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Development of chiral liquid chromatography–tandem mass spectrometry isotope dilution methods for the determination of unconjugated and total *S*-equol in human plasma and urine☆

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ABSTRACT

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods for the determination of unconjugated and total (conjugated plus unconjugated) *S*-equol in human plasma and urine were developed and validated. The separation of *R* and *S* enantiomers was achieved with a Chiracel OJ-H column operated in a normal phase mode using ethanol/hexane mobile phase components. Ionization of *S*-equol by negative ion electrospray generated the $[M-H]^-$ ion whose response was augmented by post-column addition of ammonium hydroxide. A triple stage quadrupole mass spectrometer was used to measure the ion current generated from the dissociative transitions m/z 241 → m/z 121 (*S*-equol) and m/z 245 → m/z 123 (equol-d_4). The determination of total *S*-equol included an additional deconjugation step involving incubation of the sample with sulfatase and glucuronidase. Average recovery for both unconjugated and total *S*-equol was 85% with no observable matrix effects. Linearity was established for unconjugated *S*-equol from 0.025 ng/mL to 10 ng/mL (plasma) and 0.20 ng/mL to 200 ng/mL (urine). The average coefficient of variation and accuracy per occasion was within ±15% of the theoretical concentration of *S*-equol. The method was used to measure the pharmacokinetics of *S*-equol in human plasma after an oral administration of a single 20 mg dose of *S*-equol to three normal healthy volunteers.

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1. Introduction

Equol was first isolated from mare's urine in 1932 [1] but it was not until 2005 that Setchell et al. [2] isolated equol from human and rat urine and serum and determined its structure. Using chiral-phase HPLC and mass spectrometry it was shown that it is the *S*-enantiomer of equol that is found in plasma and urine; no *R*-equol was detected. *S*-Equol is produced naturally by the gut biotransformation of daidzein in certain individuals after ingestion of soy protein. Individuals who carry out this transformation may have certain health benefits including less vasomotor symptoms, prostate cancer, osteoporosis, metabolic and cardiovascular diseases and improvement in memory and cognition [3–8]. *S*-Equol is a potent, selective estrogen receptor (ER) β agonist with 10-fold less affinity for ERα [2,9] which makes it an attractive potential therapeutic agent (Fig. 1).

Many methods have been described for measuring equol in plasma and urine utilizing GC–MS, LC–MS and LC–MS/MS [2,10–27]. Equol is highly conjugated with less than 1% present as unconjugated *S*-equol. Thus, the reported methods have used an enzymatic step to remove the conjugates of equol prior to the measurements. Therefore, the stated lower limit of quantitation in the published reports is a measure of total (conjugated plus unconjugated) equol. To support a full pharmacokinetic profile in Phase I clinical trials [28], it was necessary to establish validated methods for both unconjugated and total *S*-equol. In this report, we describe both LC and MS modifications which provide a quantifiable detection limit of 25 pg/mL for enantiomerically resolved unconjugated *S*-equol. This method was used to measure the pharmacokinetics of unconjugated and total *S*-equol in plasma from three healthy subjects after administering a single 20 mg oral dose of *S*-equol.

2. Experimental procedures

2.1. Chemicals and reagents

Reference standard *S*-equol (purity 98.8%) was obtained from Girindus America, Inc. (Cincinnati, OH) and internal standard

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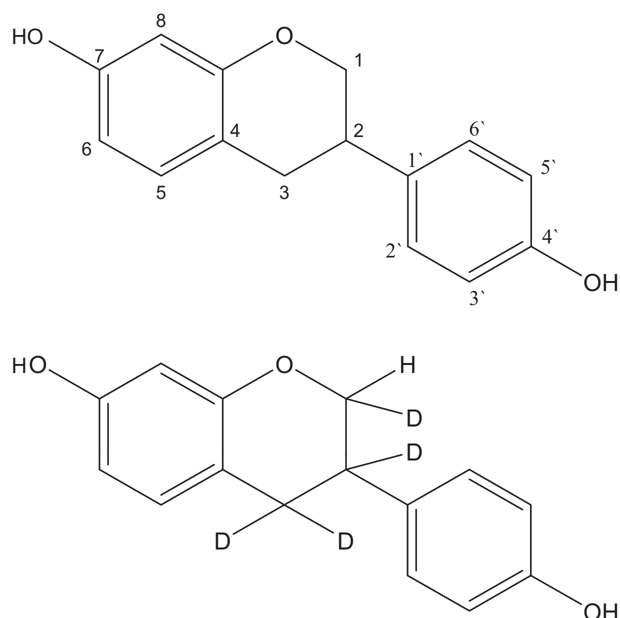

Fig. 1. Chemical structure of equol and equol-d_4 with stereocenter at C-2. The sulfate conjugate of *S*-equol is at position 7 and the glucuronide is at position 4′.

(*racemic* equol-d_4, purity 100%) from Toronto Research Chemicals (Toronto, Canada). H5 *pomatia* containing sulfatase and glucuronidase activity, phenolphthalein-β-D-glucuronide (PDG), methylumbelliferyl-sulfate (MUB), ammonium hydroxide (28%) and formic acid (LC–MS grade) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Methanol (LC–MS grade), hexane (95% *n*-hexane, HPLC grade), water (LC–MS grade) and ethanol (HPLC grade) were acquired from J.T. Baker (Phillipsburg, NJ, USA). Human plasma (K2-EDTA) and urine were purchased from Bioreclamation (Westbury, NY, USA).

