Oxidative Metabolism of the Soy Isoflavones Daidzein and Genistein in Humans in Vitro and in Vivo

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The soy isoflavones daidzein and genistein are found in high concentrations in human plasma and urine after soy consumption. However, in vitro and in vivo data regarding the oxidative metabolism of isoflavones in humans are scarce. Therefore, we have studied the oxidative metabolites of these compounds formed in human liver microsomes and excreted in urine of male and female humans ingesting soy products for 2 days. Human liver microsomes transformed the soy isoflavone daidzein to three monohydroxylated and three dihydroxylated metabolites according to GC/MS analysis. On the basis of a previous study with rat liver microsomes and with the help of reference substances, these metabolites were identified as 6,7,4′-trihydroxyisoflavone, 7,3′,4′-trihydroxyisoflavone, 7,8,4′-trihydroxyisoflavone, 7,8,3′,4′-tetrahydroxyisoflavone, 6,7,8,4′-tetrahydroxyisoflavone, and 6,7,3′,4′-tetrahydroxyisoflavone. Significant amounts of the same metabolites except 6,7,8,4′-tetrahydroxyisoflavone were also found in urine of female and male volunteers after soy intake. Genistein was metabolized by human liver microsomes to six hydroxylation products. The main metabolites were the three aromatic monohydroxylated products 5,6,7,4′-tetrahydroxyisoflavone, 5,7,8,4′-tetrahydroxyisoflavone and 5,7,3′,4′-tetrahydroxyisoflavone. The aliphatic monohydroxylated metabolite 2,5,7,4′-tetrahydroxyisoflavone and two aromatic dihydroxylated metabolites, 5,7,8,3′,4′-pentahydroxyisoflavone and 5,6,7,3′,4′-pentahydroxyisoflavone, were formed in trace amounts. The same hydroxylated genistein metabolites except the aliphatic hydroxylated one could also be detected in human urine samples. Methylated forms of the catechol metabolites, which were generated by incubations with catechol-*O*-methyltransferase in vitro could be detected only in trace amounts in the urine samples. This implies that this reaction does not play a major role in the biotransformation of the hydroxylated daidzein and genistein metabolites in vivo. Most of these oxidative metabolites are described as human in vivo metabolites for the first time. Their biological significance remains to be established.

Keywords: *Phytoestrogens; isoflavones; daidzein; genistein; metabolism; soy*

INTRODUCTION

Daidzein and genistein (Figure 1), the major isoflavones in soybeans, are associated with a broad variety of beneficial properties on human health. Several, although not all, epidemiological studies have shown that the intake of soy was inversely correlated with the incidence of hormone-related tumors, like breast and prostate cancer, as well as osteoporosis and coronary heart disease (*1*, *2*). Therefore, many studies have analyzed these compounds in food items and measured their concentrations in biological fluids such as urine, feces, and blood plasma after the intake of soy products. Furthermore, their metabolic fate in the human body has been of interest. Most bioavailability studies on isoflavones have determined the total daidzein and genistein concentration in blood plasma and urine after treatment with deconjugating enzymes and have shown that peak levels of both isoflavones in plasma and urine occurred between 6 and 12 h after the consumption of soy (*3*−*5*). Other reports focused on the formation of glucuronide or sulfate conjugates by hepatic phase II enzymes. For example, Adlercreutz et al. (*6*) found that about 80% of daidzein occurred in the urine as a monoglucuronide and 6 to 17% as a sulfoglucuronide. Genistein was mainly excreted as a monoglucuronide (53−76%) and to a much lesser extent as a diglucuronide (12−16%) and a sulfoglucuronide (2−15%). Studies regarding the phase I biotransformation of isoflavones focused almost exclusively on the reductive metabolism by intestinal bacteria. It has been reported that daidzein can be converted by the gut microflora to dihydrodaidzein, which can be further metabolized to both equol

