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Detection of Conjugated Soy Metabolites in Urinary and Tissue Samples after Methanol Extraction

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ABSTRACT

Glucuronide conjugates of soy isoflavone metabolites genistein, daidzein, glycitein and equol in urinary samples from women, female pigs and female rats were pretreated with methanol instead of solid phase extraction or liquid-liquid extraction after enzymatic digestion. The samples were analyzed using an LC-ESI-MS/MS ion trap system. Analysis of rat liver sample using this procedure was also attempted. By comparison the elution sequences with results from previous studies, glucuronide conjugates including 7-gluc-Dai, 7-gluc-Gly, 4'-gluc-Dai, 7-gluc-Gen, then 4'-gluc-Gen were identified sequentially in women urine. However, 5-gluc-Gen was also observed in female porcine urine. In rat urine, sul-Dai was observed after 7-gluc-Dai, while 7-gluc-Equ and 4'-gluc-Equ were observed after 7-gluc-Gen. The metabolite profiles in rat liver tissue samples were found with 7-gluc-Dai and 7-gluc-Gen. However, glucuronide conjugates of equol and sulfate conjugate of daidzein were only found in rat urine. Interestingly, quantification studies performed by LC-APCI/MS and LC-ECD showed that the aglycone and conjugated glycitein were both found in porcine and rat urine, but only conjugated glycitein was found in woman urine.

Key words: methanol pretreatment, urine, tissue, glucuronide conjugate

INTRODUCTION

Both beneficial and adverse effects of soy isoflavone metabolites (SIM) after soy dietary by ingesting soy products such as soy foods or soy protein isolates or by intaking of soy isoflavone supplements have been reported since last decades[1-14]. Among the SIM, genistein, daidzein, equol, and *O*-desmethylangolensin (*O*-DMA) were reported as the major SIM found in human urine after soy diet[15,16]. Other related SIM such as dihydrogenistein (DHG), dihydrodaidzein (DHD) were also reported with traceable physiological effects. Glycitein was the major soy isoflavone from food resource along with genistein and daidzein without significant recognition for its effects[15-17]. Genistein, the most studied soy isoflavone, was reported to be the major bioactive isoflavone with anti-angiogenesis activity[18,19], as a tyrosine kinase inhibitor[20], and a growth inhibitor for human breast cancer cells[21]. However, equol, the intestinal microbial metabolite of daidzein, was found the highest level with the largest variation compared to genistein, daidzein and *O*-DMA in human urine three days after soy diet consumption in human studies[22,23]. In a previous study, equol was known to be more estrogenic compared to dadizein and *O*-DMA[24]. In breast cancer resistant chimpanzees, enormous amount of urinary equol, equal to 6 times of urinary daidzein, was also detected in urine samples[25]. It was proposed that equol was a very important bioactive compound that was transformed from daidzein in equol-producing population for the evaluation for chemoprevention and clinical effectiveness of soy protein in cardiovascular, bone and menopausal health benefits[26].

Varieties of SIM including aglycones, glucuronide and sulfate conjugates were detected in the urine of human consuming soy diet[27]. It was reported that the conjugated forms were the major isoflavone metabolites excreted in men and women subjects[27]. In the previous studies, conjugated forms of isoflavones in human urine were obtained through the calculation of values after enzymatic digestion with a mixture of sulfatase and/or β-glucuronidase followed by diethyl ether extraction. This method was commonly used for finding the values of isoflavone conjugate forms in human fluids[27-39] and in rat fluids[40-49]. Conjugated forms of genistein and daidzein were observed by high performance liquid chromatography along with radioisotope chemistry and mass spectrometry in rat urine[39] and in human and rat plasma[15,44,45]. In an incubation of genistein or daidzein with either bovine hepatic or recombinant human uridine diphosphate glucuronosyltransferases (UGT) in the pres-