2.2. Calibration standards and quality control

Stock solutions of *S*-equol and internal standard (*racemic* equol-d_4) were prepared in ethanol at concentrations of 1.00 mg/mL and stored at −20 °C. For the determination of unconjugated *S*-equol, calibrants were prepared in matrix covering the theoretical concentration range of 0.025–10 ng/mL (human plasma) or 0.20–200 ng/mL (human urine). Quality control (QC) samples were prepared in blank human plasma at theoretical concentrations of 0.025, 0.0750, 4.50 and 7.50 ng/mL, and in blank urine at theoretical concentrations of 0.200, 0.600, 90.0 and 140 ng/mL. Human donors were pre-screened in order to obtain blank plasma and urine suitable for calibrant and QC preparation. For total *S*-equol, the calibrants were prepared in water covering the theoretical concentration range of 2.50–2500 ng/mL (plasma) and 2.50–2000 ng/mL (urine).

2.3. Liquid chromatography

The enantiomeric resolution of *R*,*S*-equol was achieved using a Chiracel OJ-H (Chiral Technologies Inc., West Chester, PA, USA) supercritical fluid (SFC) column (50 mm × 4.6 mm, 5 μm particle size) with hexane and ethanol as mobile phase components mixed using an Agilent 1100 series binary pump modified for normal phase solvents. Sample injections (10 μL) were performed using a Shimadzu Sil HTC autosampler. Separation of enantiomers for total *S*-equol in human urine required isocratic elution with a mobile phase composition of ethanol:hexane (60:40, v/v) while total *S*-equol from human plasma required a more polar composition of ethanol:hexane (68:32, v/v). Flow rates were 0.75 and 0.90 mL/min for urine and plasma samples, respectively.

The method for unconjugated *S*-equol involved gradient elution for 1.0 min with 20% ethanol, followed by a 2 min ramp to 60% ethanol, held isocratically for 2 min, with subsequent re-equilibration with 20% ethanol for 3 min. The flow rate was 0.75 mL/min with post-column addition of 0.4% ammonium hydroxide in ethanol, introduced at 0.2 mL/min to improve mass spectral response by negative electrospray ionization (*vide infra*).

2.4. Tandem mass spectrometry

A Sciex API 5000 triple stage quadrupole mass spectrometer (Concord, ON, Canada) was utilized for the analysis of unconjugated *S*-equol, while a Sciex API 4000 was used for total *S*-equol given the higher detection limit requirement for the latter assay. For each method, the multiple reaction monitoring (MRM) scan function was used at an optimized center-of-mass collision energy of 19 eV for the dissociative pathways m/z 241 → m/z 121 (*S*-equol) and m/z 245 → m/z 123 (*racemic* equol-d_4) with 250 ms dwell times per transition. Parent ion formation in negative ion electrospray furnished $[M-H]^-$ ions whose response was augmented by post column addition of 0.4% ammonium hydroxide in ethanol. An electrospray potential of −4.5 kV was optimal at source temperatures between 400 °C and 550 °C.

2.5. Sample preparation and extraction

2.5.1. Unconjugated S-equol

Sample preparation involved protein precipitation coupled to solid phase extraction (SPE) using a Waters HLB Oasis® sorbent (30 mg, 96-well plate format). Extraction from urine involved treatment of a 50 μL sample aliquot with 200 μL of *racemic* equol-d_4 (1.0 ng/mL in acetonitrile:methanol; 1:1, v/v). Following centrifugation (14,000 rpm, 10 min, 4 °C), the supernatant fraction (200 μL) was diluted with 0.1% formic acid (1.0 mL), vortex mixed, and loaded onto the SPE sorbent previously conditioned with methanol and 0.1% formic acid (1.0 mL each). Samples were then washed with 1.0 mL each of 0.1% formic acid, 0.1% ammonium hydroxide, and 70% aqueous methanol. Plates were next centrifuged to remove water prior to elution with methanol (1.0 mL) followed by evaporation under dry nitrogen (50 °C) and reconstitution in ethanol (200 μL).

Solid phase extraction from plasma was similar to that of urine, but the larger sample volume (400 μL) required precipitation with 1.0 mL of *racemic* equol-d_4 in a modified precipitating solvent composition (1.0 ng/mL in acetonitrile:methanol; 25:75, v/v). A subsequent supernatant dilution with 0.6 mL of 0.1% aqueous formic acid was required before SPE loading. Following SPE, extracts were reconstituted in 100 μL of ethanol.

2.5.2. Total S-equol

Urine or plasma (50 μL) was precipitated directly in a Supelco Hybrid SPE-PPT 96-well plate with 200 μL of *racemic* equol-d_4 (25 ng/mL in acetonitrile:methanol; 1:1, v/v) and vortex mixed for 2 min to ensure complete precipitation. The precipitation plate was then coupled to a collection plate containing purified H5 *pomatia* and centrifuged (2000 rpm, 10 min, room temperature). The collection plate containing both protein precipitated extract and enzyme solution was next incubated at 37 °C for 2 h in a Thermo Instruments Precision shallow form shaking bath with moderate agitation. Following deconjugation, the contents of the incubation reaction were applied to a Waters HLB Oasis® sorbent (30 mg, 96-well plate format) and *S*-equol was extracted using the procedure detailed above with a final reconstitution volume of 0.5 mL.

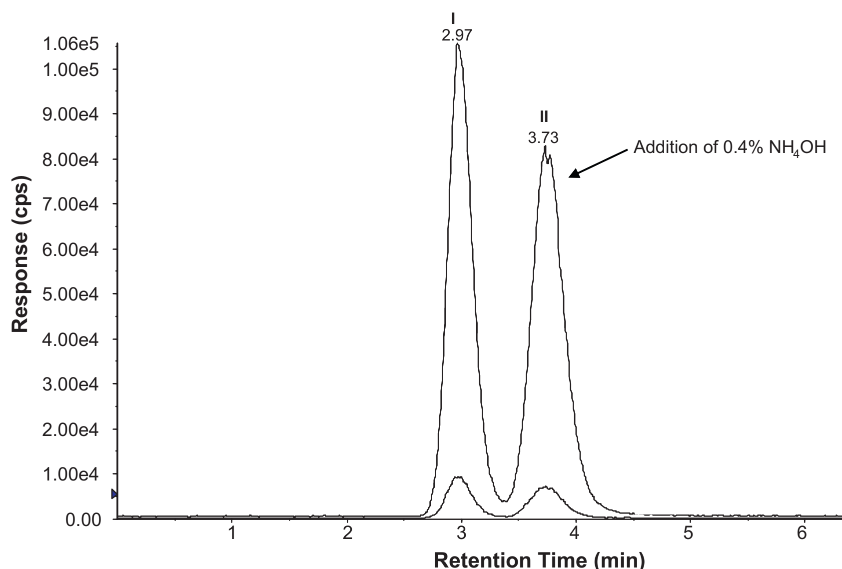

Fig. 2. Post-column addition of 0.4% ammonium hydroxide in ethanol augments *S* (I) and *R* (II)-equol response.

