JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Characterization of Isoflavones and Their Conjugates in Female Rat Urine Using LC/MS/MS

NIANBAI FANG, SHANGGONG YU, AND THOMAS M. BADGER*

Arkansas Children's Nutrition Center, 1120 South Marshall Street, Little Rock, Arkansas 72202

Isoflavone phytoestrogens found in soybeans are the most widely studied phytochemicals in human diets and soy infant formulas. The health benefits of the isoflavones daidzein and genistein have been reported, and concerns about potential adverse effects have also been raised. However, the results of direct analysis of isoflavones and their metabolites in biological fluids after consumption of soy-containing diets are scarce. This study describes an LC/MS/MS method for the analysis of isoflavones and their metabolites in the urine of female rats fed diets made with soy protein isolate. Five isoflavones (daidzein, genistein, glycitein, dihydrodaidzein, and *O*-desmethylangolensin) were identified by comparison with authentic standards. Seventeen conjugates of isoflavones were characterized in the urine, the most unusual being genistein 5-glucuronide and four glucuronide conjugates of reductive metabolites of daidzein. The application of LC/MS/MS to analyze isoflavone metabolites is simple and sensitive, and appears to be an excellent method for determining the bioavailability and metabolism of food phytochemistry.

KEYWORDS: Soy protein isolate (SPI); isoflavones; rat urine; isoflavone glucuronides; metabolism; LC/MS/MS

INTRODUCTION

Isoflavone phytoestrogens are major phytochemicals present in soybeans, which have been known as a health food in Asia for centuries and in the West more recently. Soy infant formulas are an excellent source of isoflavones and high circulating isoflavone concentrations are attained in infants (*1*). Although significant health benefits of consuming isoflavone-rich diets have been reported (*2*−*8*), some studies have raised concerns about potential adverse effects from isoflavone intake in infants as the result of early estrogenic exposure (*9*, *10*), and in adults related to breast cancer risk in women with occult tumors (*11*).

The study of isoflavone action requires a full identification of isoflavone metabolites to develop a thorough understanding of the metabolic pathways of isoflavones after consumption and bioavailability of active metabolites. Recently, a number of studies have been conducted on the analysis of isoflavones and their metabolites in biological fluids. The majority of the studies have focused on determining the concentration of isoflavone conjugates by measurement of the liberated aglycones in biological fluids after digestion with deconjugating enzymes (*12*−*15*). In other studies, LC/MS has been used to identify the isoflavones and their conjugates in biological fluids of rats administered the pure isoflavones, genistein, daidzin, and daidzein (*16*−*18*). The metabolites of these isoflavones have been reported as glucuronide and sulfate conjugates. Additionally, the monohydroxylated and dihydroxylated metabolites of daidzein and genistein have been identified by GC/MS in humans in vitro and in vivo (*19*).

The aim of this study was to develop a sensitive LC/MS/MS method to directly characterize the isoflavones and their metabolites in biological fluids as a part of our continuing study on the metabolic pathway of isoflavones. The isoflavones and their metabolites in the urine were separated by LC, and the peaks of these compounds were simultaneously identified by auto MS/MS. The application of LC/MS/MS with different ion polarities (positive and negative) is simple and sensitive, and is an invaluable technique for identification of isoflavones and their metabolites.

MATERIALS AND METHODS

Materials. Three standards, including daidzein (7,4′-dihydroxyisoflavone), genistein (5,7,4′-trihydroxyisoflavone), and biochanin A (5,7-dihydroxy-4′-methoxyisoflavone), were purchased from Indofine Chemical Company, Inc. (Belle Mead, NJ). All other standards, glycitein (7,4′-dihydroxy-6-methoxyisoflavone), dihydrodaidzein (7,4′-dihydroxyisoflavanone), dihydrogenistein (5,7,4′-trihydroxyisoflavanone), and *O*-desmethylangolensin (1-(2,4-dihydroxyphenyl)-2-2(4-hydroxyphenol)-propan-1-one) were obtained from Plantech (UK) (Reading, UK). All chemicals used for the LC/MS/MS analysis were HPLC grade. Soy protein isolate was a kind gift from Protein Technologies International (St. Louis, MO). Female Sprague−Dawley rats were purchased from Harlan−Sprague−Dawley (Indianapolis, IN).

Soy Feeding Study. Female Sprague−Dawley rats were housed in stainless steel metabolism cages and fed ad libitum diet containing SPI as the sole protein source formulated according to AIN 93G (*20*). Two SPI preparations (SPI 670 and IF-SPI) were used in the present study.

* To whom correspondence should be addressed. Phone: (501) 320-2785. Fax: (501) 320-2818. E-mail: BadgerThomasM@uams.edu.