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ence of uridine diphosphate glucuronic acid (UDPGA), glucuronide conjugates of genistein and daidzein at 7- and 4'- position were observed. These isoflavone metabolites were also identified using liquid chromatography mass spectrometry (LC/MS) in the plasma of human subjects consuming soy tablet[15]. In the pooled urine of rats fed with soy diet, a series of glucuronide conjugates of soy isoflavone metabolites were characterized[45]. An analytical method was established to measure the intact phytoestrogen conjugates in human urine using liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) with stable labeled [$^{13}C_3$] isoflavones for quantification for isoflavone metabolites[48]. General identification methods used for aglycones and conjugates of soy isoflavoens were based on mass spectrometric analysis either without pretreatment which might cause handling problems for instrumentation or with complicated procedures such as enzymatic digestion and solid phase extraction which might be time consuming and economically costly[27-49]. Methanol pretreatment may reduce the problems caused by previous mentioned methodology. In this study, methanol extraction was used to clean up samples before identification of isoflavone metabolites from woman, porcine, and rat urine and liver samples. LC-ESI-MS/MS ion trap system was also established to compare urinary soy isoflavone profiles from the subjects. Quantification of isoflavone metabolites of human, porcine and rat urine was applied by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI/MS) system and liquid chromatography and electrochemical detection (LC-ECD) system as previously mentioned[17,28,29].

MATERIALS AND METHODS

I. *Chemicals*

Daidzein (7,4'-dihydroxyisoflavone), genistein (7, 5, 4'-trihydroxyisoflavone), equol (7,4'-isoflavandiol), and glycitein (7,4'-dihydroxy-6-methoxyisoflavone) were purchased from Indofine Chemical Co., Inc. (Somerville, NJ, U.S.A.). Dihydrodaidzein (7, 4'-dihydroxyisoflavanone, DHD), dihydrogenistein (7, 5, 4'-trihydroxyisoflavanone, DHG) and *O*-desmethylangolensin (1-(2, 4-dihydroxyphenyl)-2-2(4-hydroxyphenol)-propan-1-one, *O*-DMA) were purchased from Plantech (Reading, U.K.). All other chemicals used for LC-MS/MS analysis were purchased from Sigma (St. Louis, MO, U.S.A.). Soy protein isolate (SPI) was a gift from Protein Technologies International (St. Louis, MO, U.S.A.).

II. *Sample Collection and Preparation*

Human subjects were treated as previously described[16], and three women urine samples were used in this study. Female urine samples from 9 Sprague-Dawley rats (0.5 mL of each subject) collected from a previous study[17] were also used. Porcine urine samples from two 3-days-old female pigs fed isoflavone-free diet for 14 days then changed to diet containing 24% soy protein isolates (SPI) for 4 days, were collected. The urine samples were collected between 6 and 9 am after the 4th day of SPI-diet, and were placed in tubes containing ascorbic acid and sodium azide (0.1% of each) and stored at -20°C until analysis. Human and porcine urinary samples, and pooled rat urine (2 mL for each study) were added with 8 mL of methanol, vortexed for 30 sec, and ultrasonicated for 10 min. Samples were then centrifuged at 2,000 rpm for 5 min at 4°C. This procedure was repeated once and supernatants were pooled in 45 mL tubes and dried in a SC210A Speedvac plus (Thermo Savant, Holbrook, NY, U.S.A.) for overnight at room temperature. Samples were reconstituted in 50% methanol before analyzed by LC-ESI-MS/MS.

III. *LC-ESI-MS/MS Analysis*

Concentrated urine samples with 10 µL injections were analyzed in a Bruker model Esquire LC multiple ion trap system (Bruker Daltonics Inc. Billerica, MA, U.S.A.) equipped with a 250 × 4.6 mm i.d., 5 µm, Luna C18(2) column (Phenomenex, Torrance, CA, U.S.A.). The solvent system consisted of solution A (0.1% formic acid in water, v/v) and solution B (0.1% formic acid in 1:3 acetonitrile: methanol, v/v). The solvent was delivered with flow rate of 1 mL/min by Agilent 1100 series liquid chromatographer, with splitter unit of 1:10, in a linear gradient of B from 8% to 25% B in 35 min, then to 45% in 25 min. The gradient was then changed from 45% B to 55% B in 10 min, and then to 100% B in 10 min. Solvent B was held at 100% B for 15 min and then returned to 8% B in 5 min. The metabolites were analyzed using a photodiode array detector with a wavelength range between 200 nm and 350 nm. The samples were analyzed for at least 3 times in a negative mode with a capillary voltage of 4 kV, a nebulizer pressure of 35 psi, and a drying gas flow of 10 mL/min at 350°C. The MS scan range was between 50 and 700 *m/z* with 50% of compound stability and 50% of trap drive level. The standards of glucuronide conjugates of daidzein and genistein were checked using liver cell media incubated with genistein and daidzien (unpublished data). The serial eluted conjugated metabolites were identified by referring to the results showed in previous studies[18, 45, 47, 48].