2.6. Verification of H5 pomatia sulfatase and glucuronidase activity

Plasma and urine *S*-equol is extensively conjugated [2,27,28]. Therefore, total *S*-equol was quantified following deconjugation with sulfatase- and glucuronidase derived from *Helix pomatia* type H-5 (H5 *pomatia*). As purchased, H5 *pomatia* was found to contain high endogenous levels of *S*-equol. Thus, it was necessary to purify the enzyme mixture using a Varian Bond Elute C18 sorbent (96 well plate format). Plates were conditioned with methanol (1.0 mL) followed by sodium citrate (1.0 mL, 25 mM, pH 5.0), whereupon aliquots of H5 *pomatia* solution (1.0 mL containing 11.5 units of sulfatase dissolved in 25 mM sodium citrate, pH 5.0) were loaded and collected in 96-well plates for sample incubation.

For each new batch of H5 *pomatia*, enzymatic activity (following purification) was verified by monitoring the depletion of PDG and MUB using LC–MS/MS. PDG and MUB activity markers were separated on a Waters Zorbax SB-CN column (75 mm × 4.6 mm, 5 μm particle size, Waters Corporation, Milford, MA, USA) operated at 25 °C with an acetonitrile/water mobile phase gradient at a flow rate of 1.0 mL/min. The gradient was initiated at 20% acetonitrile, ramped to 60% over 5 min and held isocratically for 1 min; the column was re-conditioned with 20% acetonitrile for 2.2 min. Acetic acid (0.5%) was added to each mobile phase component. The mass spectrometric dissociative transitions monitored for PDG and MUB were m/z 495 → m/z 319 and m/z 257 → m/z 177, respectively.

Sample preparation conditions utilized for enzyme activity verification were similar to those described for the extraction of total *S*-equol. Matrix (45 μL of human plasma or urine) was spiked with 5 μL of a solution containing 106 μg/mL of MUB and 204 μg/mL of PDG dissolved in water. Following precipitation with 100 μL of methanol:acetonitrile (1:1, v/v), the supernatant fraction (100 μL) was transferred to a 96-well plate containing purified H5 *pomatia* and incubated as detailed above. The contents of the incubation reaction were applied to a Phenomenex Strata-X SPE sorbent (30 mg, 96-well plate format) previously conditioned with methanol and 25 mM sodium citrate, pH 5.0 (1.0 mL each). Samples were then washed with 1.0 mL each of 0.1% formic acid and 20% aqueous acetonitrile, centrifuged to remove water (3000 rpm, 10 min, 4 °C), eluted with methanol (1.0 mL), evaporated (dry nitrogen at 40 °C), and reconstituted in 1.0 mL of acetonitrile:water:acetic acid (20:70:0.5, v/v/v).

2.7. Human plasma and urine samples

Plasma and urine were collected from three healthy volunteers after administration of a single oral dose of 20-mg *S*-equol. These individuals were part of a pharmacokinetics Phase 1 study carried out at Charles River Laboratories (Tacoma, WA, USA) [28]. The study protocol was approved by an independent institutional review board. The study was carried out in accordance with the Declaration of Helsinki and Good Clinical Practice and Good Laboratory Practice Guidelines. Samples were collected and stored at −80 °C prior to analysis. Plasma and urine samples anticipated to be above the upper limit of quantitation (ULOQ) were diluted with blank human plasma or urine.

3. Results and discussion

3.1. Optimization of LC–MS/MS for unconjugated S-equol extracted from human plasma

A previous report [2] showed that equol is present in plasma and urine only as the *S*-enantiomer; *R*-equol was not present indicating that *S*-equol was not converted to the *R*-enantiomer. Nonetheless, regulatory submissions for chiral entities require that the enantiomers be resolved making it necessary in the current investigation to separate the *R* and S diastereomers of equol. The enantiomeric separation of *R*,*S*-equol by normal phase LC required a mobile phase consisting of hexane and ethanol. Electrospray ionization with this solvent pair resulted in formation of the $[M-H]^-$ ion in negative ion mode with m/z 241. While the same ion was formed by negative APCI, the response was three-fold less sensitive. To augment electrospray ionization efficiency, it is common practice to add an acid or base modifier to the mobile phase such that ions are readily formed in the condensed state prior to nebulization and desolvation. However, the addition of base in the current chromatographic design had a deleterious effect on the chiral separation. Therefore, a solution of 0.4% ammonium hydroxide in ethanol was added post-column at a flow rate of 0.2 mL/min, resulting in a ten-fold increase in MS response for equol (Fig. 2). The increase in equol response was concomitant with a three-fold increase in baseline response, resulting in an overall signal-to-noise ratio improvement of 3:1.