10.1021/jf011384v CCC: $22.00
Published on Web 03/26/2002

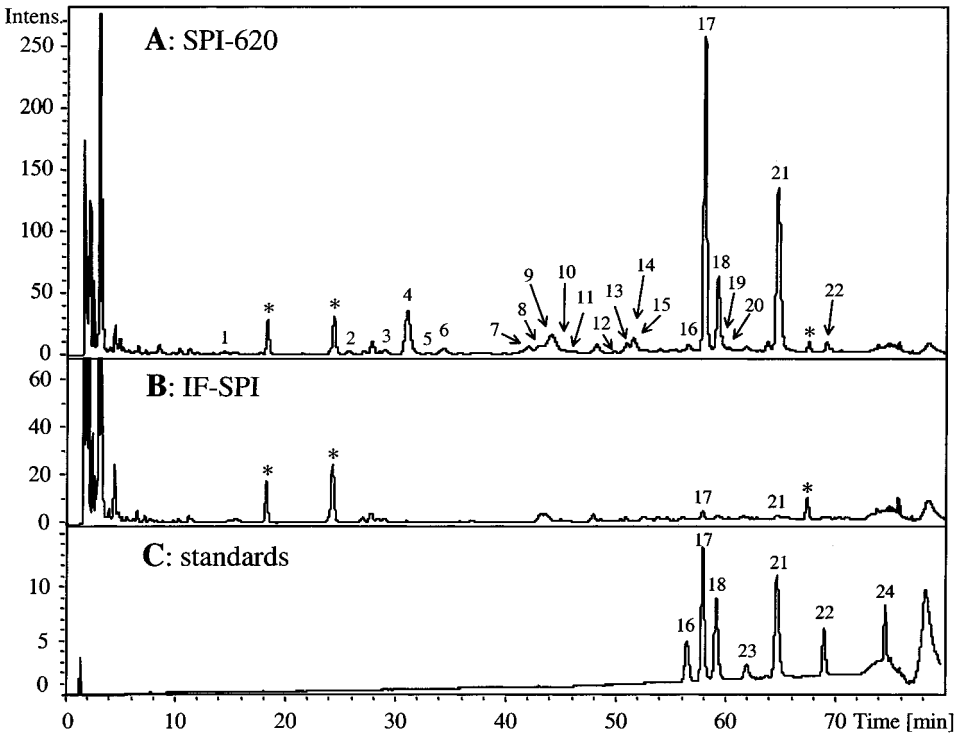


Figure 1. UV-265 nm HPLC chromatograms: (A) profile of a urine pool from rats fed an isoflavone rich diet (SPI-670); (B) profile of a urine pool from rats that were fed a diet low in isoflavones (IF-SPI); and (C) profile of a mixture of standards: dihydrodaidzein (**16**), daidzein (**17**), glycitein (**18**), genistein (**21**), *O*-desmethylangolensin (**22**), dihydrogenistein (**23**), and biochanin A (**24**). * indicates the peaks present in both urine profile A and its control profile B.

SPI 670 and IF-SPI have different quantitative levels of major isoflavones according to the analytical data from Protein Technologies International. SPI 670 contained 1.36 mg of genistein equivalents and 0.65 mg of daidzein equivalents per g of protein. Estimated daily intake of genistein and daidzein equivalents was 19.3 and 9.2 mg/kg body weight, respectively, after consumption of SPI 670. IF-SPI, used as a control for isoflavone analysis, contained less than 0.1 mg of genistein or daidzein equivalents per g of protein.

Urine was collected for 24 h after ingestion. Urine containers had ascorbic acid and sodium azide added as preservatives (0.1% concentration of each). Samples from rats were stored at −20 °C. For this study, a pool was made by combining urine from five rats per group (1 mL/rat). This protocol was approved by the Animal Care and Use Committee of the University of Arkansas for Medicine Sciences.

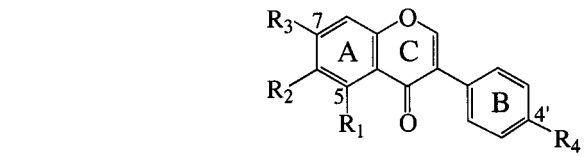
LC/MS/MS Sample Preparation. Solid phase extraction (SPE) with an LC-18 (2 g/12 mL) cartridge from Supelco (Bellefonte, PA) was used to prepare the enriched extract of rat urine for LC/MS/MS analysis. SPE procedures for the urine sample and its control were exactly the same in order to compare their LC/MS/MS results. Before a sample was applied, the cartridge was rinsed with two volumes of MeOH followed by two volumes of water. The urine sample (5 mL) was thawed and passed through the cartridge before the sorbents became dry. The cartridge was then washed with 20 mL of methanol. The methanol effluent was concentrated on a rotary evaporator under reduced pressure at room temperature, followed by drying in the freeze-dryer to give an enriched extract of interest. The enriched extract was dissolved in 250 μL of 75% aqueous MeOH (50 μL/mL urine) for LC/MS/MS analysis.

LC/MS/MS Analysis. The enriched extract was directly analyzed by LC/MS/MS with a 5-μL injection. LC/MS/MS was performed using Bruker model Esquire-LC multiple ion trap mass spectrometer equipped with an Agilent 1100 series liquid chromatograph. An Eclipse XDB-C8 column (150 × 4.6 mm i.d.) (Agilent Technologies, Wilmington, DE) was used at a flow rate of 1 mL/min. The HPLC gradient was 25% acetonitrile/methanol (solvent B) in 0.1% formic acid/H_2O (solvent A): 8−25% in 40 min, 25−55% from 40 to 70 min, 55−100% from 70 to 75 min, and finally returned to initial concentration from 75 to 80 min. HPLC profiles of isoflavones and their metabolites in the urine sample were obtained with a diode-array detector. Because UV spectra of isoflavones have band II appearing in the 245−270 nm region and the band I shoulder in the range 300−340 nm (*21*), two UV wavelengths of 265 and 315 nm were chosen for detection of isoflavones and their metabolites. A UV wavelength of 360 nm was also set in the UV detector for detection of flavones. Conditions for MS analysis of HPLC peaks included a capillary voltage of 3200V, a nebulizing pressure of 33.4 psi, a drying gas flow of 10 mL/min, and a temperature at 340 °C. The MS scan began at 60.00 and ended at 700.00 *m/z* with compound stability 50% and trap drive level 50%. Samples were analyzed by automatic MS/MS with both negative- and positive-ion modes. MS/MS conditions were the following: mass range from 100 to 700 *m/z*, isol width 4.0, frag ampl 1.00, and number of parents 1. The urine from rats after consumption of the isoflavone rich diet (SPI 670) and control urine were analyzed under exactly the same HPLC conditions. The control urine was collected from rats that were fed with IF-SPI.

