). Using MRM transitions, completion of hydrolysis was determined by the disappearance of the conjugated forms of the standards (phenolphthalein-β-D-glucuronide and *p*-nitrocatechol sulfate) and appearance of their respective deconjugated forms (phenolphthalein and *p*-nitrocatechol). After incubation for 60 min, as compared to the control, the presence of *p*-nitrocatechol sulfate (monitored by 234−154 MRM transition) was negligible (Figure 5). The disappearance of *p*-nitrocatechol sulfate was accompanied by the appearance of a new peak (156−123 MRM transition) corresponding to the MRM transition of *p*-nitrocatechol. The complete disappearance of phenolphthalein-β-D-glucuronide and the formation of its deconjugated form, phenolphthalein, were also accomplished after 60 min of incubation (data not shown).

Stability of SIL Analogues of Genistein and Daidzein. The optimized hydrolysis conditions (60 min at pH 5, 37 °C) did not impact the stability of the SIL analogues of genistein and daidzein. On the basis of the relative abundances of the ions that constitute the isotopic profiles of the SIL analogues, we observed no significant change in their intensities before and after hydrolysis, indicating the absence of H/D back-exchange in the SIL analogues of genistein and daidzein upon hydrolysis (data not shown).

Operation in the heated electrospray ionization (HESI) mode subjects analytes and their SIL analogues to high temperatures. In HESI mode, the auxiliary gas is heated to temperatures ranging between 50 to 400 °C to aid in solvent evaporation. These high temperatures can result in the loss of deuterium from the SIL analogues.[33] On the basis of the relative abundances of the ions that constitute the isotopic profiles of the SIL analogues, we observed no significant changes in the intensities for temperatures up to 300 °C. However, for temperatures greater than 300 °C, a decrease in the intensities of the monoisotopic deuterated peaks for SIL analogues (m/z = 273 for deuterated genistein and m/z 256 for deuterated daidzein) in their isotopic profiles was observed. This was accompanied by a subsequent increase in the intensities of the monoisotopic peaks of genistein (m/z = 271) and daidzein (m/z 255) indicating a loss in deuteration. However, the vaporization temperature was maintained at 150 °C for the duration of the SID−LC−MS analysis.

Proposed Changes to SID−LC−MS Methodology. One of the preferred conditions for convenient high accuracy SID−LC−MS is the absence of mass overlap between the analyte of interest and its SIL analogue. However, due to the choice of the SIL analogues for genistein and daidzein in the present study there was an overlap between the naturally occurring isotopes of genistein/daidzein (M+1 or M+2 peaks) and their respective SIL analogues. For example, the base peak of the molecular ion of daidzein-d_1 (m/z = 256 in positive ion mode) has the same mass as the naturally occurring ^{13}C isotopic peak of daidzein (M+1 peak, m/z = 256, in positive ion mode). It is thus difficult to separate these two ions during SID−LC−MS analysis when operating the MS in either single ion monitoring (SIM) mode or MRM mode. Unless taken into account, this isotopic overlap will introduce an error during the calculation of the isotopic ratio and may lead to an overestimation of the analytes of interest. The extent of the error, however, depends on the amount of the added internal standard relative to the analyte amount, and the abundance of the naturally occurring isotopic peaks that contribute to an isotopic overlap. To correct for this potential error, the isotopic profiles of the individual compounds that undergo isotopic overlap during analysis must be determined. On the basis of theoretical calculations, using the MassLynx software (Micromass, Water's Associates, Milford, MA, USA), the isotopic overlap is 16% in the case of daidzein (M+1 peak) and 2.2% in the case of genistein (M+2 peak). Experimentally, the abundances of the naturally occurring isotopic peaks were calculated relative to the abundance of the monoisotopic peak. The theoretical and the experimental isotopic profiles were similar, with standard

deviations less than 1% for the abundances of the monoisotopic as well as for the naturally occurring isotopic peaks (data not shown).

Several strategies were proposed by various researchers to correct the isotopic overlap between compounds of interest during SID−LC−MS analysis.[34−36] Of these strategies, the subtraction method was chosen for the present study. In spite of being a straightforward approach in single ion monitoring mode, the subtraction method in the MRM mode is rather challenging.[37] This is due to the fact that the distribution of the higher isotope in the [M+1] peak is random, and hence, the product ion formed upon fragmentation may or may not contain the higher isotope in its structure (Figure 6). Depending upon the product ion chosen for the analyte (the choice of which varies among researchers) and its SIL analogue, the occurrence of the higher isotope in the product ion(s) follows a probabilistic behavior. Thus, in theory, the probability of the occurrence of the higher isotope in the product ion (13/15 as shown in Figure 6) has to be multiplied by the higher isotope peak abundance (16% in case of daidzein) in order to account for the isotopic overlap. On the basis of this theoretical approach, the product ion, especially for monodeutero SIL analogues can be chosen such that the error due to the isotopic overlap is at its minimum. While a theoretical understanding can be established, we decided to experimentally eliminate the isotopic overlap.