The tandem mass spectrum of equol was characterized by predominant progeny ions with m/z 93, m/z 119, m/z 121, m/z 135,

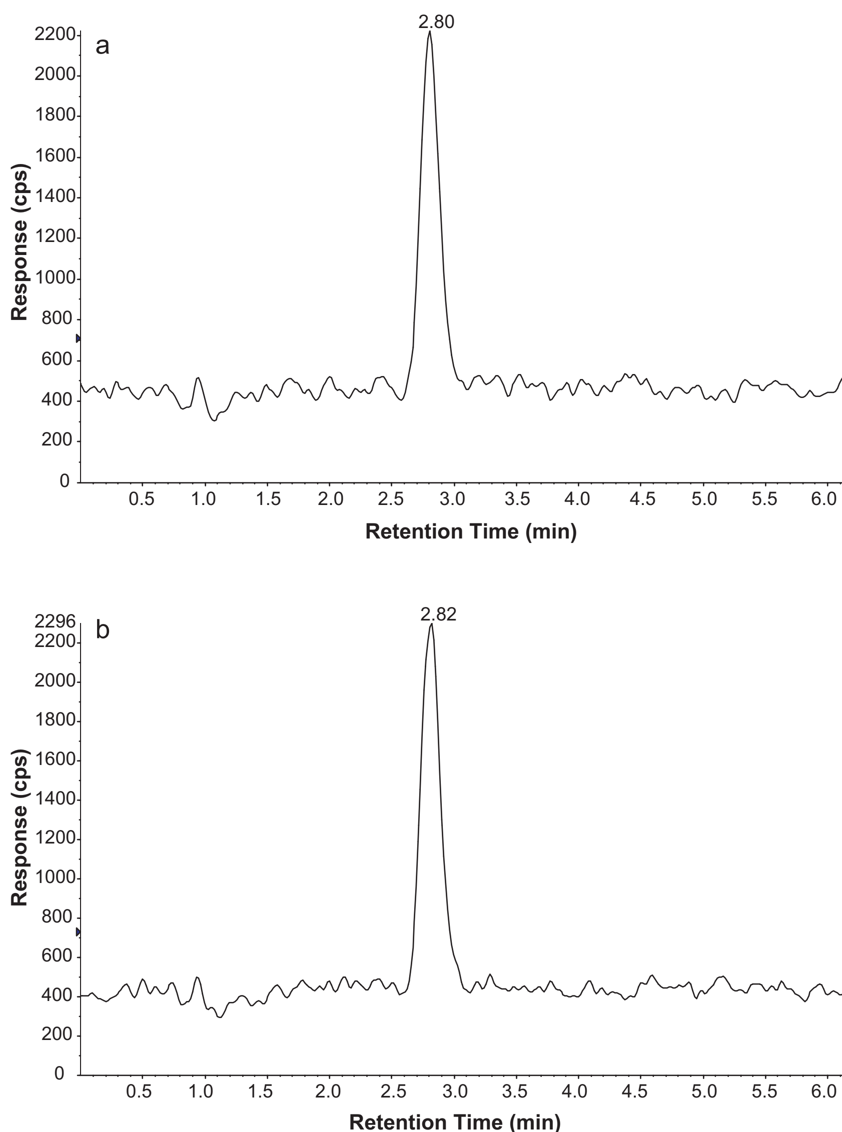

Fig. 3. Response comparison for *S*-equol spiked into (a) water and (b) urine at the LLOQ level and extracted using the method for total equol with isocratic elution (Section 2.3); equivalent recovery was noted with 1.98e^4 and 2.15e^4 area counts, respectively.

and *m*/*z* 147, formed by a retro Diels–Alder reaction. While *m*/*z* 119 and *m*/*z* 121 could be formed in equal abundance at their optimal collision energies, the *m*/*z* 121 progeny ion demonstrated better selectivity as noted by an approximate two-fold reduction in baseline noise. Therefore, the MRM transition *m*/*z* 241 → *m*/*z* 121 was used for quantitation purposes in order to achieve an optimal signal-to-noise ratio.

A previously described methodology by Setchell et al. [27] reports an LLOQ of 1.0 ng/mL for the measurement of total *S*-equol, with enantiomeric separation using a Chiracel OJ-H column (50 mm × 4.6 mm; 10 μm particle size) operated under isocratic conditions. In the current investigation, we chose the identical phase, but a smaller particle size (5 μm) to improve enantiomeric resolution while providing a narrower peak profile. The column was packed to SFC specifications in order to maintain a flow rate conducive to high throughput. In this manner, separation between enantiomers improved from a 10% valley to a 5% valley, with a reduction in base peak width from 60 s to 30 s. In addition to the improvements realized from reduced particle size, further gains in signal-to-noise ratio were realized, without loss of enantiomeric separation, using gradient elution, wherein baseline width was narrowed from 30 s by isocratic elution with ethanol:hexane (60:40, v/v) to 18 s when ramping from 20% to 60% ethanol over 2 min.

The increased gains in sensitivity from the combination of column particle size, gradient elution, and post-column addition of ammonium hydroxide resulted in an LLOQ of 0.025 ng/mL (1 pg on-column). This sensitivity was not required for total *S*-equol or unconjugated *S*-equol in urine, given the higher assay LLOQs of 2.5 ng/ml and 0.2 ng/mL, respectively. Nonetheless, the smaller particle sized column designed for SFC tolerance furnished an advantage in the isocratic mode used for these assays *via* reduced retention time without loss of enantiomeric separation. In this manner, retention times of 2.8 min and 3.26 min were achieved for *S*- and *R*-equol, respectively, with a run time of 5 min.

