Urine and Serum Biomonitoring of Exposure to Environmental Estrogens II: Soy Isoflavones and Zearalenone in Pregnant Women

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Abbreviations: BPA, bisphenol A; CDC, Centers for Disease Control and Prevention; DDZ, daidzein; EQ, equol; ER, estrogen receptor; ESI, electrospray ionization; GEN, genistein; LOD, limit of detection; LOQ, limit of quantification; MRM, multiple reaction monitoring; NHANES, National Health and Nutrition Examination Survey; TDI, tolerable daily intake; UPLC-MS/MS, ultra-performance liquid chromatography tandem mass spectrometry; ZAL, zearalanol; ZAN, zearalanone; ZEL, zearalenol; ZEN, zearalenone.

ABSTRACT

Urine and serum biomonitoring was used to measure internal exposure to selected dietary estrogens in a cohort of 30 pregnant women. Exposure was measured over a period comprising one-half day in the field (6 hours) and one day in a clinic (24 hours). Biomonitoring of the dietary phytoestrogens genistein (GEN), daidzein (DDZ) and equol (EQ), as well as the mycoestrogen, zearalenone (ZEN) and its congeners, was conducted using UPLC-MS/MS. Biomonitoring revealed evidence of internal exposure to naturally occurring dietary estrogens during pregnancy. Urinary concentrations of total GEN, DDZ and EQ were similar to levels reported for general adult U.S. population. Measurable concentrations of total (parent and metabolites) GEN, DDZ and EQ were present in 240, 207 and 2 of 270 serum samples, respectively. Six out of 30 subjects had measurable concentrations of unconjugated GEN and/or DDZ in serum between 0.6 and 7.1 nM. Urine to serum total isoflavone ratios for GEN, DDZ and EQ were 13, 47, and 180, respectively. ZEN and its reductive metabolite, α -zearalenol (α -ZEL), were present in pregnant women (11 out of 30 subjects) as conjugates at levels near the limit of quantification. The average total urinary concentration was 0.10 μ g/L for ZEN and 0.11 μ g/L for α -ZEL.

KEY WORDS

Soy isoflavones

Zearalenone

Exposure

Pregnancy

Biomonitoring

Endocrine Disruptors

1. Introduction

Total human exposure to biologically active estrogenic compounds includes at least three classes of estrogens: endogenous estrogens (e.g., estrone, estradiol and estriol); synthetic estrogens (e.g., bisphenol A (BPA), see companion paper); and naturally occurring estrogens produced by plants, fungi, and other organisms. The isoflavones genistein (GEN) and daidzein (DDZ) are important members of naturally occurring estrogens (Figure 1). DDZ and GEN are present as their β -glucosides, genistin and daidzin, in various plants (soy beans, red-clover) used to produce food products and dietary supplements. The highest concentrations occur in soy-based products.

Figure 1. Chemical structures of 17 β -estradiol, the soy isoflavones genistein, daidzein and equol and the mycoestrogen zearalenone and its metabolites zearalenol, zearalanone and zearalanol.

GEN and DDZ and its metabolite EQ have structural similarities to endogenous estrogens (Figure 1) and bind preferentially to the estrogen receptor beta (ER β) with relative binding affinities in the order 17 β -estradiol>GEN>EQ>DDZ (Muthyala *et al.*, 2004). The reported biological effects of soy isoflavone exposure in humans include potential beneficial health effects (e.g. amelioration of menopause symptoms, osteoporosis, and serum lipids related to

cardiovascular health) and potential adverse effects similar to those attributed to synthetic estrogens affecting mammary carcinogenesis (EFSA, 2015). Multiple targets, lifestage differences, epigenetic changes, differences in metabolism between individuals, and variable dosage forms in clinical trials contribute to uncertainty regarding mode of action. Rodent studies with GEN demonstrate that timing of exposure is a critical determinant of mammary carcinogenesis, with studies in adults showing growth promotion of existing tumors while prepubertal exposure can be protective and fetal exposure can enhance susceptibility later in life (Ju *et al.*, 2001; Ju *et al.*, 2006; Messina and Hilakivi-Clarke, 2009).