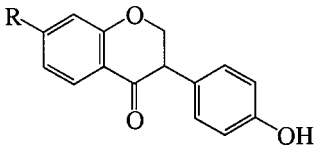
RESULTS AND DISSCUSSION

Comparison of HPLC Profiles. To detect isoflavones and their metabolites, the profile of urine from rats fed the SPI-670 diet was compared to that of rats fed the IF-SPI diet. Several peaks were present in HPLC profiles of urine from both groups. The only difference between sample diet SPI-670 and its control IF-SPI were the concentrations of isoflavone equivalents. The levels of isoflavone equivalents in the SPI-670 diet were more than 10 times higher than those in the IF-SPI control diet. More than twenty peaks were present in the HPLC profile of urine from the rat fed SPI-670, and representative profiles of isoflavones and their metabolites are shown in **Figure 1**.

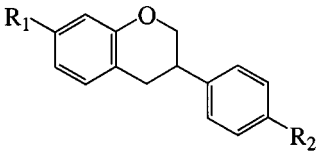
Isoflavone Aglycons. Isoflavone aglycones, including genistein, daidzein, glycitein, dihydrogenistein, dihydrodaidzein, and



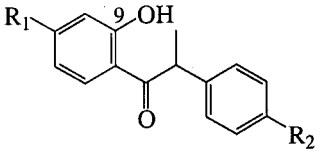
No.	metabolite	R_1	R_2	R_3	R_4
17	daidzein	H	H	OH	OH
4	daidzein 7-glucuronide	H	H	* OGlcUA	OH
8	daidzein 4'-glucuronide	H	H	OH	OGlcUA
7	daidzin	H	H	* OGlc	OH
1	daidzin-glucuronide	H	H	OGlc	OGlcUA
12	6"-*O*-malonyldaidzin	H	H	Omalonyl	H
21	genistein	OH	H	OH	OH
14	genistein 5-glucuronide	OGlcUA	H	OH	OH
3	genistein 7-glucuronide	OH	H	OGlcUA	OH
9	genistein 4'-glucuronide	OH	H	OH	OGlcUA
2	genistein 7,4'-diglucuronide	OH	H	OGlcUA	OGlcUA
18	glycitein	H	OMe	OH	OH
6	glycitein 7-glucuronide	H	OMe	OGlcUA	OH
11	glycitein 4'-glucuronide	H	OMe	OH	OGlcUA
10	glycitin	H	OMe	OGlc	OH



No.	metabolite	R
16	dihydrodaidzein	OH
5	dihydrodaidzein 7-glucuronide	OGlcUA



No.	metabolite	R_1	R_2
	equol	OH	OH
**13	equol glucuronide	OGlcUA	OH
**15	equol glucuronide	OGlcUA	OH



No.	metabolite	R_1	R_2
22	*O*-desmetylangolensin	OH	OH
**19	*O*-desmetylangolensin glucuronide	OGlcUA	OH
**20	*O*-desmetylangolensin glucuronide	OGluUA	OH

* GlcUA: β-glucuronic acid; Glc: β-glucose.

** conjugation position could not be determined by LC/MS/MS.

Figure 2. Structures of the isoflavones and their metabolites in female rat urine.

O-desmethylangolensin, have been previously identified in rat urine after treatment with deconjugating enzymes (*12*). A mixture of authentic standards was subjected to HPLC. Under the same conditions, the retention times of components **16**, **17**, **18**, **21**, and **22** in the urine were identical to those of their corresponding standards (**Figure 1A** and **1C)**. These results indicate that the structures of compounds **16**, **17**, **18**, **21**, and **22** are dihydrodaidzein, daidzein, glycitein, genistein, and *O*-desmethylangolensin, respectively (**Figure 2**). The same daughter spectra of standards and their corresponding isoflavones in the urine confirm these structural assignments of **16**, **17**, **18**, **21**, and **22** (**Table 1**).

Isoflavone Conjugates. Compounds **1**, **4**, **7**, **8**, and **12**, in their daughter spectra with positive-ion mode, gave the same base peak at *m*/*z* 255 representing daidzein aglycon as the mother nucleus. This diagnostic fragment indicates that these compounds are daidzein conjugates (**Figure 3**). The molecular ion $[M - 1]^-$ at *m*/*z* 429 of **4** yielded two ions at *m*/*z* 253 and 175 in the daughter spectrum (MS/MS) corresponding to fragments of daidzein and glucuronide groups, respectively. A weak peak at *m*/*z* 385 resulted from loss of CO_2 from glucuronide. The daughter spectra in both positive- and negative-ion modes suggest a daidzein−glucuronide structure for **4**. The daughter spectrum with positive-ion mode of **8** is identical to that of **4**, suggesting that **4** and **8** are daidzein−glucuronide isomers. The daidzein 7-*O*-β-D-glucuronide is a major conjugate metabolite of daidzein after daidzein administration (*16*, *17*). In the present study, **4** is the largest peak in the 265 nm HPLC profile, except for the peaks of isoflavone aglycones (**Figure 1A**). Thus, it is reasonable to assume that **4** is daidzein 7-*O*-β-D-glucuronide. There are two conjugation sites, hydroxy groups at C-7 and 4′, on daidzein (**Figure 2**), therefore **8** is daidzein 4′-*O*-β-D-glucuronide. Compound **8** is a minor component in the urine and is often ignored in the analysis of isoflavone metabolites in biological fluids. To confirm the assignments of **4** and **8,** the urine sample was analyzed under the HPLC condition previously described for the analysis of daidzein and daidzein 7-*O*-β-D-glucuronide (*17*). The retention behaviors of **4** (16.84 min), **8** (19.41 min), and **17** (daidzein, 24.03 min) support **4**, but not **8**, as daidzein 7-*O*-β-D-glucuronide on the basis of the reported retention times of daidzein and daidzein 7-*O*-β-D-glucuronide (*17*). Compound **7** gave a