3.2. Optimization of deconjugation for the quantitation of total S-equol

The conjugates of *S*-equol were identified using the precursor ion scan function of the triple stage quadrupole mass spectrometer. The parent ion masses that form *m*/*z* 241 (i.e. the deconjugated

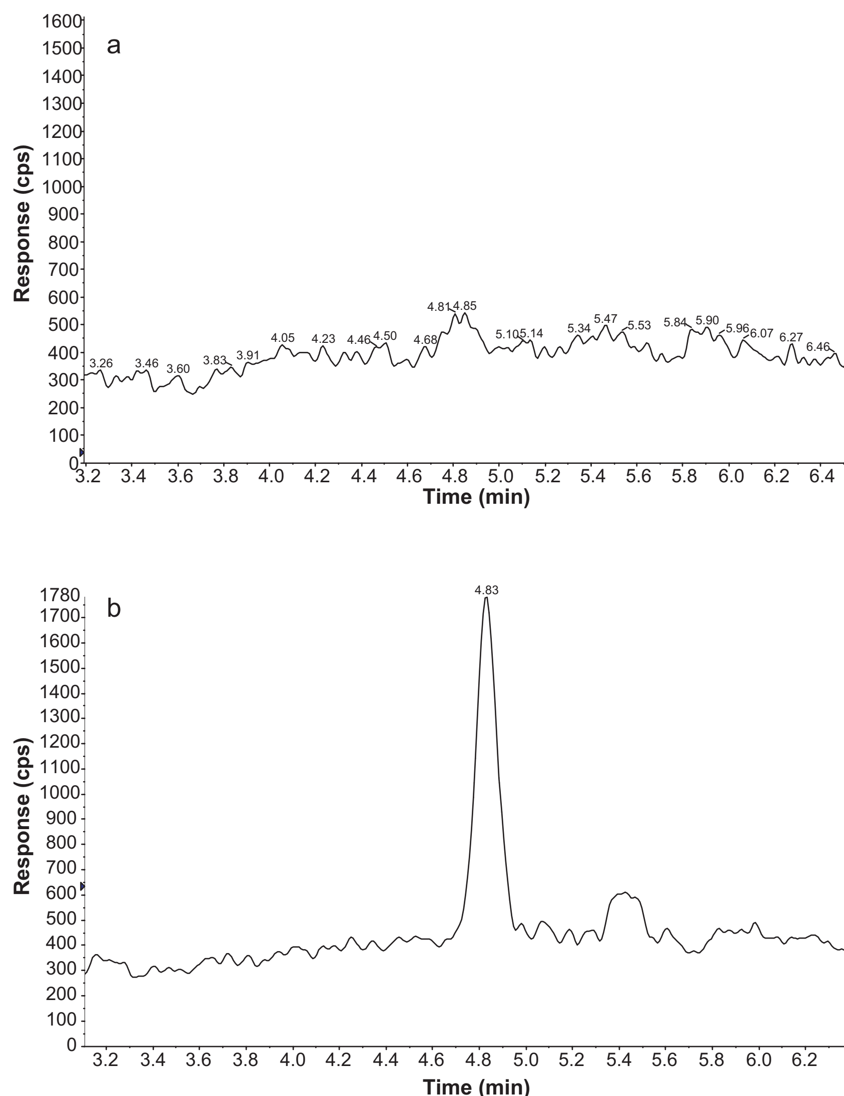

Fig. 4. Representative ion chromatograms using gradient separation (Section 2.3) for free *S*-equol extracted from human plasma samples for (a) double blank and (b) the lower limit-of-quantitation (25 pg/mL), achieved with a root mean square signal-to-noise (3σ noise) ratio of 7:1.

$[M-H]^-$ ion corresponding to *S*-equol), were identified at *m*/*z* 417, *m*/*z* 321, and *m*/*z* 248, corresponding to the theoretical masses for the glucuronide, sulfate, and doubly charged glucuronide + sulfate conjugates, respectively. Depletion of the conjugates was measured by monitoring the MRM transitions *m*/*z* 321 → *m*/*z* 241 (sulfate), *m*/*z* 417 → *m*/*z* 241 (glucuronide), and *m*/*z* 248 → *m*/*z* 241 (glucuronide + sulfate diconjugate).

The concentration of purified H5 *pomatia* required for deconjugation and incubation time was investigated with optimization defined as the minimal units of sulfatase activity required for maximal deconjugation (given sulfatase is the least abundant component in H5 *pomatia*). Incubation of rat plasma obtained from dosing at 2000 mg/kg with purified H5 *pomatia* at concentrations of 1, 2, and 4 mg/mL for 1 and 2 h at 37 °C demonstrated optimal deconjugation at 2 mg/mL (11.5 units sulfatase, 1170 units glucuronidase activity) with a 2 h incubation period. At a purified H5 *pomatia* concentration of 2 mg/mL, the amount of *S*-equol in the enzyme source was reduced to 0.15 ng/mL, or 6% of the LLOQ, which is consistent with validation guidelines [29].

Having established the optimal enzyme concentration and incubation period for deconjugation of total *S*-equol in plasma, these conditions were used on MUB and PDG substrates to verify H5 *pomatia* activity from lot-to-lot during validation and sample analysis. Concentrations of PDG and MUB used for verification of enzymatic activity corresponded to an equimolar equivalence of sulfate and glucuronide equol conjugates ten times the upper limit of quantitation (ULOQ) for total-*S*-equol. In this manner, a more accurate assessment of the dilution factors was possible for those samples exceeding the calibration range.

3.3. Establishment of standard addition technique

To determine plasma total equol concentrations in a general population, plasma and urine were obtained from twelve donors; concentrations of plasma total *S*-equol ranged from non-detectable to ~1 ng/mL, while in urine, concentrations were as high as 9 ng/mL in some donors. Consequently, in the absence of conjugated *S*-equol reference standards, it was necessary to use the technique of standard addition, wherein *S*-equol calibrants were prepared in water and or plasma from donors along with the addition of the internal standard *racemic* equol-d_4, the latter to determine matrix candidacy for QC preparation. The measurement of the total *S*-equol in screened matrix, based upon the mean concen-

tration derived from triplicate extracts per donor, was added to the theoretical concentration of the QC sample, with the criteria that endogenous concentrations not exceed 10% of the lowest QC. Since the recovery of *S*-equol was identical from aqueous calibrants and control matrix, and matrix effects were not observed from either plasma or urine, the preparation of calibrants in water is a valid approach for quantifying total *S*-equol in study samples (Fig. 3).