The mycotoxin zearalenone (ZEN) (Figure 1), produced by *Fusarium* fungi frequently contaminating grains, especially corn, is another dietary estrogen. The estrogenic activity of ZEN was confirmed in several *in vitro* and *in vivo* assays after earlier findings that pigs fed with *Fusarium*-infected grains had symptoms specific to estrogen exposure including hypertrophy of the vulva and mammary glands (Stob *et al.*, 1962). A number of epidemiological studies link the presence of ZEN in food and/or biological samples to adverse effects in the reproductive system, such as precocious puberty and premature development of the breasts (Saenz de Rodriguez *et al.*, 1985; Freni-Titulaer *et al.*, 1986; Szuets *et al.*, 1997; Massart *et al.*, 2008). Studies in pigs showed an impact on the reproductive capacity of dams fed a naturally contaminated diet (0.2-1 mg ZEN/kg feed). Other studies reported delayed fetal development in rats administered high doses (48.5-146 mg ZEN/kg feed) (Schoevers *et al.*, 2012; Zhang *et al.*, 2014). In contrast to the isoflavones, ZEN binds to both ER isoforms with similar affinity (reviewed in Metzler *et al.*, 2010). Reduction of the keto group to zearalenol (ZEL) and/or the double bond to zearalanol (ZAL) and zearalanone (ZAN) by fungal or mammalian metabolism or chemical reaction increases the estrogenic potential of ZEN. The α -epimers of ZEL and ZAL have estrogen

receptor binding affinities for α and β isoforms ~ 10 fold higher than for ZEN (Metzler *et al.*, 2010). The relative receptor binding potency for ER α is 17 β -estradiol > α -ZEL > ZEN > GEN > DDZ and for ER β 17 β -estradiol > α -ZEL > GEN > ZEN > DDZ (Bovee *et al.*, 2004).

The recent focus on endocrine disruption, particularly during the period of fetal development, raises the important question about the contribution of this group of naturally occurring estrogens to internal estrogen exposures, especially in women of child-bearing age or during pregnancy. There is pharmacokinetic evidence that the soy isoflavones, and ZEN reach the fetal compartment.

Ingested genistin and daidzin reaching the gastro-intestinal tract are hydrolyzed by intestinal and microbial β -glucosidases. The released aglycones, GEN and DDZ, are absorbed and conjugated with glucuronic acid or sulfate in the intestine and liver (Doerge *et al.*, 2000; Rowland *et al.*, 2003). Excretion of the conjugates occurs mainly through urine and to a lesser extent in feces (<5%) (Vitale *et al.*, 2013). The recovery of DDZ and GEN as conjugates in human urine is approximately 30-40% and 10-15%, respectively (Bloedon *et al.*, 2002; Setchell *et al.*, 2003). In the presence of specific intestinal bacteria and under optimum conditions, DDZ is metabolically activated to equol (EQ). The production of EQ varies widely among individuals. Whereas in the western population only 25-30% are able to produce EQ, the frequency among adults from Asian countries can be higher (50-60%) (Setchell and Clerici, 2010).

Exposure of the developing fetus through placental transfer of isoflavones has been shown for GEN in animal models and in humans, producing total GEN concentrations in fetal and maternal serum that are similar. The corresponding concentrations of the biologically active aglycones are reportedly higher in the fetus (Adlercreutz *et al.*, 1999; Doerge *et al.*, 2001; Jarrell *et al.*, 2012).

Limited data are available about the toxicokinetics of ZEN in humans. Biotransformation studies in animals reveal several pathways, including reduction of the keto group by hydroxysteroid dehydrogenases to α - and β -ZEL, cytochrome P450-catalyzed hydroxylation on aromatic and aliphatic positions, and Phase II conjugation to glucuronides and sulfates (Metzler *et al.*, 2010). Two studies, each in a single man, reported the pattern of urinary metabolites after ingestion of either a high dose of ZEN or naturally contaminated corn-based food (Mirocha *et al.*, 1981; Warth *et al.*, 2013). Urinary excretion accounts for approximately 10% of the administered dose, with glucuronides of ZEN and α -ZEL as the main metabolites and minor amounts of β -ZEL. Placental transfer of ZEN and α -ZAL was shown in rats and rabbits, leading to the conclusion that fetal exposure to these estrogenic compounds might also be likely in humans (Bernhoft *et al.*, 2001; Lange *et al.*, 2002).