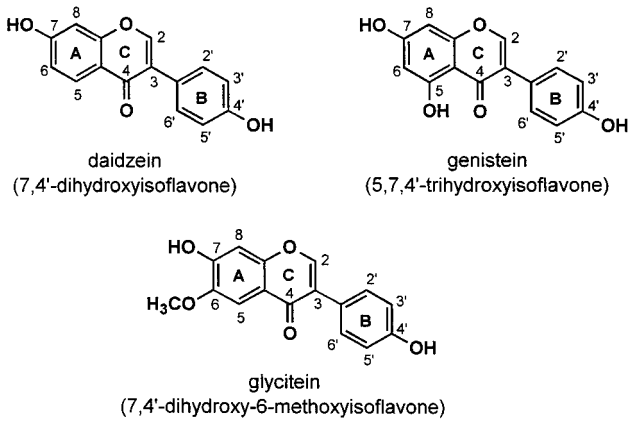

Figure 1. Chemical structures of the soy isoflavones daidzein, genistein, and glycitein.

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and *O*-desmethylangolensin. Genistein is first transformed by gut bacteria to dihydrogenistein, followed by a cleavage of the C-ring to form 6′-hydroxy-*O*-desmethylangolensin, which can be further degraded by the colonic microflora to 4-hydroxyphenyl-2-propionic acid. Decarboxylation can then lead to the putative metabolic end product 4-ethylphenol (*7*−*9*).

To date, however, in vivo data regarding the oxidative metabolism of isoflavones in humans are scarce. In a recent study from our laboratory, it has been reported that daidzein and genistein were readily metabolized to several mono- and di-hydroxylated products by liver microsomes from aroclor-induced male Wistar rats (*10*). The aim of the present study was to clarify the significance of these hydroxylated metabolites in humans. Therefore we have investigated the oxidative metabolism of daidzein and genistein in vitro using human liver microsomes and in vivo by analyzing the urine of three male and three female volunteers after the consumption of soy products.

MATERIAL AND METHODS

Chemicals. Daidzein, genistein, 6,7,4′-trihydroxyisoflavone, 7,3′,4′-trihydroxyisoflavone (purity >99% according to GC/MS analysis), *S*-adenosyl-L-methionine (SAM), $NADP^+$, NADH, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), and catechol-*O*-methyltransferase (COMT) were obtained from Sigma-Aldrich Chemical Co. (Deisenhofen, Germany). Glucuronidase/arylsulfatase (isolated from *Helix pomatia*) was purchased from Roche Diagnostics Co. (Mannheim, Germany). Equol was synthesized in our laboratory as described by Wessely and Prillinger (*11*). The purity was >99% according to GC/MS analysis.

Preparation and Characterization of Human Liver Microsomes. Microsomes were prepared from a liver sample of a male human according to standard procedures as described by Lake (*12*). Protein concentrations were estimated by using Pierce bicinchoninic reagent (*13*). Cytochrome P450 concentrations were measured as described by Omura and Sato (*14*).

Incubation with Microsomes and Extraction. Standard incubation mixtures contained 4 mg of microsomal protein, 50 nmol isoflavone dissolved in 40 μL of DMSO, and a NADPH-generating system (3 mM $MgCl_2$, 1 mM $NADP^+$, 8 mM D,L-isocitrate, and 0.5 U isocitrate dehydrogenase) in a final volume of 2 mL of 0.05 M potassium phosphate buffer pH 7.4. After 2 min of preincubation at 37 °C in a shaking water bath, the reaction was started by adding the NADPH-generating system. The reaction was stopped after 60 min by extraction with 4 × 2 mL of ice-cold ethyl acetate. Controls were carried out by omitting the NADPH-generating system or by using heat-inactivated microsomes. The organic solvent was evaporated to dryness under reduced pressure at room temperature. The residue was dissolved in 50 μL of methanol and 3950 μL of water and applied to a RP-18 cartridge. The column was first rinsed with 5 mL of 20% aqueous methanol and the metabolites were then eluted with 5 mL of 70% aqueous methanol. The eluate was evaporated to dryness, dissolved in 200 μL of aqueous 50% methanol, and subjected to HPLC.