3.4. Sample preparation

Due to the propensity for *S*-equol to undergo protein binding, it was necessary to perform a seminal precipitation prior to SPE. In the case of total *S*-equol, precipitation was performed directly in a Supelco phospholipid 96-well removal plate to facilitate direct collection of extract into a 96-well plate containing purified H5 *pomatia*. In this manner, the number of sample transfer steps was minimized, thereby increasing sample processing efficiency.

In the absence of precipitation, recovery of *S*-equol by SPE was only 30%; when precipitation and SPE were coupled, average recovery increased to ∼85%. There were no differences in recovery when comparing protein precipitated supernatant fraction processed with and without the phospholipid removal plate. Due to the high affinity of *S*-equol for the polymeric HLB Oasis® sorbent, it was possible to wash extracts with up to 70% methanol without breakthrough. This level of extract cleanliness resulted in column longevities on the order of ∼1500 injections. Further, ion suppression was not observed for either the unconjugated or total *S*-equol assays as determined by comparison of a fortified blank matrix extract with the equivalent amount of standard in solution. A lack of H/D exchange for the internal standard was inferred by a consistent response for equol-d_4 within and between sample batches. In addition, control matrix fortified with equol-d_4 demonstrated a null response for equol.

3.5. Method validation

The validation of unconjugated *S*-equol in human urine and plasma included an assessment of selectivity, linearity, carry-over, inter- and intra assay precision and accuracy, stock solution stability, short and long-term matrix stability, freeze–thaw matrix stability, injection medium integrity, and dilution integrity. The assay for total equol was qualified with the assessment of linearity, inter- and intra assay precision and accuracy, injection medium integrity, and dilution integrity; notably, in the absence of *S*-equol glucuronide and sulfate reference standards, a full validation compliant with regulatory specifications was not feasible for these metabolites.

3.5.1. Assay selectivity

Method selectivity was assessed for unconjugated *S*-equol analyzing plasma and urine from six control donors. In addition, one donor was analyzed in duplicate in the prescence of equol-d_4, and one donor in the presence of *S*-equol, the latter fortified at the upper limit of quantitation. As is shown in Fig. 4a, a representative chromatogram of control plasma demonstrated the absence of interfering peaks. Similar selectivity was observed for control urine. In comparison, a lower limit of quantitation of 25 pg/mL could be achieved with a root mean square (3σ) signal-to-noise ratio of 7:1 (Fig. 4b). For blank samples spiked with equol-d_4, there were no significant interfering peaks at the retention time of *S*-equol; similarly, blank samples spiked with *S*-equol revealed no significant interfering peaks at the retention time of equol-d_4. Method selectivity for total *S*-equol extracted from plasma was indeterminable due to the variability in endogenous content of conjugated equol amongst donors. However, blank aqueous extracts demonstrated a lack of *S*-equol response when accounting for H5 *pomatia* contribution.

3.5.2. Calibration and linearity

The concentration range for unconjugated *S*-equol was 0.025–10.0 ng/mL in plasma and 0.200–200 ng/mL in urine. The differences between nominal standard concentrations and the back-calculated concentrations from the weighted linear regression line were less than or equal to 5% for each point on the standard curve, indicating that the linear regression analysis weighted $1/x^2$ provided an adequate fit for the data. The quantitation range for total *S*-equol in plasma was 2.5–2500 ng/mL and 2.5–2000 ng/mL for urine. Calibration curves for both unconjugated and total *S*-equol were linear and weighted $1/x^2$ with coefficients of determination (r^2) > 0.98.

3.5.3. Carry-over

The Shimadzu Sil HTC autosampler needle was rinsed both before and after aspiration of sample using a wash solution of isopropyl alcohol. Carry-over was minimized with a needle rinsing volume of 420 μL in addition to a single valve wash. Carry-over was assessed by injecting a blank sample extract immediately after an injection of the highest concentration standard. Response for *S*-equol in the blank extract was found to be <5% of the LLOQ standard response while the equol-d_4 response was <5% of the mean response of the internal standard in the calibration standards.

3.5.4. Precision and accuracy at the lower-limit of quantitation

Six LLOQ replicates from a single control donor were evaluated for precision and accuracy. Unconjugated *S*-equol in human plasma furnished a mean concentration of 0.025 ng/mL with 4.8% CV and 99.2% accuracy; in human urine, a mean concentration of 0.213 ng/mL with 3.7% CV and 106% accuracy was obtained. Total *S*-equol in human plasma furnished a mean concentration of 2.61 ng/mL (104% accuracy) with 6.5% CV; in urine, the mean concentration was 2.41 ng/mL (96.4% accuracy) with 5.2% CV.

3.5.5. Intra- and inter-accuracy and precision

The within and between batch precision and accuracy for unconjugated and total *S*-equol was determined using six replicate QC samples analyzed on three separate occasions using a different donor for each analysis. The average CV (%) and accuracy per occasion was within ±15% of the theoretical concentration of *S*-equol; the % CV and accuracy for the inter-day results similarly met acceptance criteria (Tables 1 and 2). These precision and accuracy data exceeded the guidance for industry on the validation of bioanalytical methods [29].

3.5.6. Stock solution stability, short and long-term matrix stability, freeze–thaw stability

Solution stability of the *S*-equol stock preparation at a concentration of 1.0 mg/mL in ethanol was evaluated *via* dilution against a freshly prepared stock. In this manner, it was determined that *S*-equol stock solution was stable for at least 120 days when stored at −20 °C.