IV. *Quantification of Isoflavone Metabolite*

A modified LC-ECD method[28,29] and an LC-APCI/MS method[16,17,29] were applied to quantify the isoflavone metabolites in sample extracts after enzymatic digestion as mentioned previously[29]. The detector settings for LC-ECD analysis were 400, 450, 480, 520, 580, 630, 680, and 730 mV. Genistein, daidzein and glycitein were monitored at 480 mV and equol at 580 mV.

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RESULTS AND DISCUSSION

In this study, the profiles of isoflavone metabolites in woman, female porcine and rat urine samples were analyzed using an LC-ESI-MS/MS ion trap system with negative ion mode and monitored with UV (λ=260 nm for genistein, daidzein and equol; λ=280 nm for glycitein) detector (Figure 1A). Two isoflavone metabolites eluted at 46.2 and 52.8 minutes with extracted ion chromatogram (EIC) of *m/z*=429 in a negative mode from woman urine samples were shown in Figure 1B. LC-ESI-MS/MS profile appeared that the molecular ion $[M-H]^-$ at *m/z* =429 observed in both peaks yielded two product ions at *m/z* =253 and *m/z* =175, which represent glucuronide-conjugate of daidzein, fragments of daidzein aglycone and glucuronic acid, respectively (data not shown). These two eluted compounds were identified as 7-glucuronide-daidzein (7-gluc-Dai) and 4'-glucuronide-daidzein (4'-gluc-Dai)[45, 48] (Table 1).

Another compound eluted at 48.3 min with EIC of *m/z* =459 (Figure 1C) from the woman urine sample yielded two product ions with *m/z* = 283 and 175 (data not shown). These ions represent glucuronide-conjugated glycitein, glycitein and glucuronic acid in a negative mode. This compound was thus identified as 7-glucuronide-glycitein (7-gluc-Gly) according to the fragmentation results[45, 48].

The compounds eluted at 54.2 min and 59.6 min with EIC of *m/z* =445 (Figure 1D) from urinary samples were identified as glucuronide-conjugates of genistein. The parent compound at *m/z* 445 yielded two product ions at *m/z* =269 and 175 (data not shown) which represented genistein and glucuronic acid respectively. These compounds were identified as 7-glucuronide-genistein (7-gluc-Gen) and 4'-glucuronide-genistein (4'-gluc-Gen)[48]. Results from a quantification study showed that glucuronide conjugates of daidzein, glycitein and genistein were identified along with their aglycone formed by calculation after enzymatic hydrolysis in woman urine.

In female porcine and rat urine, the patterns of urinary profile were found similar to those of woman urinary profile. Glucuronide conjugates of daidzein, genistein and glycitein were identified as 7-gluc-Dai, 4'-gluc-Dai, 7-gluc-Gly, 7-gluc-Gen and 4'-gluc-Gen. Interestingly, a minor peak eluted at 60.4 min which was not clearly observed in woman urine was also identified with EIC of *m/z* =445 and product ions with *m/z* =269 and *m/z*