Table 1. ESI MS/MS Data for Free Isoflavone in Rat Urines and Their Standards

isoflavonoids	Rt. min.	ion polarity	MS, *m/z* $[M-1]^-$	MS, *m/z* $[M+1]^+$	ion, *m/z* in MS/MS		
16	56.4	negative	255		149[a]	135	121
std. dihydrodaidzein	56.5	negative	255		149[a]	135	121
16	56.2	positive		257	*b*		
std. dihydrodaidzein	56.5	positive		257	163[a]	123	95
17	57.8	negative	253		224[a]	208	135
std. daidzein	57.8	negative	253		224[a]	208	135
17	57.7	positive		255	137[a]	227	199
std. daidzein	57.8	positive		255	137[a]	227	199
18	59.0	negative	283		268[a]	240	
std. glycitein	59.0	negative	283		268[a]	240	
18	59.1	positive		285	270[a]	229	145
std. glycitein	59.2	positive		285	270[a]	229	145
21	64.6	negative	269		181[a]	225	
std.	64.7	negative	269		181[a]	225	
21	64.3	positive		271	153[a]	243	215
std. genistein	64.8	positive		271	153[a]	243	215
22	69.0	negative	257		109[a]	239	163
std. desmethylangolensin	69.0	negative	257		109[a]	239	163
22	69.1	positive		*c*			
std. desmethylangolensin	69.1	positive		*c*			
dihydrogenistein		negative	*c*				
std. dihydrogenistein	62.0	negative	271		165[a]		
dihydrogenistein		positive		*c*			
std. dihydrogenistein	62.0	positive		273	*b*		
biochaninA		negative	*c*				
std. biochaninA	74.6	negative	283		268[a]		
biochaninA		positive		*c*			
std. biochaninA	74.5	positive		285	240[a]	170	

[a] Base peak in daughter mass spectra. [b] Because positive ion polarity was not a good ionization mode for these compounds, no daughter mass spectrum was obtained during auto MS/MS analysis. [c] No mass spectra were obtained during MS analysis.

$[M + 1]^+$ at *m/z* 417 in accord with a $C_{21}H_{20}O_9$ formula of daidzin that is a principal isoflavone component in SPI (*22*). The fragment at *m/z* 255 in its daughter spectrum was formed by loss of a glucoside. Thus, we assign **7** as daidzin, daidzein 7-*O*-*β*-D-glucopyranoside. Compound **1** is more polar than **4** and **7** according to their retention behavior in reversed-phase HPLC (**Figure 1**). Compound **1** with $[M + 1]^+$ at *m/z* 593 produced three diagnostic fragments: a daidzein−glucuronide moiety at *m/z* 431; a daidzein−glucoside moiety at *m/z* 417; and a daidzein moiety at *m/z* 255 in the MS/MS. The fragments at *m/z* 431 and 417 resulted from loss of glucoside and glucuronide, respectively. The fragment at *m/z* 255 was derived from losses of glucoside and glucuronide. The MS/MS data indicates that **1** is a glucoside and glucuronide diconjugate of daidzein. Considering daidzin (daidzein 7-glucoside) is a principal isoflavone component in SPI (*22*) and detected as **7** in the urine, the structure of **1** should be daidzin−glucuronide. There is only one conjugation site (C-4′) on daidzin, suggesting structure daidzin 4′-*O*-*β*-D-glucuronide for **1**. In the daughter spectrum of **12** with a $[M + 1]^+$ at *m/z* 503, the base peak at *m/z* 255 for the daidzein aglycone moiety might be derived from loss of a malonylglucosyl, suggesting *O*-malonyl daidzin for **12**. Because 6″-*O*-malonyldaidzin is present in SPI (*22*), the structure of **12** was assigned as 6″-*O*-malonyldaidzin.

The molecular ion $[M - 1]^-$ at *m/z* 445 of compounds **3** and **14** yielded a base peak at *m/z* 269 in the daughter spectrum corresponding to the diagnostic genistein fragment (**Table 2**). The value (176 Da) of the lost fragment suggests the structure genistein−glucuronide for both **3** and **14**. The fragment at *m/z* 175 from $[M - 1]^-$ at *m/z* 445 in the daughter spectrum of **14** (**Figure 4**) also supports the presence of a glucuronide group. For compound **9**, auto MS/MS with negative-ion mode failed to produce a daughter spectrum. Using positive-ion mode, the