Short-term matrix stability for unconjugated *S*-equol was assessed by analyzing QC samples, in triplicate, at low and high concentrations after storage in matrix at room temperature. Stability was deemed acceptable if a minimum of two out of three QC samples at each concentration were within ±15% of their theoretical concentrations. The determination of long-term matrix stability used similar criteria for acceptance with QC samples stored at −80 °C. The results shown in Table 3 indicate *S*-equol stability was

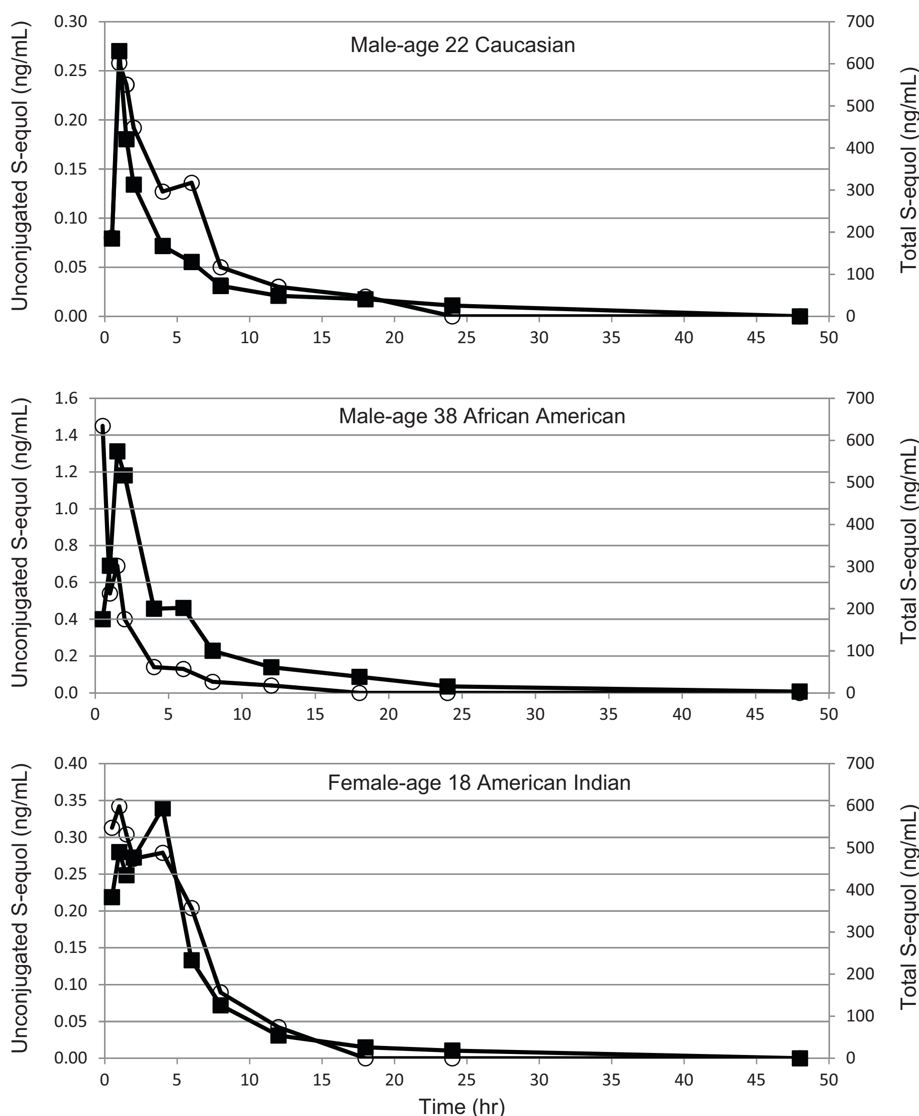

Fig. 5. Plasma concentrations of unconjugated *S*-equol (-○-) and total *S*-equol (-■-). The subjects were given a single oral dose of 20 mg *S*-equol.

greater in plasma than urine with short term stability of 23 h *vs* 19 h, and long term stability of 194 days *vs* 130 days, respectively.

Freeze–thaw stability was examined by exposing QC samples in triplicate at low and high concentrations of *S*-equol to a minimum of three freeze–thaw cycles at −80 °C. By comparing the initial values of QC concentration after one freeze–thaw cycle to those concentrations after subsequent freeze–thaw cycles, the effect of freeze–thawing on the stability of *S*-equol in plasma and urine was determined. Freeze–thaw stability was considered acceptable after three cycles as each measured concentration was within ±15% of the theoretical concentration.

3.5.7. Injection medium integrity

Following storage at 4 °C, the integrity of *S*-equol in the injection medium was determined by re-injection of standards and QC samples that previously met all acceptance criteria. Injection medium integrity (IMI) was considered acceptable if the calibration curve and QC samples met routine acceptance criteria following re-injection. Samples of unconjugated and total *S*-equol in human plasma furnished five days and four days of IMI, respectively, while in urine, IMI was conferred for six days (unconjugated) *S*-equol and five days (total *S*-equol).

3.5.8. Dilution integrity

The determination of dilution integrity establishes the sample dilution factor which conserves linearity in response. In the current study, a 100-fold dilution factor was required. Therefore, triplicate QC samples were prepared at theoretical concentrations of 10,000 ng/mL. Each sample was diluted 100-fold, in duplicate, with blank matrix to give a theoretical concentration of 100 ng/mL. The acceptance criteria for accuracy and relative standard deviation were met (±15%) indicating sample integrity was not compromised upon dilution. For total *S*-equol, measured concentrations were corrected for H5 *pomatia* endogenous contribution.