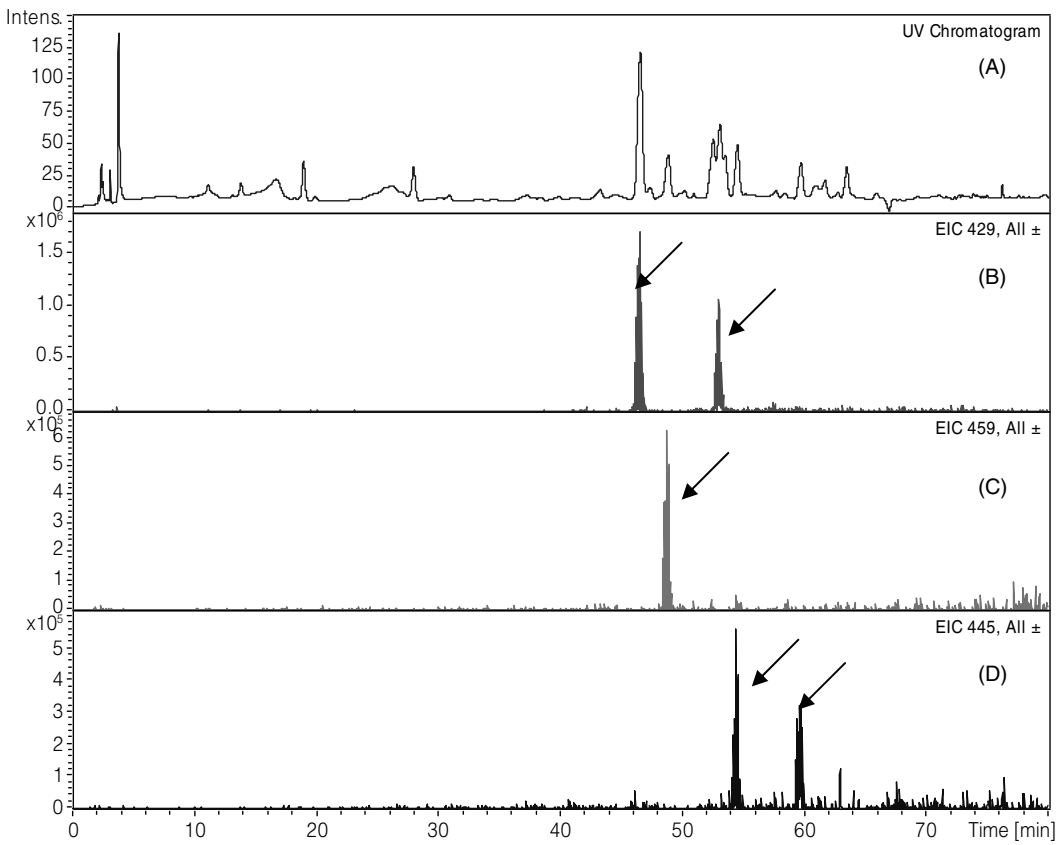

Figure 1. LC-ESI- MS/MS profile of woman urine.
(A) Woman urinary samples were monitored by UV absorbance at λ=260 nm, and were identified using extracted ion chromatogram (EIC) and automatic MS/MS with negative mode, (B) Two glucuronide conjugates of daidzein at EIC 429 were eluted at 46.2 and 56.8 min, (C) Glucuronide conjugate of glycitein (EIC 459) was eluted at 48.3 min (monitored at λ=280nm), (D) Two glucuronide conjugates of gensitein (EIC 445) were eluted at 54.2 and 59.6 min.

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Table 1. Retention times, mass of extracted ion chromatogram (EIC) and automatic MS/MS of observed urinary conjugated metabolites in women, female pigs and rats

Isoflavone metabolite conjugates	RT (min)	EIC MS (m/z)	Product ions (m/z)	Women	Pigs	Rats
7-glucuronide-Daidzein	46.2 (260 nm)	429	253,175	+	+	+
Sulfate-Daidzein	46.7 (316 nm)	333	253	N/A	N/A	+
7-glucuronide-Glycitein	48.3 (280 nm)	459	283,175	+	+	+
4'-glucuronide-Daidzein	52.8 (260 nm)	429	253,75	+	+	+
7-glucuronide-Genistein	54.2 (260 nm)	445	269,175	+	+	+
7-glucuronide- Equol	56.5 (260 nm)	417	241,175	N/A	N/A	+
4'-glucuronide- Equol	56.9 (260 nm)	417	241,175	N/A	N/A	+
4'-glucuronide-Genistein	59.6 (260 nm),	445	269,175	+	+	+
5-glucuronide-Genistein	60.4 (260 nm)	445	269,175	N/A	+	+