Daidzein was analyzed separately using the same calibration protocol, and the MRM transition of daidzein-d_1, which is equivalent to the M+1 peak of the naturally present ^{13}C isotope, was monitored. The area response obtained was subtracted from the corresponding area responses obtained from the calibration assay that included the SIL analogues. Following this approach, we successfully eliminated the error caused by the isotopic overlap, and the linearity of the standard curve for daidzein was improved from R^2 = 0.95 before compensating for isotopic overlap to a R^2 value >0.99. A similar protocol was employed to compensate for the error due to the isotopic overlap between genistein and its SIL analogue, genistein-d_2. The obtained data strongly supported the viability of the isotopic correction strategy that was employed and subsequently provided a basis for the use of mono- or dideuterated internal standards for the quantification of isoflavones.

Validation of the Analytical Assay. *Linearity, Accuracy, and Precision.* All calibration curves were linear with R^2 values >0.99 within the concentration range tested. Accuracy (in terms of % E_{rel}) for all the analytes of interest varied between −4.55% and 5.91% (Table 2). Intra-assay precision (in terms of % RSD) also varied between 0.95% and 6.66% (Table 2). Instrument precision (reinjection repeatability) was also tested with % E_{rel} < 5% and % RSD < 8% (Table 3). Results indicated that the analytical method is both accurate and precise within the concentration range tested.

Stability. Working standards of the analytes were stable at room temperature (25 °C) after 3 h with % E_{rel} < 5% and % RSD < 5% (Table 4). Working standards of the SIL analogues were also stable, as there was no significant change in their isotopic profiles before and after holding them at room temperature for 3 h (data not shown). All validation standards were stable in the autosampler after 12 h at 4 °C, with % E_{rel} < 5.5% and % RSD <5% (Table 5).

Carryover. No carryover was observed. The concentration of the analytes observed after running a blank plasma extract was <5% of that of the standard with the lowest concentration (1 μg/L) (data not shown).

Method Application. The proposed analytical method was successfully applied in a rat system to quantitate the analytes of

Table 2. Accuracy and Precision of the Developed Analytical Method Determined upon Analysis of Three Validation Standards at 10, 200, and 750 μg/L[a]

	nominal concn (μg/L)	calcd concn (μg/L)	accuracy (% E_{rel})	precision (% RSD)
genistein	10	9.63	−3.63	4.69
	200	193.29	−3.35	4.44
	750	787.99	5.03	3.66
daidzein	10	10.33	3.37	0.95
	200	196.59	−1.71	1.98
	750	786.52	4.86	3.21
equol	10	9.74	−2.56	2.88
	200	192.11	−3.94	5.43
	750	733.72	−2.17	5.42
tamoxifen	10	10.56	5.67	1.43
	200	199.62	−0.186	6.66
	750	794.37	5.91	1.65
raloxifene	10	10.47	4.77	1.56
	200	190.89	−4.55	3.58
	750	771.38	2.85	2.23

[a]% E_{rel}, percent relative error; % RSD, percent relative standard deviation.

Table 3. Re-Injection Reproducibility Data to Determine Instrument Precision[a]

	first injection (μg/L)	second injection (μg/L)	accuracy (% E_{rel})	precision (% RSD)
genistein	9.95	9.92	−0.64	0.45
	190.95	190.21	−0.39	0.27
	780.33	798.3	2.23	1.56
daidzein	10.26	9.67	−2.91	4.23
	193.38	195.99	0.67	0.94
	804.03	781.18	−1.42	2.03
equol	9.54	10.63	5.71	7.63
	181.42	183.45	0.56	0.78
	727.24	691.39	−2.46	3.57
tamoxifen	10.67	10.52	−1.41	2.03
	188.31	202.89	3.87	5.27
	808.35	770.29	−2.35	3.41
raloxifene	10.59	10.51	4.77	1.56
	191.31	188.99	−0.61	0.86
	777.55	802.66	1.61	2.24

[a]% E_{rel}, percent relative error; % RSD, percent relative standard deviation.