Internal exposures to natural, synthetic and endogenous estrogens have been measured independently in separate cohorts, but not simultaneously in a single cohort, preventing a comparison of the contribution of these sources of estrogens in the context of total estrogenicity. The goal of this study was to characterize total and internal exposure to a select group of naturally present dietary estrogens during pregnancy, a developmental period that may be uniquely sensitive to the presence of estrogens. This objective was met through urine and serum biomonitoring in a cohort of 30 pregnant women from the Salt Lake City, UT metropolitan area. Biomonitoring was conducted during a field exposure phase and a 24-hour clinical exposure phase. Biomarkers for exposure to the naturally occurring dietary estrogens GEN, DDZ, EQ, ZEN, α -/ β -ZAL, ZAN, α -/ β -ZAL as well as their conjugated metabolites were analyzed by using UPLC-MS/MS.

2. Materials and methods

All human subject research activities were conducted in accordance with protocols approved by the Pacific Northwest National Laboratory Institutional Review Board (IRB # 2014-14). The participation of the National Center for Toxicological Research was reviewed and approved by the FDA Research Involving Human Subjects Committee (RIHSC#10-119T) and determined not to constitute engagement in human subjects research because it was limited to analysis of anonymized samples.

2.1 Protocol

The protocol was reviewed by an international panel of academic and governmental scientists with expertise in human biomonitoring. Complete descriptions of the cohort selection and demographics as well as the study protocol and the serum and urine collection procedure can be obtained in the companion paper (Teeguarden et al., FCT under review). Briefly, thirty pregnant volunteers were recruited for the study from the Salt Lake City, UT metropolitan area in 2014. Volunteers were healthy non-smoking females 18 to 40 years of age, between approximately 15 and 35 weeks of a normal, low-risk pregnancy with normal GI-tract, kidney, and liver function, as determined by clinical laboratory tests, medical history and physical examination. Two cohorts were recruited from a pool of 116 candidates, a 20 member cohort selected for the highest potential for exposure to BPA (The focus of Teeguarden et al., FCT under review) and a 10 member cohort selected based on answers to survey questions with indicated higher dietary exposure potential for soy isoflavones. The full study exposure period comprised a field exposure period and a clinical exposure period. The field exposure period started with the first morning void on Day 1 and ended with collection of the 0-hour urine sample collected upon admittance to the clinic, usually immediately after lunch of the first day.

All urine was self-collected during this period. Sample times and volumes were recorded electronically via a text-to-email system. BPA accumulating in the bladder during the overnight period following the last void would be present in this urine sample. The clinical exposure period started immediately after the 0-hour urine sample was collected and completed with the final study urine sample collected ~ 24 hours later.

2.2 Sample analysis criteria

Pooled urine samples from 6-h field exposure were first analyzed for a screening of total (aglycones and conjugates following quantitative enzymatic hydrolysis of conjugates) GEN, DDZ and EQ. Total isoflavones were also measured in all clinical phase urine and serum samples. Aglycone levels were measured in serum samples with total GEN, total DDZ, or total EQ concentrations greater than 20x limit of quantification (LOQ) for the 24-h clinical period (i.e., samples with detectable levels assuming <5% aglycone content). Urine concentrations and total urine volumes were used to calculate the cumulative excretion obtained from each individual during the 24-h clinical period as a lower bound on total daily exposure.

Urinary levels of total ZEN (aglycones and conjugates) and total ZEL, ZAN and ZAL as additional metabolites were measured in pooled samples collected over the 6-h field exposure period for all subjects and in selected spot urine samples collected during the 24-h clinical period from subjects ingesting corn-containing food items identified from dietary survey data and photographs of food items and packaging. All serum samples of subjects with a positive urine sample during field or clinical exposure were analyzed for the total content of zearalenone and its congeners following quantitative enzymatic hydrolysis of conjugated forms.

2.3 Analytical methods

For the measurement of soy isoflavones and ZEN together with their metabolites, validated methods using isotopically labeled internal standards, supported liquid extraction and ultra-performance liquid chromatography coupled with tandem mass spectrometry detection (UPLC-MS/MS) and electrospray ionization (ESI) were used. The UPLC separation was performed using a Waters Acquity UPLC System (Waters, Milford, MA). A Waters Quattro Premier XE mass spectrometer equipped with an electrospray interface was used with an ion source temperature of 100°C, a capillary voltage of 3 kV, and a desolvation temperature of 350°C. Depending on the matrix, negative or positive ions were acquired in multiple reaction monitoring mode (MRM) for MS/MS measurements. The parameters for the monitored transitions are summarized in [Supplemental Table 1](#).