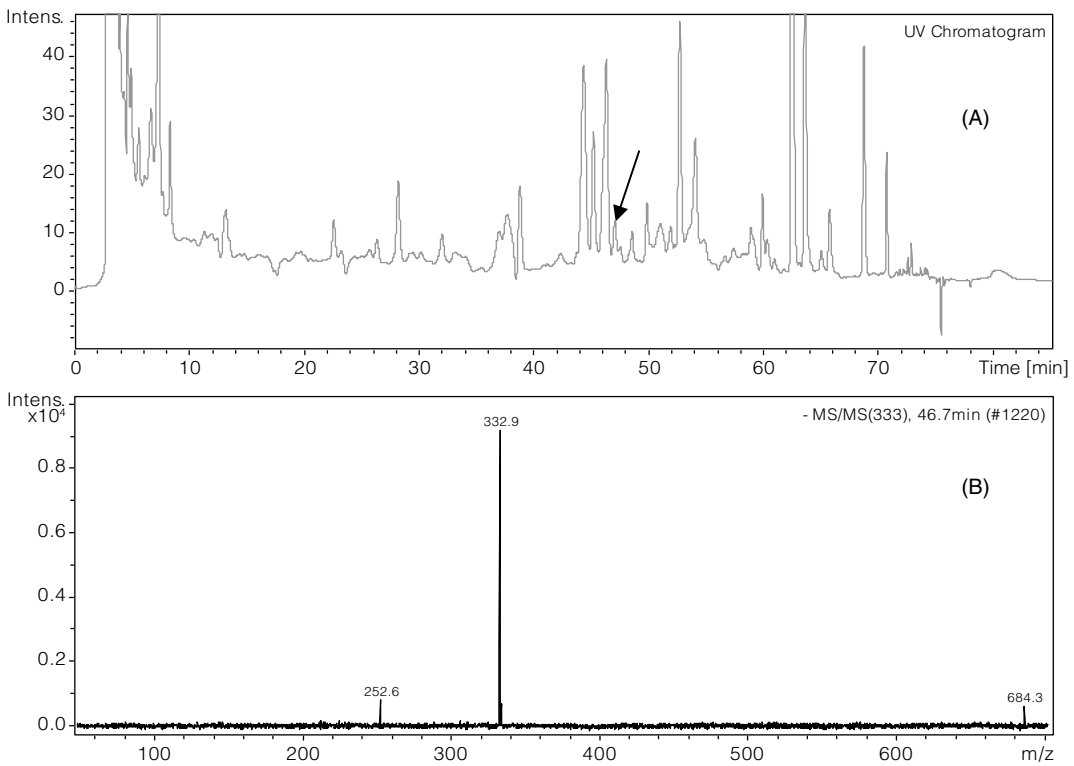

Figure 2. LC-ESI- MS/MS profile of female rat urine.
(A) Female rat urine samples were monitored by UV absorbance at λ=316 nm, and were identified using extracted ion chromatogram (EIC) and automatic MS/MS with negative mode. Sulfate conjugates of daidzien at EIC 332.9 was eluted at 46.7 min, (B) Product ion mass spectra of sul-daid (m/z=332.9).

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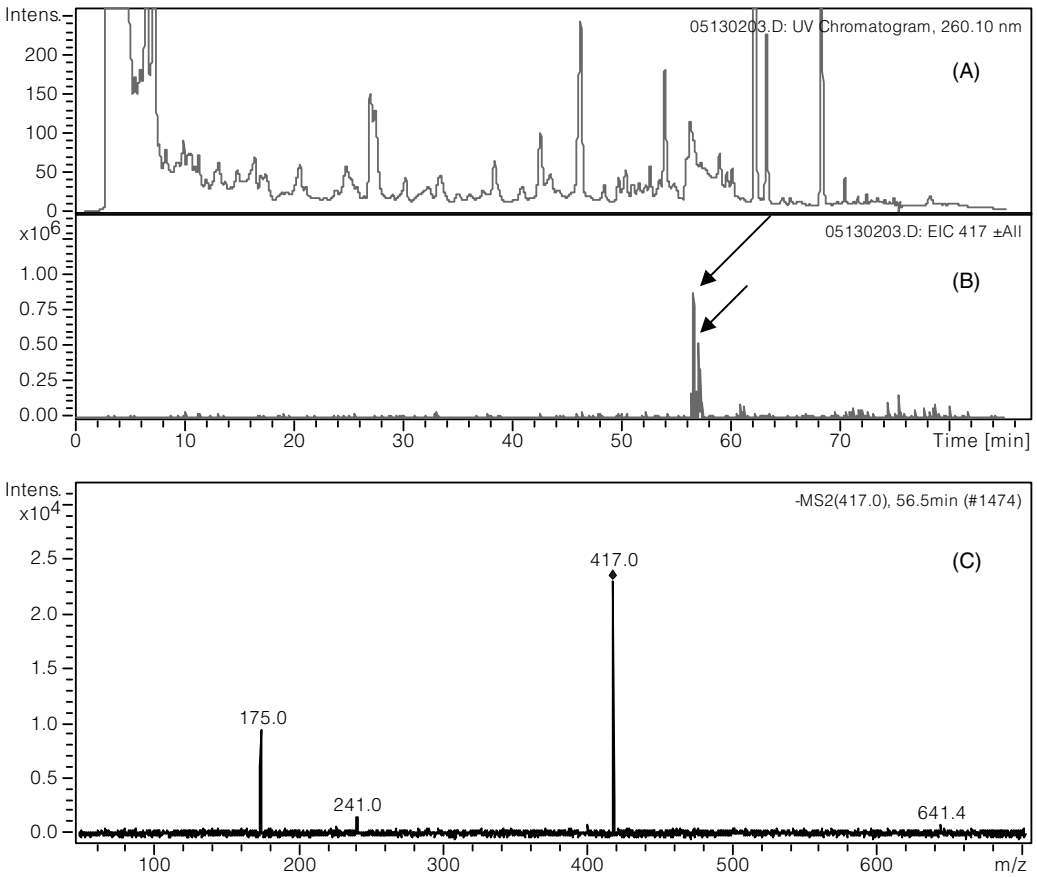