Table 4. Stability of Working Standards of Analytes (10 μg/L) Held at Room Temperature (25°C) for 3 h[a]

	concn at 0 h (μg/L)	concn after 3 h (μg/L)	accuracy (% E_{rel})	precision (% RSD)
genistein	10.11	9.89	−2.22	1.56
daidzein	5.04	4.86	−3.70	2.57
equol	10.15	10.03	−1.20	0.84
tamoxifen	10.23	10.11	−1.19	0.83
raloxifene	9.96	9.84	−1.22	0.86

[a]% E_{rel}, percent relative error; % RSD, percent relative standard deviation.

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Table 5. Stability of the Validation Standards Held in the Autosampler at 4°C for 12 h[a]

	concn at 0 h (μg/L)	concn after 12 h (μg/L)	accuracy (% E_{rel})	precision (% RSD)
genistein	9.63	9.47	−1.63	1.16
	193.29	187.44	−3.02	2.17
	787.74	756.41	−3.97	2.87
daidzein	10.33	10.41	0.75	0.52
	196.59	200.03	1.75	1.22
	786.52	819.22	4.15	2.88
equol	9.74	10.01	2.73	1.96
	193.11	195.39	1.71	1.19
	733.72	732.98	−0.11	0.07
tamoxifen	10.56	10.96	3.81	2.63
	199.62	189.56	−5.04	3.65
	794.37	785.53	−1.12	0.79
raloxifene	10.47	10.53	0.51	0.36
	190.89	187.14	−1.96	1.41
	771.38	729.58	−5.41	3.93

[a]% E_{rel}, percent relative error; % RSD, percent relative standard deviation.

interest at concentrations within the linear range tested. Peak plasma concentration of daidzein (2.61 nmol/L) and genistein (9.11 nmol/L) was reached 4 h post-ingestion of daidzin and genisten, respectively (Figure 7). Plasma concentration of equol continued to increase over time. The rate of disappearance of daidzein in the plasma was slower than that of genistein.

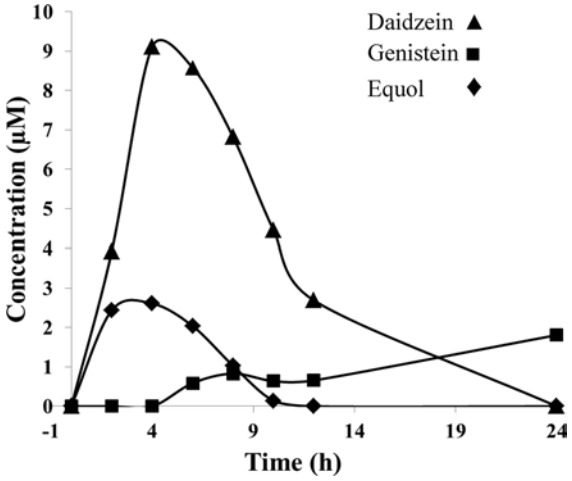

Figure 7. Plasma concentrations of daidzein, genistein, and equol obtained from two male Wistar rats at 0, 2, 4, 6, 8, 10, 12, and 24 h after being gavaged with a single dose of either genistein or daidzein at a concentration of 100 μmol/kg body weight.

Two deuterated SIL analogues of daidzein and genistein were successfully produced using a novel and simple approach. The deuteration approach followed in this study greatly reduced the efforts and costs associated with the preparation of SIL analogues following a syntheses-based approach using predeuterated starting materials. Results based on computational chemistry coupled with MS and NMR data confirmed the site and mechanism of deuteration. The SIL analogues produced, mono- and dideutero substituted at the ortho positions, exhibited minimal deuterium isotope effects and were stable under the employed sample preparation protocol and MS analysis. Differential matrix effects due to the slight differences in retention times between SIL analogues and their respective analytes were minimal. A strategy to eliminate errors due to the isotopic overlap between the synthesized SIL analogues of isoflavones and their respective analytes of interest was developed in the MRM mode, thereby improving the accuracy of the proposed analytical method. Applying this unique isotopic overlap correction strategy will allow for the expanded use of similar SIL analogues in SID−LC−MS analysis. This work provided, for the first time, a validated analytical SID−LC−MS method to detect natural and known synthetic SERMs in a single analytical assay. The method proved to be sensitive, selective, rapid, and accurate. Such an analytical method is useful to quantify SERMs in some biological matrices and could be valuable for research focused on determining the bioavailability of individual SERMs and the effect of isoflavones on tamoxifen/raloxifene metabolic pathways and vice versa.

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Notes

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