Incubations with Catechol-*O*-methyltransferase. The methylation reaction catalyzed by catechol-*O*-methyltransferase (COMT) was used to generate the methoxy derivatives of several catechol metabolites. The isolated metabolite was incubated with 3.5 μM *S*-adenosyl-L-methionine, 50 μM ascorbic acid, 10 μM $MgCl_2$, 70 μM dithiothreitol, and 50 U COMT in a final volume of 1 mL of 0.05 M phosphate buffer pH 7.4 at 37 °C for 45 min. The incubation mixture was extracted with ethyl acetate and analyzed by HPLC and GC/MS after derivatization with BSTFA as decribed below.

Collection and Cleanup Procedures of Urine Samples. Three female and three male healthy volunteers (age 25 to 33 years, body weight ranged from 55 to 83 kg) ingested two capsules with soy extract (containing 30 mg of isoflavones) and 0.8 L of soy milk (containing about 70 mg of isoflavones) per day for 2 days. Urine samples were collected in the morning of day 1 (prior to the first soy consumption), in the afternoon of day 1, in the morning and in the afternoon of day 2 ,and in the morning of day 3. Samples were stored at −30 °C after adding 0.1% sodium azide and 0.1% ascorbic acid to retard bacterial growth and to stabilize the isoflavonoid metabolites. Frozen urine aliquots were warmed to room temperature, vortex-mixed and centrifuged at 3000*g* for 20 min. Aliquots of 10.0 mL of clear supernatant were mixed with 1 mL of sodium acetate buffer (1.5 M, pH 3.0) and applied to a RP-18 cartridge preconditioned with 10 mL of methanol and 10 mL of sodium acetate buffer (0.15 M, pH 3.0). The column was washed with 10 mL of acetate buffer, and the phytoestrogens were eluted with 3 mL of methanol. The methanol extract was evaporated to dryness; the residue was dissolved in 3 mL of 0.15 M sodium acetate buffer pH 5.0, mixed with 10 mg of ascorbic acid, and incubated with 4500 Fishman units of β-glucuronidase/arylsulfatase (from *Helix pomatia*) for 12 h at 37 °C. The hydrolyzed samples were extracted with 3 × 2 mL of ice-cold ethyl acetate and the combined organic phase was evaporated to dryness. The residue was dissolved in 20 μL of methanol and 3.95 mL of water and purified on a RP-18 cartridge rinsed with 5 mL of 20% aqueous methanol and eluted with 3 mL of 80% aqueous methanol. The methanol eluate was evaporated to dryness and dissolved in 200 μL of 50% aqueous methanol, vortex-mixed, and centrifuged at 10000*g* for 5 min prior to HPLC injection. For GC/MS analysis, an aliquot of 20 μL was dried under a stream of nitrogen and derivatized with 10 μL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) for 2 h at room temperature.

HPLC Analysis of Metabolites. The HPLC analysis was performed on an HP1100 series HPLC equipped with a binary HPLC pump, a photodiode array detector, and an HP Chem Station for data collection and handling. The HPLC column and the gradient profile were modified from those of our previous paper (*10*): HPLC separation of the metabolites was carried out on a Prontosil (250 mm × 4.6 mm i.d., particle size 3 μm) reversed-phase column (Bischoff, Germany) with the following linear gradient: from 15% to 35% acetonitrile in water (pH 3) in 50 min, from 35% to 50% in 10 min, and from 50% to 60% in 30 min. The flow rate was 0.9 mL/min and the eluent was recorded with a diode array detector at 260 nm. Observed peaks were scanned between 190 and 400 nm.

GC/MS Analysis of Metabolites. GC/MS was carried out on a Finnigan MAT system (gas chromatograph model GCQ) connected to an ion trap mass detector. The TMS-derivatives were analyzed using a nonpolar capillary column (MDN-5S, 30 m × 0.25 mm i.d., 0.25 μm film thickness, Supelco) and a linear temperature gradient (60 °C for 1 min, then 30 °C/min to 250 °C, hold for 10 min, then 1 °C/min to 275 °C and hold for 5 min). The injector port temperature was 50 °C. Mass spectra were obtained by electron impact ionization at 70 eV and an ion source temperature of 150 °C. Full scan spectra (mass range 50−750 amu) were recorded at a rate of 2 spectra per sec. The selected ion mode (SIM) was used for the detection of daidzein, genistein, and their hydroxylated metabolites.