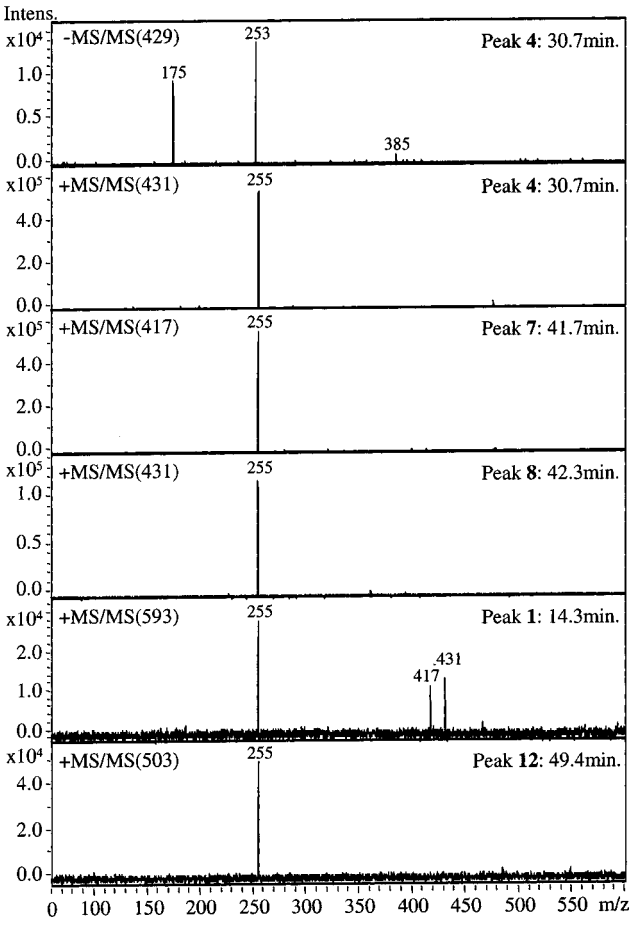


Figure 3. Daughter mass spectra of daidzein and its conjugation metabolites.

Table 2. ESI MS/MS Data for Isoflavone Conjugates in Rat Urine

conjugates[a]								
no.	structures	Rt. min.	ion polarity	MS $[M-1]^-$	MS $[M+1]^+$	ion in MS/MS *m/z*		
daidzein (**17**)								
1	GlcUA-Glc	14.1	negative	591		415[b]	253	175
		14.3	positive		593	255[b]	431	417
4	7-GlcUA	30.7	negative	429		253[b]	175	
		30.7	positive		431	255[b]	199	
7	7-Glc	41.9	negative	415		*c*		
		41.9	positive		417	255[b]		
8	4′-GlcUA	42.3	negative	429		*c*		
		42.3	positive		431	255[b]		
12	malonylGlc		negative	*d*				
		49.4	positive		503	255[b]		
genistein (**21**)								
2	diGlcUA	25.4	negative	621		445[b]	269	
		25.4	positive		(623)447[e]	271[b]		
3	7-GlcUA	29.0	negative	445		269[b]		
			positive		*d*			
9	4′-GlcUA	44.0	negative	445		*c*		
		44.0	positive		447	271[b]		
14	5-GlcUA	51.2	negative	445		269[b]	175	
		51.2	positive		(447)271[e]	153[b]	243	
glycitein (**18**)								
6	7-GlcUA	33.6	negative	459		283[b]	175	
		33.8	positive		461	285[b]		
10	7-Glc		negative	*d*				
		44.8	positive		447	285[b]		
11	4′-GlcUA		negative	*d*				
		45.5	positive		461	285[b]		
equol								
13	GlcUA	50.4	negative	417		175[b]	241	
		50.6	positive		(419)243[e]	123[b]	107	
15	GlcUA	51.5	negative	417		175[b]	241	
		51.4	positive		(419)243[e]	107[b]	123	
O-desmethylangolensin (**22**)								
19	GlcUA	59.5	negative	433		175[b]	257	
			positive		*d*			
20	GlcUA	60.0	negative	433		257[b]	175	
			positive		*d*			
dihydrodaidzein (**16**)								
5	GlcUA	32.1	negative	431		175[b]		
			positive		*d*			

[a] Because appropriate standards were not available, compounds were identified tentatively only on the basis of MS/MS data. [b] Base peak in daughter mass spectra. [c] Because molecular ion was not a base peak in MS, no daughter spectrum was obtained during auto MS/MS. [d] Because positive ion polarity could not ionize the compound, no mass spectra was obtained in MS. [e] The daughter spectra derived for base peak in MS instead of molecular ion in auto MS/MS.

molecular ion $[M+1]^+$ at *m/z* 447 of **9** gave a genistein fragment at *m/z* 271 (**Table 2**), suggesting that **9** is a genistein−glucuronide. The MS/MS data from **3**, **9**, and **14** indicate that they are genistein−glucuronide isomers. The 7- and 4′-monoglucuronide conjugates of genistein have been identified in the blood of rats receiving dietary genistein. The HPLC retention times of genistein 7-glucuronide (7.10 min), genistein 4′-glucuronide (8.08 min), and genistein (12.85 min) have been reported (*18*). Under the same HPLC conditions as those described in ref *18*, the elution order and retention times are as follows: 7.09 min for **3**, 7.99 min for **9**, 9.33 min for **14,** and 12.83 min for **21** in the present study. Comparison of the retention times for **3**, **9**, **14**, and **21** and those previously reported for genistein and its conjugates (*18*) indicates that **3** and **9** are genistein 7-glucuronide and genistein 4′-glucuronide, respectively. There are three conjugation sites on genistein at C-5, 7, and 4′, which implies that **14** is a 5-glucuronide conjugate of genistein. Compound **2** gave a $[M-1]^-$ at *m/z* 621 in accord with a formula of genistein diglucuronide. The base peak at *m/z* 445 and the genistein fragment at *m/z* 269 were resulted from losses of two glucuronide groups in sequence. The data of MS/MS suggest the structure genistein−diglucuronide for **2**, but do not provide evidence for the conjugation sites on genistein.