Figure 3. (A) LC-ESI- MS/MS profile of glucuronide conjugates of equol (gluc-Equ), (B) Female rat urinary sample eluted at 56.5 and 56.9 min were monitored by extracted ion chromatogram (EIC) mode at m/z=417.0 (middle panel), (C) Product ion mass spectra of gluc-Equ (m/z=417.0) monitored by automatic MS/MS with negative mode.

=175. This compound was putatively identified as 5-glucuronide-genistein (5-gluc-Gen)[45, 48].

In rat urine, a compound monitored under λ=316 nm and eluted at 46.7 min with *m/z* =332.9, yielded a product ion at *m/z* =252.6 was identified as a sulfate conjugate of daidzein (Figure 2). Interestingly, glucuronide-conjugated equol (gluc-Equ) observed at 56.5 and 56.9 min with *m/z* =417 were only found in female rat urine. In an automatic MS/MS monitoring with negative mode, these compounds yielded two daughter ions at *m/z* =241 (equol) and *m/z* =175 (glucuronic acid). These metabolites were putatively identified as 7-glucuronide–equol (7-gluc-Equ) and 4'-glucuronide–equol (4'-gluc-Equ) (Figure 3).

After enzymatic digestion, quantitative studies were performed by LC-ECD and LC/MS analysis[16,17,28,29]. Results showed that daidzein metabolites (71.1%), compared with genistein (14.2%), glycitein (14.8%), were the major components of total soy isoflavones found in woman urine. Daidzein, genistein and glycitein conjugates were about 97.6%, 91.4% and ~100% of total aglycones, respectively in women urine. In female porcine urine, daidzein metabolites (41.5%) and genistein metabolites (39.7%) were considered as the major components of total isoflavones. Isoflavone conjugates of daidzein, genistein and glycitein were 80.5%, 82.4%, and 84.5% of total aglycones respectively. Equol was not detected in either woman or female porcine urine. In female rat urine, equol was a dominant isoflavone metabolite (~72.0% of total isoflavones); daidzein, genistein and glycitein were found with 15.7%, 6.6% and 5.8% of total isoflavones respectively. The glucuronide conjugated equol was also observed in rat liver samples after the same sample preparation procedure mentioned previously. The conjugated forms of equol, daidzein, genistein and glycitein were 52.7%, 43.0%, 69.5%, and 27.6% of total aglycones respectively as previously shown[49]. UV chromatograms showed that indeed aglycones were not observed in woman urinary samples, however, aglycones of gensitein, daidzein, glycitein and equol were observed in pig urinary samples, and in rat urinary and liver samples. The urinary profile of isoflavone metabolites in woman was very similar to that in female pig in characterization (data not shown).