RESULTS AND DISCUSSION

Identification of Daidzein and Genistein Metabolites. The identification of the hydroxylated daidzein and genistein metabolites detected in the incubations with human liver microsomes and in the urine samples was performed on the basis of a previous study (*10*) which reported nine hydroxylated daidzein and six hydroxylated genistein metabolites formed by liver microsomes from aroclor-treated rats. Characterization of the isolated metabolites was carried out by using high performance liquid chromatography (HPLC) with diode array detection, gas chromatography/mass spectrometry (GC/MS), and HPLC/atmospheric pressure ionization−

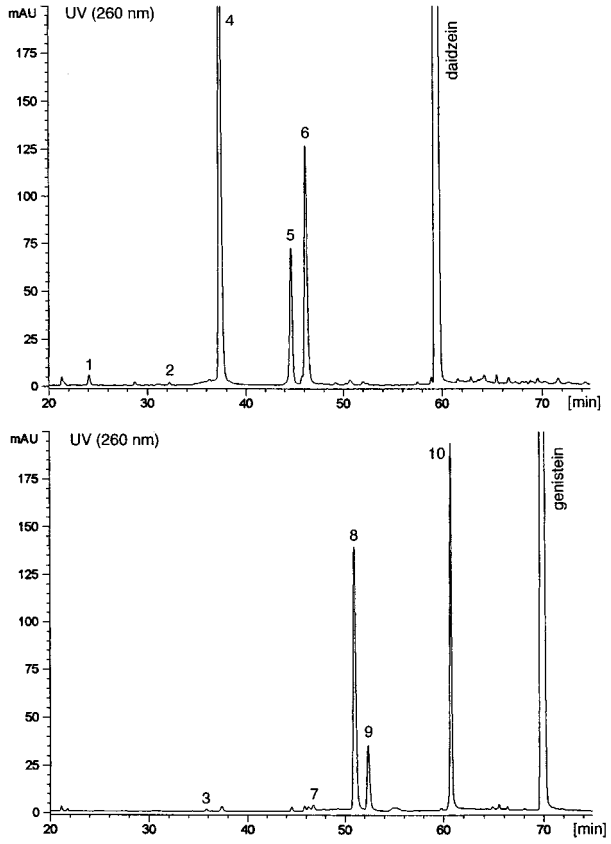


Figure 2. HPLC profile of daidzein (top) and genistein (bottom) metabolites formed by human liver microsomes. Peak numbers refer to Table 1.

electrospray mass spectrometry (API−ESMS), as well as by reference substances. Therefore, the chromatographic data (retention time in HPLC and GC as well as the mass and the UV spectra) of several hydroxylated metabolites of daidzein and genistein were known.

Human Microsomal Metabolites of Daidzein and Genistein. Daidzein and genistein (Figure 1) were incubated with human liver microsomes, and the organic extract of each incubation was first analyzed by reversed-phase HPLC with diode array detection at 260 nm. Representative HPLC chromatograms are shown in Figure 2. In the case of daidzein, three main (peaks 4, 5, and 6) and two minor metabolites (peaks 1 and 2) were detected. Five products (peaks 3, 7, 8, 9, and 10) were also observed in the HPLC chromatogram of the genistein incubation extract (Figure 2). Two of the products (peaks 3 and 7) were found only in very small amounts. All these metabolites were absent in the respective chromatograms obtained from control incubations without NADPH or when heat-inactivated microsomes were used. The metabolites were identified on the basis of their retention times and of their UV absorbance scans in comparison to reference compounds and the above-mentioned characterized microsomal metabolites from a previous study (*10*). The UV maxima of the daidzein and genistein metabolites are summarized in Table 1.