The analysis of total soy isoflavones and zearalenone in serum and urine was conducted with enzymatic deconjugation using *Helix pomatia* glucuronidase/sulfatase (2U/100 µl sample, Sigma, St. Louis, MO) after adding 50 µl the isotopically labeled internal standard (0.4 pmol/µl). Depending on the concentration of the samples, determined by a screening analysis of individually pooled samples, between 5 and 100 µl serum or urine were used for the incubation in 25 mM citrate buffer (pH 5.0) for 24 h or 2 h, respectively. Aglycone analysis was conducted without enzymatic hydrolysis. Sample clean-up was performed using Isolute SLE+ supported liquid extraction in a 96-well plate format (Biotage, Charlotte, NC) for the soy isoflavones and Novum SLE simplified liquid extraction plate (Phenomenex, Torrance, CA) for zearalenone and its congeners. Samples were loaded onto the plate and allowed to completely absorb after a short pulse of positive pressure (3 psi, Biotage Pressure+). Elution was achieved with 2x900 µl methyl tert-butyl ether, waiting 5 min between each elution step before applying positive pressure for complete elution. The extracts were evaporated to dryness under a heated stream of nitrogen (Biotage SPE Dry), reconstituted in 100 µl MeOH/H₂O (50/50, v/v) and then injected onto the UPLC-MS/MS system.

For the soy isoflavones, chromatography was performed using a Waters Acquity BEH C18 column (100x2.1 mm, 1.7 µm) with a VanGuard BEH C18 column (1.7 µm) at 45°C and with different elution profiles depending on the sample matrix. Plasma samples were analyzed using an isocratic elution with 60% H₂O and 40% acetonitrile and a flow rate of 0.4 ml/min. Total runtime including an organic flush was 5.5 min. Due to matrix effects, the analysis of soy isoflavones in urine samples was conducted under modified chromatographic conditions: Water was substituted with 0.1% formic acid to provide better ionization in positive ion mode and the flow rate was decreased to 0.1 ml/min to prevent coelution with interfering matrix components.

Zearalenone and its congeners were separated at 50°C on a Supelco Titan C18 (100x2.1 mm, 1.9 µm) with a Supelco Titan C18 UPLC guard cartridge (Supelco, Bellefonte, PA).

Separation was achieved starting with 20% acetonitrile and 80% H₂O and increasing it to 58% acetonitrile over 4.2 min. An organic flush with reconstitution to initial conditions prepared the column for the next injection. Total run time was 7.5 min.

The isotopically labeled standards used in this study were (3,4,1'-¹³C₃)-GEN (kindly provided by Dr. Nigel Botting, St. Andrews Univ., dec.), (3',5',8-D₃)-DDZ (Cambridge Isotopes, Tewksbury, MA), (4-¹³C-2,2-D₂)-EQ (Obiter Research, Champaign, IL), and ¹³C₁₈-ZEN (Sigma-Aldrich, St. Louis, MO). Labeled standards of α - and β -ZEL were derived from (2,2,2,3,4,4-D₆)-zearalenone (Santa Cruz Biotechnology, Dallas, TX) through reduction of the ketone group with sodium borohydride. The mixture was separated into the α - and β -isomers with preparative HPLC on a Phenomenex Luna C18(2) (250x10.00 mm, 5 μ m) (Phenomenex, Torrance, CA) in a total run time of 24 min. The flow rate was 5 ml/min and the water/acetonitrile gradient started with holding 20% acetonitrile for 5 min, linearly increasing to 60% within 16 min and flushing the column with 100% acetonitrile for 2 min before returning to initial conditions. The chemical and isotopical purities were confirmed with UV and MS analysis and yielded $\geq 99\%$. Labeled ZEN was used as a surrogate standard for ZAN and labelled ZEL as a surrogate for ZAL.

3. Results

3.1 Soy isoflavones

3.1.1 Urinary excretion

Urinary excretion of total GEN, total DDZ and total EQ varied widely within subjects and across the study period (**Figure 2**). Peak excretion occurred most frequently in first morning voids (between hours 15-20), reflecting exposure through dinner meals and post-dinner snacks. Another peak of excretion was observed in some subjects after lunch or breakfast meals (study

hours 4-6, **Figure 2**). The geometric mean total urinary concentration of all positive individual spot urine samples (12 to 22 samples per subject) was 105.4 µg/L for total GEN (GSD 3.68, 95% CI 87.0-127.7, range LOD-5469 µg/L, n=180), 42.2 µg/L for total DDZ (GSD 5.96, 95% CI 35.4-50.5, range LOD-9105 µg/L, n=389) and 74.7 µg/L for total EQ (GSD 3.35, 95% CI 40.2-139.1, range LOD-891 µg/L, n=17). Total DDZ was detected in all samples, whereas total GEN was not detected in 7 subjects (23.3%) and the formation of EQ (as total EQ) was observed in 4 subjects (13.3%).