The daughter spectra in positive-ion mode of compounds **6**, **10**, and **11** yielded the same base peak at *m/z* 285 in accord with the diagnostic glycitein fragment, indicating that **6**, **10**, and **11** are the conjugates of glycitein. In the daughter spectra of **6** and **11**, the ion at *m/z* 285 derived from the same $[M+1]^+$ at *m/z* 461 after loss of a glucuronide fragment (176 Da) suggests that **6** and **11** are glycitein−glucuronide isomers. Considering that 7- glucuronides of daidzein (Rt 30.7 min) and genistein (Rt 29.0 min) are retained on the C8 sorbent much less than those of their 4′-isomers (42.3 min for daidzein−glucuronide and 44.0 min for genistein−glucuronide), the HPLC retention behaviors of **6** (Rt 33.6 min) and **11** (Rt 45.5 min) suggest that **6** and **11** are glucuronated at the 7- and 4′-hydroxy groups, respectively. Compound **10** is a glycitein glucoside because the mass difference of 162 Da between $[M+1]^+$ at *m/z* 447 and the glycitein fragment at *m/z* 285 implies a glucoside group in the structure of **10**. Because glycitin (glycitein 7-glucoside) is a component in SPI diet (*22*), it is expected that **10** is glycitin, an unmetabolized SPI ingredient in the urine.

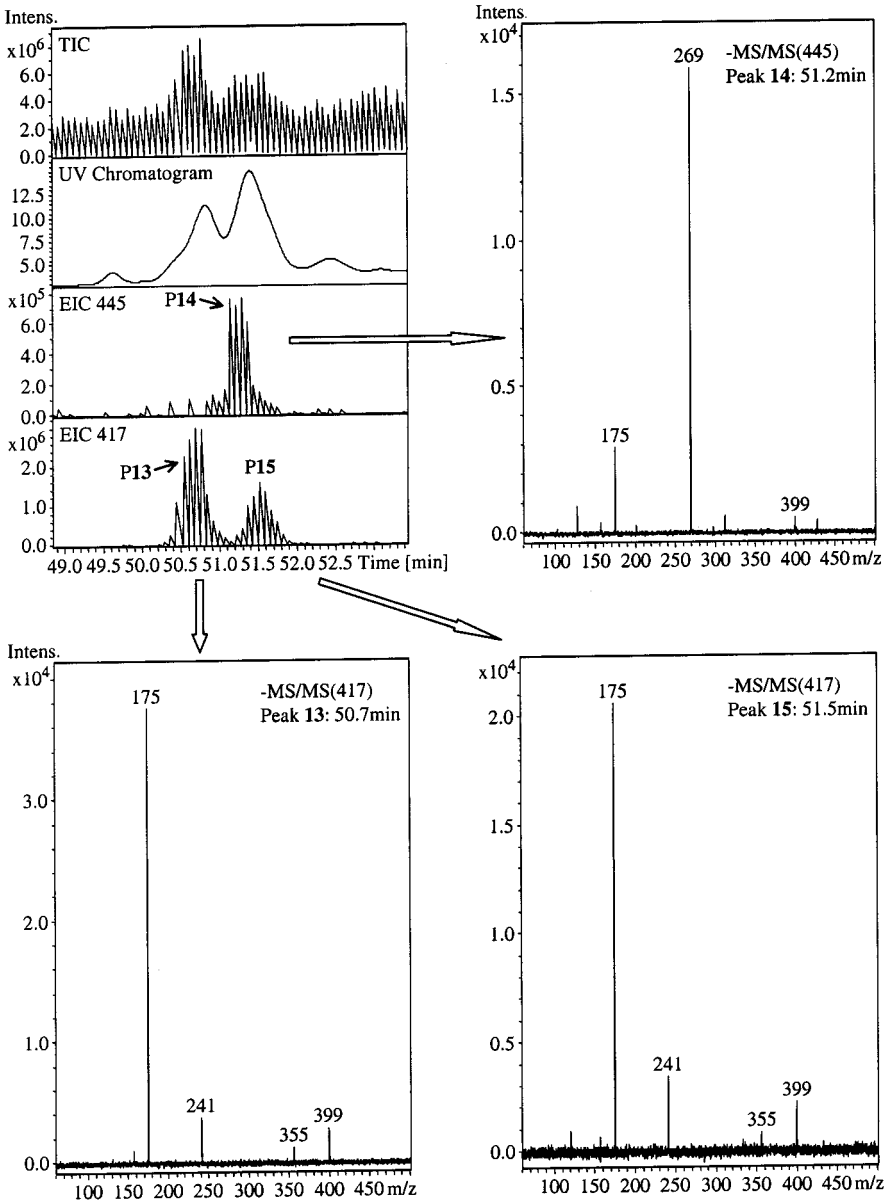


Figure 4. Daughter mass spectra of genistein 5-glucuronide (peak **14**) and two glucuronide conjugates of equol.