In addition to the HPLC analysis, the incubation extracts containing all metabolites were trimethylsilylated and were further analyzed by GC/MS which gave rise to six daidzein and six genistein hydroxylation products (Figure 3). For the correlation of HPLC and GC peaks, each HPLC peak was isolated and analyzed by GC/MS after trimethylsilylation with BSTFA. Furthermore, the human metabolites were co-chromatographed with the above-mentioned characterized metabolites from rat liver microsomes (*10*). Comparison of the HPLC and GC data shows that only one metabolite of daidzein (GC peak p), as well as of genistein (GC peak m), could not be detected by HPLC and were found only in trace amounts with GC/MS using the selected ion mode (Figure 3). The HPLC and GC chromatographic data of the metabolites and their assigned chemical structures are summarized in Table 1.

Daidzein and Genistein Metabolites in Human Urine. As the isoflavones and their metabolites occur in urine predominantly as glucuronide and sulfate conjugates and mixtures thereof, the urine samples were hydrolyzed with the enzyme glucuronidase/arylsulfatase, which has been described as the most gentle and efficient method (*15*). Hydrolysates were purified and concentrated by ethyl acetate and solid-phase extraction as described under Material and Methods. In a control experiment, the overall recovery of daidzein and genistein added to blank urine was found to be about 80% and 60%, respectively. In each experiment, 10 mL of urine was used and the same aliquot of the purified extract was analyzed by GC/MS.

The control urine samples of the three male and the three female volunteers, which had been collected prior to the first consumption of soy food, were analyzed with both HPLC and GC/MS for the presence of the major

Table 1. Chromatographic and Spectroscopic Data of the Oxidative Metabolites of Daidzein and Genistein Formed by Human Liver Microsomes

HPLC peak[a]	RT in HPLC (min)[a]	UV maxima (nm)	GC peak[b]	RT in GC (min)	M^+,GC/MS TMS−derivative	isoflavone substitution pattern
1	24.1	260	o	38.1	574	7,8,3′,4′-tetrahydroxyisoflavone
nd[c]	-	-	p	38.3	574	6,7,8,4′-tetrahydroxyisoflavone
2	32.2	258/325	q	39.4	574	6,7,3′,4′-tetrahydroxyisoflavone
3	37.3	268	n	37.1	662	5,7,8,3′,4′-pentahydroxyisoflavone
4	35.8	258	i	32.4	486	7,8,4′-trihydroxyisoflavone
nd[c]	-	-	m	36.5	662	5,6,7,3′,4′-pentahydroxyisoflavone
5	44.6	249/292	g	31.4	486	7,3′,4′-trihydroxyisoflavone
6	46.1	256/290/324	k	34.2	486	6,7,4′-trihydroxyisoflavone
7	46.7	258	a1	23.0	502	2,5,7,4′-tetrahydroxyisoflavone
			a2	23.4	574	
8	51.0	268/351	h	32.5	574	5,7,8,4′-tetrahydroxyisoflavone
9	52.3	270	f	31.0	574	5,6,7,4′-tetrahydroxyisoflavone
daidzein	59.2	249/303	c	25.2	398	7,4′-dihydroxyisoflavone
10	60.7	262/(287)	j	32.5	574	5,7,3′,4′-tetrahydroxyisoflavone
genistein	69.7	262	d	26.2	486	5,7,4′-trihydroxyisoflavone

[a] According to Figure 2. [b] According to Figures 3 and 5. [c] nd, not detected.