Figure 2. Time course of urinary elimination (in μg) of total GEN (all subjects), total DDZ (all subjects) and total EQ (subjects with measurable urinary concentrations). For all subjects, 0h

marks the first urine sampling period of the clinical phase of the study (after lunch), not time of day.

The average total isoflavone concentrations in the urine collected within the 24-h clinical phase were calculated for each subject from individual samples and corresponding urine volumes (Table 1). There was no significant difference in the geometric mean concentrations of soy isoflavones between the 10 selected soy-consuming individuals and the overall cohort of 30.

The mean urinary concentrations of total GEN, DDZ and EQ were similar to the values obtained from a cohort of 1974 adults (20 years and older) from the U.S. sampled between 2009 and 2010 during the National Health and Nutrition Survey (NHANES) by the U.S. Centers for Disease Control and Prevention (CDC) (CDC, 2015).

Table 1. Geometric mean concentrations of total GEN, total DDZ and total EQ in 24-h urine samples calculated from individual samples and corresponding urine volumes ($\mu\text{g/L}$) and comparison to mean U.S. exposure values for adults (NHANES, 2009/2010), ^a(CDC, 2015), CI, confidence interval, GSD, geometric standard deviation.

	Concentration in 24h-urine ($\mu\text{g/L}$)		
	GEN	DDZ	EQ
Total Cohort n=30			
Geometric Mean	24.0	42.3	12.2
95% CI	8.4-68.5	21-85.2	0.4-344.3
GSD	10.61	6.51	8.15
Range	LOD-1214	LOD-1389	1.4-167
Soy Cohort n=10			
Geometric Mean	21.2	43.0	-
95% CI	1.1-395.1	9.9-186.5	-
GSD	16.26	7.76	-
Range	LOD-785	3.4-1389	-
U.S. adult population (NHANES 2009/2010)^a n=1974			
Geometric Mean	30.5	63.0	6.97
95% CI	26.7-34.8	54.7-72.5	6.07-8.00

The distribution of urine total isoflavone concentrations across individual spot urine samples for each subject is shown in Figure 3 along with 50th, 75th, 90th and 95th percentiles for the corresponding urine concentrations for U.S. adults (CDC, 2015). Median values in the 30

member pregnancy cohort exceed the U.S. adult 50th percentile in 18 (60%) subjects for total GEN, 11 (36.6%) for total DDZ and 4 (13.3%) for total EQ. Individual samples exceed the 95th percentile for 3 (10%) subjects in case of total GEN and 5 (16.6%) in case of total DDZ. Subject 210 showed some urine samples above the 95th percentile for total EQ and subject 222 had detectable levels of total EQ in 5 spot urine samples that were all above the 95th percentile.

Figure 3. Urinary concentrations of total GEN, total DDZ and total EQ in individual spot urine samples. Solid lines represent selected percentiles from NHANES U.S. exposure data for adults from 2009/2010, dotted lines the corresponding 95% confidence intervals. Boxes represent 25th and 75th percentiles (min. of 3 data points). Median values of each subject are depicted as solid lines within the box. Whiskers represent 10th and 90th percentiles (min. of 9 data points). Outliers are marked as open circles. Because of the limited number of positive samples, urinary concentrations of total EQ in spot urines are shown as individual spots. LOQ levels are marked as a cross on the right axis.

3.1.2 Serum concentrations

Measurable concentrations of total GEN were observed in 240 of the 270 analyzed serum samples (88.9%) and 207 serum samples were positive for total DDZ (76.6%). Total EQ was only detected in 2 serum samples from different subjects (0.7%) with concentrations of 3.6 and 7.8 nM. The geometric mean of serum total concentrations were 8.7 nM for total GEN (GSD 4.82, 95% CI 7.0-10.8, range LOD-467 nM, n=240) and 4.4 nM for total DDZ (GSD 4.56, 95% CI 3.6-5.4, range LOD-235 nM, n=207).