Two minor components, **13** and **15**, with the same $[M - 1]^-$ at *m*/*z* 417 have nearly identical daughter spectra (**Figure 4**). The base peak at *m*/*z* 175 is a typical fragment for glucuronide, and the ion at *m*/*z* 241 is formed from an equol moiety. Thus, **13** and **15** are equol glucuronide isomers. Like compounds **13** and **15**, the daughter spectra of minor components **19** and **20** from the same $[M - 1]^-$ at *m*/*z* 433 are essentially identical (**Table 2**). Two ions at *m*/*z* 175 and 257 are derived from glucuronide and *O*-desmethylangolensin moieties, respectively. Thus, **19** and **20** are *O*-desmethylangolensin glucuronide isomers. For compound **5**, the negative ion at *m*/*z* 175 in daughter spectrum from $[M - 1]^-$ at *m*/*z* 431 suggests glucuronide moiety for **5**. The molecular ion at *m*/*z* 431 of **5** corresponds to the formula of dihydrodaidzein glucuronide, but no ion at *m*/*z* 255 for dihydrodaidzein moiety is shown in its daughter spectrum. Dihydrodaidzein is the one of five aglycones found in the urine sample. As glucuronide conjugates of all four other aglycones are detected in the urine, the structure for **5** is identified as dihydrodaidzein glucuronide tentatively. The glucuronide conjugates of equol, *O*-desmethylangolensin, and dihydrodaidzein are new isoflavone metabolites, not previously reported. The exact regiochemistry of conjugates for **5**, **13**, **15**, **19**, and **20** could not be determined by LC/MS/MS in the present study because appropriate standards are not available for LC/MS/MS and no pure separated glucuronide conjugates are available for NMR.

Metabolism of Isoflavones. The principal isoflavone constituents in SPI are isoflavone glucosides, including daidzin and genistin (*22*). Although rats in this study were in metabolism cages that kept food, urine, and feces separate, it is possible that isoflavone glucosides, daidzin (**7**) and glycitin (**10**), in the urine resulted from contamination of the SPI in the food. However, genistin was not detected in the urine and daidzin and glycitin were detected. If contamination were the cause of daidzin and glycitin, one would expect to find genistin also. Thus, these data suggest that these glucosides were not food contaminants in the urine, but that they were absorbed in small amounts. Also, a trace of 6″-*O*-malonyldaidzin was detected in urine. Both 6″-*O*-malonyl-genistin and -glycitin are present in SPI in higher concentrations than that of 6″-*O*-malonyldaidzin, and no 6″-*O*-malonyl -genistin and -glycitin were detected in

the urine. Therefore, the trace amount of 6″-*O*-malonyldaidzin detected in the urine may be due to direct absorption by female rats.

Identification of aglycones dihydrodaidzein, equol, and *O*-desmethylangolensin in the urine suggests a reductive metabolic pathway for daidzein. Interestingly, the reductive metabolites of genistein and glycitein were not found in the urine. The glucuronide and sulfate conjugates are the major metabolites in the urine of male rats administered isoflavones daidzein and daidzin (*16*, *17*), but glucuronide conjugate is reported to be the only conjugate metabolite in the urine of female rats receiving daidzein (*17*). In the present study, the glucuronides are the predominant conjugate metabolites in the urine collected from female rats, in agreement with this latter study (*17*).

Application of LC/MS/MS. For analysis of isoflavones and their metabolites in the urine by LC, seven isoflavone aglycones were exhibited as well-separated peaks using a C8 column (**Figure 1C**). Furthermore, there were no significant non-isoflavone peaks to interfere with the peaks of isoflavones and their metabolites in 265 nm profile (**Figure 1A** and **1B**). The different conjugation sites of the glucuronide group result in the different retention behaviors in this HPLC condition. The 7-glucuronide conjugates of daidzein, genistein, and glycitein have the retention time around 29 to 34 min with their corresponding 4′-isomers around 42 to 45 min. Their retention behaviors indicate that the 4′-glycuronide isoflavones are retained longer on the C8 sorbent than their 7-isomers, and then are eluted at a higher organic percentage. Equol and *O*-desmethylangolensin are the metabolites of daidzein with a reducted C-ring. In contrast to the isoflavones above with their own C-ring intact, glucuronide conjugation regiochemistry (7- or 4′-OH) of equol and *O*-desmethylangolensin does not affect their retention behaviors (**Table 2**).

In addition to the different retention behaviors, 7-glucuronide conjugates of isoflavones are different from their 4′-isomers in their ionization behaviors. MS with negative-ion mode increases sensitivity for detection of 7-glucuronide conjugates. On the other hand, 4′-glucuronide conjugates yield good daughter spectra in the positive-ion mode (**Table 2**). Isoflavones contain both hydroxy and keto groups, which can be ionized in negative- and positive-ion modes, respectively. While ions of aglycone moieties from isoflavone-glucuronides are produced in MS/MS with both negative- and positive-ion modes, fragments corresponding to the acidic glucuronide only exhibits in daughter spectrum with negative-ion mode.

In conclusion, 22 isoflavones and their metabolites were characterized in the urine of female rats that consumed diets made with soy protein isolate. This study has for the first time demonstrated that the formation of glucuronide conjugates could occur at any hydroxy group of isoflavones including the 5-hydroxy group of genistein. Furthermore, this is the first identification of additional conjugated metabolites of isoflavones such as glucuronides of equol and daidzin. LC/MS/MS with an ESI source is a simple and sensitive method for direct identification of isoflavones and their metabolites in rat urine. However, the procedure used in the present study is not practical for large studies because it takes 80 min for each LC/MS/MS analysis (**Figure 1**). Further study is under way to optimize analytical methods for large studies. Also, LC/MS/MS methods are being developed for quantitative analysis of conjugated isoflavones in biological fluids.

ABBREVIATIONS USED

SPI, soy protein isolate; IF-SPI, isoflavone-free soy protein isolate; HPLC, high-performance liquid chromatography; GC, gas chromatography; MS, mass spectrometry; ESI, electrospray ionization; SPE, solid-phase extraction; UV, ultraviolet and visible absorption; NMR, nuclear magnetic resonance.

ACKNOWLEDGMENT

We thank Drs. Martin J. J. Ronis, Reza Hakkak, Ronald L. Prior, and Liwei Gu for helpful comments.