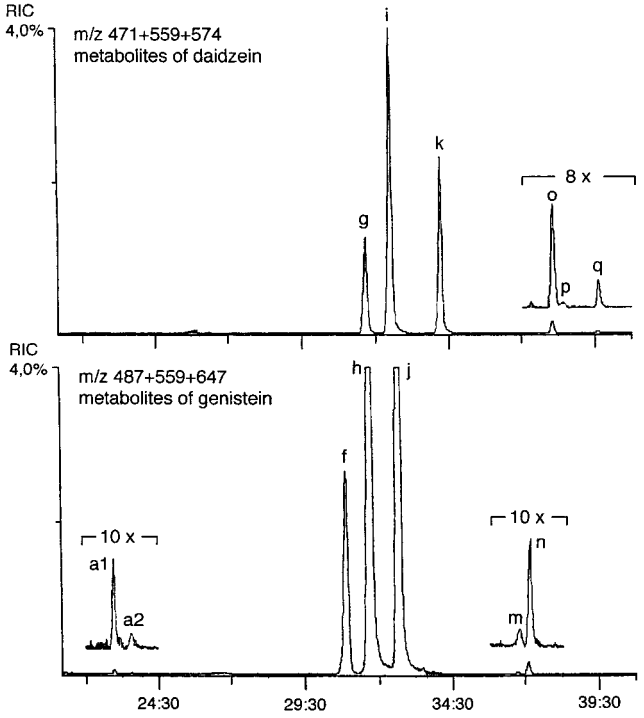


Figure 3. GC selected ion chromatograms of hydroxylated metabolites of daidzein and genistein from human liver microsomes. Peak labels refer to Table 1. The peaks are numbered according to the system used for rat microsomal metabolites (*10*).

soy isoflavones daidzein, genistein, and glycitein, and the isoflavan equol. In two of the six urine samples, none of the mentioned compounds were detected. In three other urine samples only minute amounts of daidzein and genistein were found. Only one control urine sample of a female volunteer contained considerable amounts of the three isoflavones and a marginal amount of equol.

After the consumption of soy products, concentrations of daidzein, genistein, and glycitein were significantly increased in the urine samples of all six subjects and at all time points of urine collection. Representative HPLC chromatograms from before and after the intake of soy are shown in Figure 4.Besides daidzein, genistein, and glycitein the five major human microsomal metabolites (peaks 4, 5, 6, 8, and 10, Figure 2) could be tentatively identified on the basis of their retention time and UV spectra as in vivo metabolites in several urine samples.

In addition, a more sensitive detection method for the hydroxylated daidzein and genistein metabolites was carried out by GC/MS using the selected ion mode. Characteristic ions for monohydroxylated daidzein metabolites were *m/z* 486 (M^+, TMS derivative) and 471 ($M^+ - CH_3$; loss of phenolic silyl ether group). Specific ions for the detection of dihydroxylated daidzein or monohydroxylated genistein metabolites were *m/z* 574 (M^+, TMS derivative) and 559 ($M^+ - 15$; loss of CH_3 radical), and for dihydroxylated genistein metabolites were *m/z* 662 (M^+, TMS derivative) and 647 ($M^+ - 15$; loss of CH_3 radical). When the GC/MS data of the human urine samples were searched for these typical