Measurement of unconjugated isoflavone levels in serum was conducted in samples with total concentrations higher than 20-times the LOQ of the aglycones method (99 samples). Six serum samples of 3 different subjects had measureable levels of GEN (10% of the cohort) and 14 serum samples of 6 different subjects for DDZ (20% of the cohort). The samples with measurable total EQ did not meet the inclusion criterion for the analysis of the unconjugated compound. Geometric means of aglycone serum concentrations of all positive samples was 1.8 nM for GEN (GSD 2.05, 95% CI 0.9-2.2, range LOD-6.6 nM, n=6) and 1.4 nM for DDZ (GSD 2.00, 95% CI 0.9-3.8, range LOD-7.1 nM, n=14).

The geometric mean of the ratio of aglycones to total concentrations in serum was 0.6% (95% CI 0.4, 1) for GEN and 1.4% (95% CI 1, 1.9) for DDZ. Individual ratios over the time course of sampling are depicted in [Figure 4](#). The percentage of DDZ aglycones in the serum is significantly higher than the ratio of GEN (student's t-test, two-tailed distribution).

3.1.3 Urine-to-serum relationships

The time course of urinary excretion compared to measured serum total levels for two different individuals is depicted in [Figure 5](#). Consistent with previous reports (Setchell *et al.*,

2003), total GEN serum concentrations were higher than serum total DDZ concentrations while the opposite was true for urine. The urinary excretion and serum concentrations time courses for total GEN and DDZ showed similar patterns, but peak urinary excretion lagged serum peak concentrations by 5 to 6 h (GEN) and 1 to 3 h (DDZ). Two peak concentrations were observed consistent with absorption through lunch and dinner, which are more pronounced for total DDZ due to a more rapid elimination in urine. Subject 222 was one of four EQ producers in the cohort. The long lag time between peak DDZ urinary elimination and the appearance of total EQ in urine, approximately 10 h after a meal, is probably the result of slow production of EQ from DDZ by microbiota during transit through the GI tract.

Figure 4. Urine/serum ratios of total genistein, daidzein and equol and fraction of aglycones in serum. Lines represent the corresponding geometric means.

Figure 5. Time course of urinary excretion (μg , solid lines, closed symbols) and serum concentrations (nM, dashed lines, open symbols) of total GEN, DDZ and EQ in subjects 222 and 226. Lunch (L) and dinner (D) times are marked with arrows.

Urine-to-serum ratios are used to estimate internal exposure concentrations (serum) from more accessible urine biomonitoring data. Urine to serum ratios of total isoflavone measurements over time are shown in [Figure 4](#). Geometric means are 13 for total GEN and 47 for total DDZ, relating to a more efficient urinary excretion for total DDZ. Total EQ was only detected in 2 matched urine and serum samples from 2 individuals. Urine to blood ratios were calculated as 472 and 69 for total EQ.

3.2 Zearalenone

Concentrations of total ZEN (ZEN plus conjugate metabolites) and total α -ZEL (α -ZEL plus conjugate metabolites) as the major reductive metabolite, were measured in selected urine samples corresponding to individuals eating corn-based products and in pooled preclinical samples from all subjects. We identified eight subjects who consumed corn-based snacks during the 24-h clinical period, seven of which had detectable urinary levels of total ZEN and/or α -ZEL near the limit of quantification. Four additional subjects with measurable total ZEN in the pooled urine samples collected during the 6-h field exposure of the study were also included in the

analysis. Examining photographs of the meals and packaging materials, we found no consistent dietary source of ZEN in these subjects. Ten urine samples from three subjects had total ZEN levels above the LOQ. The urinary excretion of total ZEN and total α -ZEL is summarized in **Table 2**. Average total urinary concentration of all positive samples, including samples above the LOD but below LOQ, was 0.10 $\mu\text{g/L}$ for total ZEN (STD 0.068, range LOD-0.31 $\mu\text{g/L}$, n=41) and 0.11 $\mu\text{g/L}$ for total α -ZEL (STD 0.057, range LOD-0.25 $\mu\text{g/L}$, n=10). The ZEN congeners β -ZEL, α - β -ZAL and ZAN were not detected in any sample post deconjugation.

All serum samples of the eleven subjects with measurable urinary total ZEN concentrations were analyzed for total ZEN. One sample was positive for total ZEN with a concentration near the limit of quantification. Because no corresponding urine sample was available for the same time point, a tentative urine-to-serum ratio of 0.3 was calculated with the urine sample 2-h after the serum sampling.