In conclusion, the elution sequence of conjugates of isoflavone metabolites was assigned as following: 7-gluc-Dai (46.2 min; λ=260nm), sul-Dai (46.7 min; λ=316nm), 7-gluc-Gly (48.3 min; λ=280nm), 4'-gluc-Dai (52.8 min; λ=260nm), 7-gluc-Gen (54.2 min; λ=260nm), 7-gluc-Equ (56.5 min; λ=260nm), 4'-gluc-Equ (56.9 min; λ=260nm), 4'-gluc-Gen (59.6 min; λ=260nm), then 5-gluc-Gen (60.4 min;

λ=260nm) (Table 1). Similar pattern was also supported by other studies[45, 48]. As shown in previous study, isoflavone aglycones were rarely observed in woman urine. However, these algycones were observed in animal samples after enzymatic hydrolysis using sulfatase H-5[17] as accounted for total aglycones. These results were consistent with previous studies[16,17,20,35,49]. These glucuronide conjugated isoflavone metabolites including 7-gluc-Dai, 4'-gluc-Dai, 7-gluc-Gly, 7-gluc-Gen, and 4'-gluc-Gen were also identified in rat urine[45] and human urine[48]. 7-glucuronide conjugated gensitein (7-gluc-Gen), 4'-sulfate-genistein (4'-sulp-Gen) and a mixed conjugated genistein 4'-sulfate 7-glucuronide (4'-sulp-Gen-7-gluc-Gen) in urine were obtained from rats 24 hr after oral administration of 100 ppm genistein as described previously[17]. Isomers of genistein glucuronide conjugates (7-, 4'-, and 5-gluc-Gen), daidzein glucuronide conjugates (7- and 4'-gluc-Dai), glycitein glucuronide conjugates (7- and 4'-gluc-Gly), and equol glucuronide conjugates (7- and 4'-gluc-Equ) in rat urine were also obtained from female rat 24 hr after ingestion of soy protein isolate (SPI) [45]. 7-gluc-Dai, 4'-gluc-Dai; 7-gluc-Gen and 4'-gluc-Gen were observed and identified from the incubations of bovine hepatic UGT with genistein and daidzein by LC/MS and ^{1}H-NMR[18,47]. It was shown that daidzein and genistein glucuronide conjugates eluted with a sequence of 7-gluc-Dai, 4'-gluc-Dai, 7-gluc-Gen, and 4'-gluc-Gen[18,48]. Interestingly, a 5-glucuronide-genistein conjugate (5-gluc-Gen) was eluted after 4'-gluc-Gen and identified in this study and also in rat urine[45]. However, a peak eluted with similar flow sequence was reported as an unknown compound[48]. Conjugates of *O*-desmethylangolensin (*O*-DMA), dihydrogenistein (DHG) or dihydrodaidzein (DHD) was not observed.

Glucuronide-conjugated genistein and daidzein observed in the present study were also identified and confirmed from the cell media of liver cell cultures with incubations of gensitein and daidzein for 24 hr (unpublished data). In human studies, glucuronide and/or sulfate conjugates of genistein and daidzein were observed in human urinary samples only by quantification analysis after enzymatic hydrolysis[16-18,48]. A sulfate conjugate of daidzein was observed in rat urine without enzymatic treatment in this study, however, this compound was only monitored at λ=316 nm by UV detector. Although sulphate conjugated genistein and daidzein were identified as mentioned in previous studies, these compounds were not able to be elucidated directly without enzymatic treatment in biological fluid. For rat urinary samples, serial glucuronide conjugates of isoflavone metabolites were characterized but the samples were not quantified[45]. Clarke *et al.* applied stable isotope labeled genistein, daidzein and equol as internal standards to identify and quantify glucuronide and sulfate conjugates of isoflavones from human urine without sample pretreatment. Their results showed that glucuronide conjugates were the major isoflavone metabolites in human consuming soy diet[48]. However, the mentioned method can not apply on tissue samples due to detection limits and hardness of sample cleaning. However, glucuronide conjugates of equol from urinary and liver samples were observed and