3.6. Application of validated methods to clinical samples

The LC–MS/MS methods were utilized to measure unconjugated and total *S*-equol in plasma and urine from three healthy volunteer after administration of a single oral dose of 20 mg *S*-equol in an immediate release capsule. In these subjects, there were no measurable levels of endogenous unconjugated or total *S*-equol present in plasma before dosing. As is shown in Fig. 5, both unconjugated and total *S*-equol were measurable 30 min after dosing indicating rapid absorption of the compound. The concen-

Table 1
Intra- and inter assay precision and accuracy for unconjugated *S*-equol.[a]

Theoretical concentration (ng/mL)	Measured concentration (mean ± S.D.; *n* = 6)	Coefficient of variation (%)	Accuracy (% difference)
Human urine: intra-day			
0.60	0.594 ± 0.02	2.9	99.0
	0.590 ± 0.02	3.4	98.3
	0.577 ± 0.04	7.2	96.1
90.0	90.7 ± 2.90	3.2	100.7
	91.7 ± 2.84	3.1	101.8
	88.6 ± 4.61	5.2	98.4
140	139.8 ± 3.91	2.8	99.8
	137.1 ± 1.37	1.0	97.9
	142.1 ± 4.83	3.4	101.5
Human urine: inter-day			
0.60	0.587 ± 0.03	4.4	97.8
90	90.3 ± 3.45	3.8	100.3
140	139.7 ± 3.37	2.4	99.8
Human plasma: intra-day			
0.075	0.074 ± 0.008	11.0	98.9
	0.074 ± 0.002	3.6	98.4
	0.079 ± 0.004	5.5	105.0
4.50	4.65 ± 0.21	4.6	103.3
	4.37 ± 0.05	1.1	97.1
	4.36 ± 0.16	3.7	96.9
7.50	7.49 ± 0.52	6.9	99.9
	7.02 ± 0.08	1.1	93.6
	7.08 ± 0.07	1.0	94.4
Human plasma: inter-day			
0.075	0.0756 ± 0.005	6.7	100.8
4.50	4.46 ± 0.14	3.2	99.1
7.50	7.19 ± 0.22	2.9	95.9

[a] Samples were obtained by spiking unconjugated *S*-equol at the theoretical concentration into plasma or urine using six replicates.

tration of unconjugated *S*-equol was rapidly maximal (C_{max}) at 1 h after dosing; the levels continued to decline linearly over the next 12 h. Total *S*-equol levels increased during the first 2 h after dosing and then increased or leveled off from hours 2 through 4 which is likely the result of the enterohepatic recirculation of *S*-equol. The LLOQ (0.025 ng/mL) for the methodology described in this report represents a significant advantage when defining the pharmacokinetics of unconjugated *S*-equol. As is illustrated in Fig. 5, most data points fall below 1.0 ng/mL, the lowest LLOQ reported for plasma total *S*-equol [27]. Urine unconjugated *S*-equol X_u (total urinary excretion) and $Fe_{(0-24)}$ (calculated fraction of administered dose excreted) were 11,736 ng and 0.0586%, respectively while total *S*-equol X_u and $Fe_{(0-24)}$ were 202,579 ng and 1.0123%, respectively.

Table 2
Intra- and inter assay precision and accuracy for total *S*-equol.[a]

Theoretical concentration (ng/mL)	Measured concentration (mean ± S.D.; *n* = 6)	Coefficient of variation (%)	Accuracy (% difference)
Human urine: intra-day			
7.5	7.30 ± 0.10	1.4	97.3
	8.12 ± 0.02	2.1	108.3
	8.51 ± 0.29	3.4	113.4
1000	989.2 ± 14.8	1.5	98.9
	997.2 ± 15.9	1.6	99.7
	1062 ± 31.8	3.0	106.2
1800	1776 ± 21.3	1.2	98.7
	1701 ± 30.6	1.8	94.5
	1822 ± 41.9	2.3	101.2
Human urine: inter-day			
7.5	7.97 ± 0.14	1.7	106.3
1000	1016 ± 20.8	2.0	101.6
1800	1766 ± 31.3	1.7	98.1
Human plasma: intra-day			
7.5	8.43 ± 0.24	2.9	112.4
	7.45 ± 0.62	8.3	99.3
	8.06 ± 0.41	5.1	107.5
1000	1056 ± 14.8	1.4	105.6
	1073 ± 20.4	1.9	107.3
	1032 ± 24.8	2.4	103.2
1800	1834 ± 12.8	0.7	101.9
	1860 ± 27.9	1.5	103.3
	1851 ± 35.2	1.9	102.8
Human plasma: inter-day			
7.5	7.98 ± 0.42	5.3	106.4
1000	1053 ± 20.0	2.0	105.3
1800	1848 ± 25.3	1.4	102.7

[a] Samples were obtained by spiking unconjugated *S*-equol at the theoretical concentration into plasma or urine using six replicates.

Table 3
Short- and long term matrix stability for unconjugated *S*-equol.

Theoretical concentration (ng/mL)	Measured concentration (ng/mL)	Relative error (%)
Urine: short term stability (19 h, rt)		
0.600	0.577	−3.8
	0.603	0.5
	0.635	5.8
140	127	−9.4
	123	−12.5
	126	−10.2
Urine: long term stability (130 days, −80 °C)		
0.600	0.516	−14
	0.495	−17.5
	0.513	−14.5
140	124	−11.6
	125	−11.0
	121	−13.9
Plasma: short term stability (23 h, rt)		
0.075	0.080	6.7
	0.073	−2.5
	0.079	6.2
7.5	7.08	−5.7
	6.89	−8.2
	7.05	−6.0
Plasma: long term stability (194 days, −80 °C)		
0.075	0.074	−0.7
	0.076	2.0
	0.079	5.3
7.50	7.15	−4.6
	7.17	−4.4
	7.28	−3.0

4. Conclusions

The LC–MS/MS methods described in this report for the determination of unconjugated and total *S*-equol in human plasma and urine met the validation guidelines for drug analysis in clinical studies [29]. The methods proved to be selective, precise, accurate, and sensitive, with the LLOQ significantly lowered to ensure accurate measurement of the pharmacokinetics of unconjugated *S*-equol. The validated method has been used to determine the pharmacokinetics of unconjugated and conjugated *S*-equol in normal subjects after an oral dose of 20 mg *S*-equol.

Financial disclosure/conflicts of interest

RLJ and RJS hold equity in Ausio Pharmaceuticals, LLC.

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