Table 2. Urinary excretion (μg) and serum levels (nM) of total zearalenone and its metabolite α -zearalenol: n.d., not detected, -, no corresponding sample available, *, measured values below LOQ.

Subject	Meal		Sampling Time	Urinary total excretion (μg)		Serum Concentration (nM)
	Time	Type		ZEN	α -ZEL	ZEN
205		unknown	08:20-11:00	0.08*	n.d.	-
211		unknown	01:15-10:42	0.14*	n.d.	-
214	15:40	Corn chips	08:39	0.02*	n.d.	-
			12:15	0.02*	n.d.	-
217		unknown	09:00-11:39	0.03*	n.d.	-
221	13:54	Corn crisps	21:36	0.02*	0.03*	n.d.
			23:20	0.02*	0.03*	-
			02:44	n.d.	0.05*	-
			13:07	0.02*	n.d.	n.d.
223	16:36	Corn chips	01:48	0.04*	n.d.	-
	20:01		13:57	0.003*	n.d.	n.d.
224	15:00	Corn crisps	23:15	0.01*	n.d.	-
			08:46	n.d.	0.01*	-
			13:23	n.d.	0.01*	n.d.
227	14:42	Corn cereal	03:00-11:20	0.19	n.d.	-
			13:07	0.02*	n.d.	n.d.
			14:07	0.02*	n.d.	n.d.
			15:37	0.02*	n.d.	n.d.
			16:41	0.04	0.03*	-
			18:40	0.03*	n.d.	n.d.
			19:40	0.03*	n.d.	n.d.
			00:46	0.02*	n.d.	-
			08:11	0.08	n.d.	-
			11:09	0.02*	n.d.	-
			13:07	0.03	n.d.	n.d.
229		unknown	04:00-13:50	0.11	n.d.	-
			15:35	-	-	0.39*
			17:35	0.01*	n.d.	n.d.
			23:08	0.01*	n.d.	n.d.
			00:58	0.01*	n.d.	-
			06:39	0.04*	n.d.	-
			10:32	0.02*	n.d.	-
			12:59	0.02	n.d.	-
			13:51	0.01*	n.d.	-
			15:05	0.02	n.d.	n.d.
232	15:10	Corn chips	07:47-09:46	0.05	n.d.	-
			17:50	n.d.	0.04*	-
			18:32	0.004*	0.004*	n.d.
			19:31	0.004*	n.d.	n.d.
			21:31	0.01*	n.d.	n.d.
			23:20	0.02*	n.d.	-
			08:23	0.05*	n.d.	-
			10:55	0.02*	0.03*	-
			12:10	0.01	n.d.	-
			13:05	0.01	0.01	n.d.
233	15:36	Corn chips	08:08	0.03*	n.d.	-

4. Discussion

Human exposure to the soy isoflavones GEN, DDZ and EQ has been addressed by CDC's NHANES program annually from 1999 to 2010 by measuring the amounts eliminated in urine (CDC, 2015). The geometric mean isoflavone concentrations in the 30 member cohort are similar to those reported in larger U.S. cohorts (NHANES), likely reflecting common consumption of a Western diet with an emphasis on whole grains and legumes, rather than soy products as the main source of isoflavones. Several urine samples had GEN, DDZ and/or EQ concentrations exceeding the 95th percentile of the NHANES reference values, similar to mean levels found in Japanese women consuming a soy-based Asian diet, which are ~50 times higher than the average in men and women in the United States (Arai *et al.*, 2000). Besides typical soy-based foods, such as tofu and soy milk, more and more processed foods use soy flour or protein isolates as supplements and fillers, which can serve as alternative sources of isoflavones in the Western diet (Lusas and Riaz, 1995; Lampe *et al.*, 1999). Kinetic profiles of the urinary excretion show that the majority of GEN and DDZ was excreted in the first morning void (Figure 2), reflecting consumption of the soy isoflavones with dinner and a reported half-life of approximately 8-h (Setchell *et al.*, 2003). The metabolism of DDZ to EQ in this study was only detected in 13% of the subjects and is lower than the more typical 25 to 30% in Western countries, possibly due to the small size of the cohort. Calculated urine-to-serum ratios of 13 (GEN), 47 (DDZ) and 180 (EQ) can be used to estimate the total isoflavone serum concentrations from urine biomonitoring data. Urine biomonitoring has advantages over serum biomonitoring, which is more invasive for example. Geometric mean serum concentrations of total isoflavones are in the same range as in individuals consuming a Western diet reported in a cohort of 215 pregnant Canadian women (Valentin-Blasini *et al.*, 2003; Jarrell *et al.*, 2012). However, some serum samples with higher concentrations may indicate exposures more similar to Japanese women (Morton *et al.*, 2002) ingesting a higher soy diet. Circulating levels of