Table 2. Quantification of soy isoflavone metabolites (SIM) in urinary samples

Isoflavone	Woman (nmol/mL)		Female Piglet (nmol/mL)		Female Rat (nmol/mL)	
	Aglycone[a]	(Aglycone + Conjugates)[b]	Aglycone	(Aglycone + Conjugates)	Aglycone	(Aglycone + Conjugates)
Daidzein % [c]	0.14 ± 0.04 2.4	5.82 ± 0.33	3.49 ± 0.18 19.5	17.93 ± 0.96	45.36 ± 2.00 57.0	79.52 ± 2.29
Glycitein %	0.00 0	1.21 ± 0.07	1.26 ± 0.08 15.5	8.13 ± 0.09	24.15 ± 0.36 72.4	33.34 ± 1.41
Genistein %	0.01 ± 0.00 8.6	1.16 ± 0.11	3.14 ± 0.04 17.6	17.18 ± 0.75	8.91 ± 0.22 30.5	29.20 ± 0.63
Equol[d] %	ND	ND	ND	ND	172.83 ± 14.08 47.3	364.94 ±14.10
Sum of Observed SIM	0.15	8.19	7.89	43.24	251.25	537.00
Sum of calculated Total conjugates	8.04 (98.2%)		35.35 (81.75%)		287.75 (53.58%)	

[a] Aglycones were measured without enzymatic digestion.
[b] (Aglycones + Conjugates) were measured after enzymatic digestion[29] using 100 units of Sulfatase H-5 for 3 hours at 37°C; (b - a) = concentration of calculated conjugates.
[c] Percentage of aglycone = (a/b) × 100%.
[d] Equol was analyzed using LC-ECD system[28, 29] .

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Table 3. Percentage of total soy isoflavone metabolites (SIM) in woman, pig and rat urine

	Woman (%)[a]	Female Pig (%)	Female Rat (%)
Daidzein	71.1	41.5	15.7
Glycitein	14.8	18.8	6.6
Genistein	14.2	39.7	5.8
Equol	0	0	72.0

[a] % of SIM was calculated by (aglycone + conjugates)/(sum of observed SIM) *100%; data were subtracted from Table 2.

identified without considerable troublesome in this study. Results from previous studies with enzymatic hydrolysis showed that aglycones and conjugates of equol were only observed in female rat and monkey urine. Conjugates of daidzein and genistein were found as the major metabolites in woman and porcine urine, aglycones of these two isoflavones were mainly found in monkey urine[49,50], and there was no significant difference between aglycone and conjugate contents in rat urine[49,50]. In this study, results from the quantitative analysis by LC-ECD and/or LC-APCI/MS following the previous methods[16,17,28,29] showed that almost all of the SIM found in woman urine (98.2%) were conjugates, ~81.8% of SIM were found conjugates in porcine urine, and ~46.4% of SIM in rat urine were aglycone (Table 2). Percentages of glycitein metabolites (aglycone + conjugates) observed in woman, porcine and rat urine were 14.8%, 18.8% and 6.6% of total observed soy isoflavone metabolites, respectively (Table 3). Interestingly, the conjugated glycitein was 100% in human urine, 84.5% in porcine urine, and only 27.6% in rat urine (Table 2). A further study is needed to explore this phenomenon.

In a human study, genistein, glycitein and daidzein were observed in 24-hr urine pool after single soy meal intake[51]. Daidzein, glycitein, and genistein are uncompetitive inhibitors of recombinant cytochrome P450 2A6 (CYP2A6) catalyzed nicotine *C*-oxidation[52]. However, the significance of glycitein and its potential estrogenic role is an interesting topic to study. The previous sample treatment employed sulfates H5 to hydrolyze the metabolites and to quantify the samples under LC-APCI/MS system in order to identify the conjugated forms. In this study, methanol treatment offered a rapid and economical clean up method for urine sample preparation. This method also offered a rapid and easier clean- up for tissue samples such as liver samples. In conclusion, methanol treatment used in this study offered a simple and rapid way which could be applied for preparation of either biofluids or other biomatrices before mass spectrometric study. An LC-ESI-MS/MS ion trap system to build mass fragmentation profiles, in combination with photodiode array system to monitor the elution sequence under various absorbance wavelengths would be an applicable analytical methodology to analyze glucuronide conjugated metabolites, but not always the sulphate conjugates, in biofluid and tissue samples by merely an HPLC system. LC-ESI-MS/MS ion trap system was not attempted for the quantification analysis for glucuronide or sulphate conjugates of soy isoflavones since standards of conjugated metabolites were not technically and economically available up-to-date. This study did offer a rapid, easier and economical substitute LC-ESI-MS/MS methodology to perform identification study, however, along with an established quantification methodology either using an LC/MS system[15-17, 29] or an LC-ECD system[28,29] .

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