LITERATURE CITED

(1) Setchell, K. D. R.; Zimmer-Nechemias, L.; Cai, J.; Heubi, J. E. Exposure of infants to phyto-oestrogens from soy-based infant formula. *Lancet* **1997**, *350*, 23−27.

(2) Knight, D. C.; Eden, J. A. A review of the clinical effects of phytoestrogens. *Obstet. Gynecol.* **1996**, *87*, 897−904.

(3) Messina, M. J.; Persky, V.; Setchell, K. D. R.; Barnes, S. Soy intake and cancer risk: a review of the in vitro and in vivo data. *Nutr. Cancer* **1994**, *21*, 113−131.

(4) Ingram, D.; Sanders, K.; Kolybaba, M.; Lopez, D. Case-control study of phyto-oestrogens and breast cancer. *Lancet* **1997**, *350*, 990−994.

(5) Adlercreutz, C. H.; Goldin, B. R.; Gorbach, S. L.; Hockerstedt, K. A.; Watanabe, S.; Hamalainen, E. K.; Markkanen, M. H.; Makela, T. H.; Wahala, K. T.; Adlercreutz, T. Soybean phytoestrogen intake and cancer risk. *J. Nutr.* **1995**, *125*, 757S−770S.

(6) Hakkak, R.; Korourian, S.; Shelnutt, S. R.; Lensing, S.; Ronis, M. J.; Badger, T. M. Diets containing whey proteins or soy protein isolate protect against 7,12-dimethylbenz(*a*)anthracene-induced mammary tumors in female rats. *Cancer Epidemiol., Biomarkers Prev.* **2000**, *9*, 113−117.

(7) Lamartiniere, C. A.; Moore, J.; Holland, M.; Barnes, S. Neonatal genistein chemoprevents mammary cancer. *Proc. Soc. Exp. Biol. Med.* **1995**, *208*, 120−123.

(8) Hawrylewicz, E. J.; Zapata, J. J.; Blair, W. H. Soy and experimental cancer: animal studies. *J. Nutr.* **1995**, *125*, 698S−708S.

(9) Irvine, C.; Fitzpatrick, M.; Robertson, I.; Woodhams, D. The potential adverse effects of soybean isoflavones in infants feeding. *N. Z. Med. J.* **1995**, *108*, 208−209.

(10) Sheehan, D. M. Herbal medicines, Phytoestrogens and toxicity: Risk:benefit considerations. *Exp. Biol. Med.* **1998**, *217*, 379−385.

(11) Bouker, K. B.; Hilakivi-Clarke, L. Genistein: Does it prevent or promote breast cancer? *Environ. Health Perspect.* **2000**, *108*, 701−709.

(12) Cimino, C. O.; Shelnutt, S. R.; Ronis, M. J. J.; Badger, T. M. An LC-MS method to determine concentrations of isoflavones and their sulfate and glucuronide conjugates in urine. *Clin. Chim. Acta* **1999**, *287*, 69−82.

(13) King, R. A.; Bursill, D. B. Plasma and urinary kinetics of the isoflavones daidzein and genistein after a single soy meal in humans. *Am. J. Clin. Nutr.* **1998**, *67*, 867−872.

(14) Watanabe, S.; Yamaguchi, M.; Sobue, T.; Takahashi, T.; Miura, T.; Arai, Y.; Mazur, W.; Wähälä, K.; Adlercreutz, H. Pharmacokinetics of soybean isoflavones in plasma, urine and feces of men after ingestion of 60 g baked soybean powder (kinako). *J. Nutr.* **1998**, *128*, 1710−1715.

(15) Lu, L.-J.; Anderson, K. E. Sex and long-term soy diets affect the metabolism and excretion of soy isoflavones in humans. *Am. J. Clin. Nutr.* **1998**, *68* (suppl), 1500S−1504S.

(16) Yasuda, T.; Kano, Y.; Saito, K., Ohsawa, K. Urinary and biliary metabolites of daidzin and daidzein in rats. *Biol. Pharm. Bull.* **1994**, *17*, 1369−1374.

(17) Bayer, T.; Colnot, T.; Dekant, W. Disposition and biotransformation of the estrogenic isoflavone daidzein in rats. *Toxicol. Sci.* **2001**, *62*, 205−211.

(18) Holder, C. L.; Churchwell, M. I.; Doerge, D. R. Quantification of soy isoflavones, genistein and daidzein, and conjugates in rat blood using LC/ES-MS. *J. Agric. Food Chem.* **1999**, *47*, 3764−3770.

(19) Kulling, S. E.; Honig, D. M.; Metzler, M. Oxidative metabolism of the soy isoflavones daidzein and genistein in humans in vitro and in vivo. *J. Agric. Food Chem.* **2001**, *49*, 3024−3033.

(20) Reeves, P. G.; Nielsen, F. H.; Fahey, G. C. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J. Nutr.* **1993**, *123*, 1939−1951.

(21) Markham, K. R.; Mabry, T. J. Ultraviolet−visible and proton magnetic resonance spectroscopy of flavonoids. In *The Flavonoids, Part I*; Harborne, J. B., Mabry, T. J., Mabry, H., Eds.; Academic Press: New York, 1975; pp 45−77.

(22) Wang, H.; Murphy, P. A. Isoflavone content in commercial soybean foods. *J. Agric. Food Chem.* **1994**, *42*, 1666−1673.

Received for review October 19, 2001. Revised manuscript received January 17, 2002. Accepted January 18, 2002. Funding has been provided in part from the U.S. Department of Agriculture/Agricultural Research Service under Project #0501-00044-001-01S. The contents of this publication do not necessarily reflect the views or policies of the U.S. Department of Agriculture.

JF011384V