receptor-active aglycones in the blood in this study represent 0.6% of total GEN and 1.4% of total DDZ and are consistent with literature after controlled oral dosing of women (Bloedon *et al.*, 2002). In contrast to endogenous estrogens, aglycones of the soy isoflavones are not tightly bound to serum proteins (e.g., SHBG), and can in addition be the principal (100% unconjugated) form present in target tissues such as brain, liver, and reproductive tissues (Milligan *et al.*, 1998; Chang *et al.*, 2000). Placental transfer results in similar blood concentrations of total isoflavones in the fetus and the mother but with a higher amount of aglycones, possibly due to immature conjugation metabolism (Doerge *et al.*, 2001; Jarrell *et al.*, 2012). The possibility for impacts by soy isoflavones on fetal development and reproductive health in later life has been hypothesized based on some support from studies in animals, if not in humans (NTP, 2008; Jefferson *et al.*, 2012). Although the quantitative implications remain unclear, early developmental exposure to increased levels of endogenous estrogens or an altered hormonal response might have consequences in later life (Innes and Byers, 1999; Kaijser *et al.*, 2001; Fenton, 2006; Hilakivi-Clarke *et al.*, 2013). Compared to the levels of potent endogenous hormones like estradiol, estrone, and estriol during pregnancy, which change throughout gestation and show a steep increase of unconjugated forms in maternal blood up to 40-, 20- and 250-fold, respectively (De Hertogh *et al.*, 1975), impact on the fetus from additional exposure to phytoestrogens from a Western diet could well be minimal.

The present study investigates for the first time the exposure of pregnant women in the U.S. to ZEN and its reductive metabolite α -ZEL. The detection of urinary excretion of conjugated metabolites of these mycoestrogens in over one third of the cohort without any consistent dietary sources suggests that exposure seems to be common, albeit producing low concentrations in urine (LOD-0.3 $\mu\text{g/L}$) and serum (LOD-0.4 nM). Two recent exposure studies from Sweden and Italy with 252 and 100 subjects, respectively, reported fractions of urine samples positive for total ZEN and α -ZEL and urine concentrations, $\sim 0.1 \mu\text{g/L}$, similar to those

in our study (Solfrizzo *et al.*, 2014; Wallin *et al.*, 2015). These urine concentrations can be used to place the exposures in the context of regulatory reference levels. A tolerable daily intake (TDI) of 0.25 µg ZEN/kg bw per day was established by the European Food Safety Authority (CONTAM, 2011). Consumption of a ZEN dose of ~ 83% of the TDI, in a corn-rich diet over 4 days, resulted in 24-h-urine concentrations of 0.3 to 0.6 µg/L (Warth *et al.*, 2013). Urine concentrations of ZEN over the course of the current study were ~ 1/10th of those reported by Warth *et al.*, evidence that exposures in our study might be lower than the TDI, but it should be noted that no particularly corn-rich diet was consumed and exposure occurred most likely through consumption of a minor amount of corn snacks or cereals. Urinary elimination of ZEN estimated from just two studies, each from a single individual, is 10-20% of the administered dose, therefore low ZEN concentrations in urine are expected (Mirocha *et al.*, 1981; Warth *et al.*, 2013).

5. CONCLUSION

The biomonitoring data for the soy isoflavones GEN, DDZ, and EQ in pregnant women show that the urinary and serum levels vary across several orders of magnitude between individuals, presumably due to differences in consumption, absorption, and other pharmacokinetic factors. Daily exposures were consistent with exposures to Western diet low in soy-based foods. Results of serum and urine biomonitoring were consistent with internal exposure to GEN, DDZ, and ZEN, and to a lesser extent EQ, at concentrations and biologically active fractions greater than some synthetic estrogens. The serum concentrations, and urine:serum ratios, could be used together with similar data for natural and synthetic estrogens, to evaluate the potential contribution each of these estrogens to daily estrogen levels during pregnancy.

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