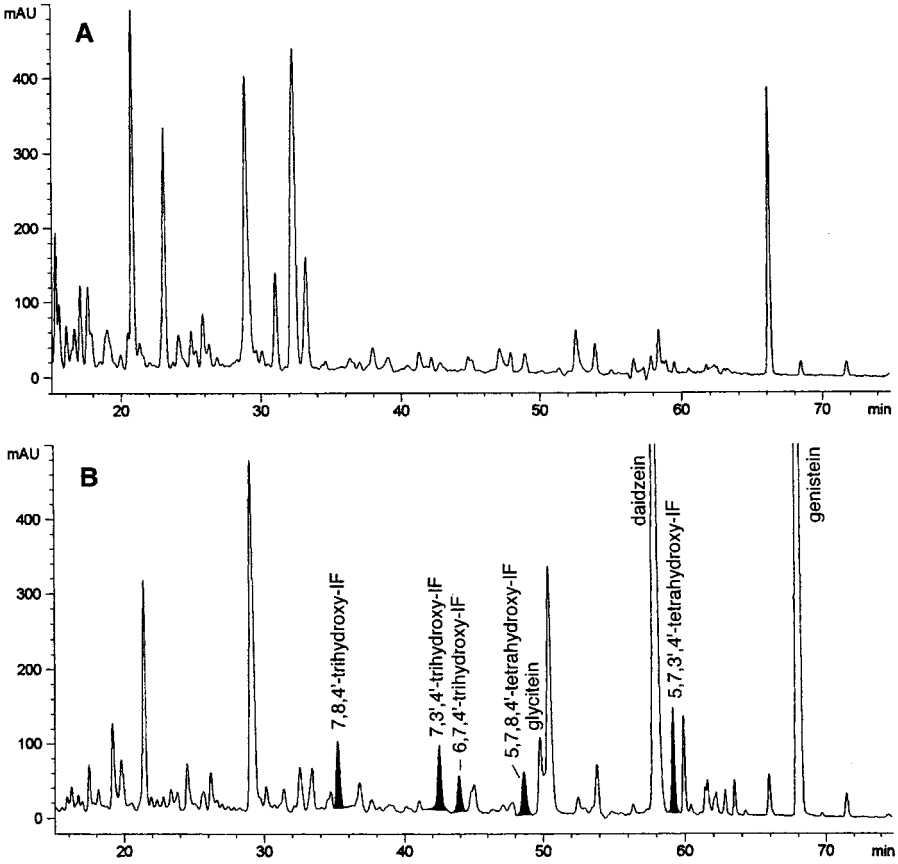


Figure 4. Characteristic HPLC chromatograms of extracts of human urine before (A) and after (B) the intake of soy products. The UV absorbance scans of the main microsomal metabolites (peaks 4, 5, 6, 8, 10, Figure 2) are identical with those of the black-marked HPLC peaks in chromatogram B. IF, isoflavone.

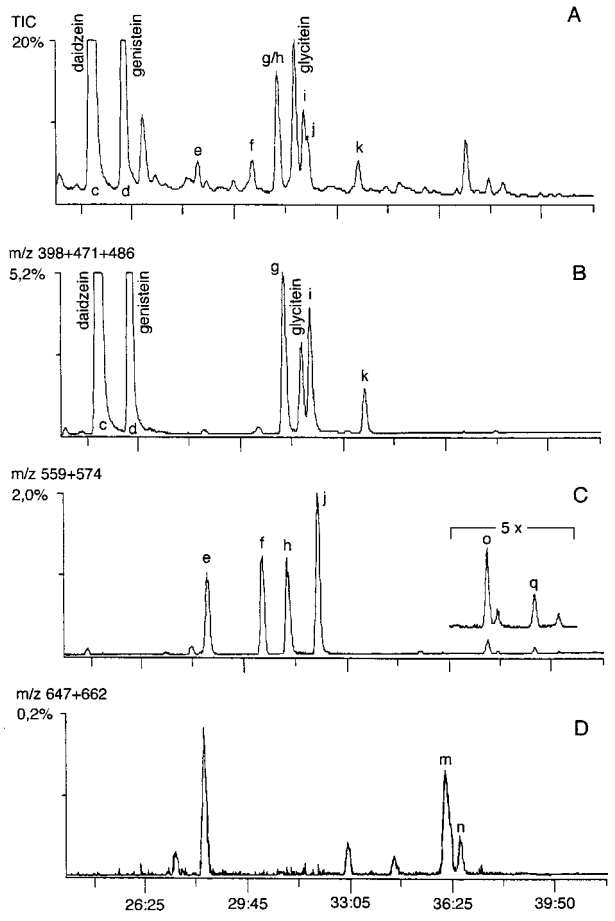


Figure 5. Capillary-GC/MS analysis of the urine of subject HN (female) after ingestion of soy products. The urinary sample was analyzed after cleanup and trimethylsilylation: (A) total ion current; (B) ion current reconstructed from typical ions of monohydroxylated daidzein metabolites; (C) ion current reconstructed from typical ions of dihydroxylated daidzein or monohydroxylated genistein metabolites; (D) ion current reconstructed from typical ions of dihydroxylated genistein metabolites. The peaks are numbered according to the system used for rat microsomal metabolites (*10*).

ions, three monohydroxylated daidzein products (peaks g, i, and k, Figure 5B), six dihydroxylated daidzein or monohydroxylated genistein metabolites (peaks e, f, h, j, o, and q, Figure 5C), and two dihydroxylated genistein metabolites (peaks m and n, Figure 5D) were clearly detected.

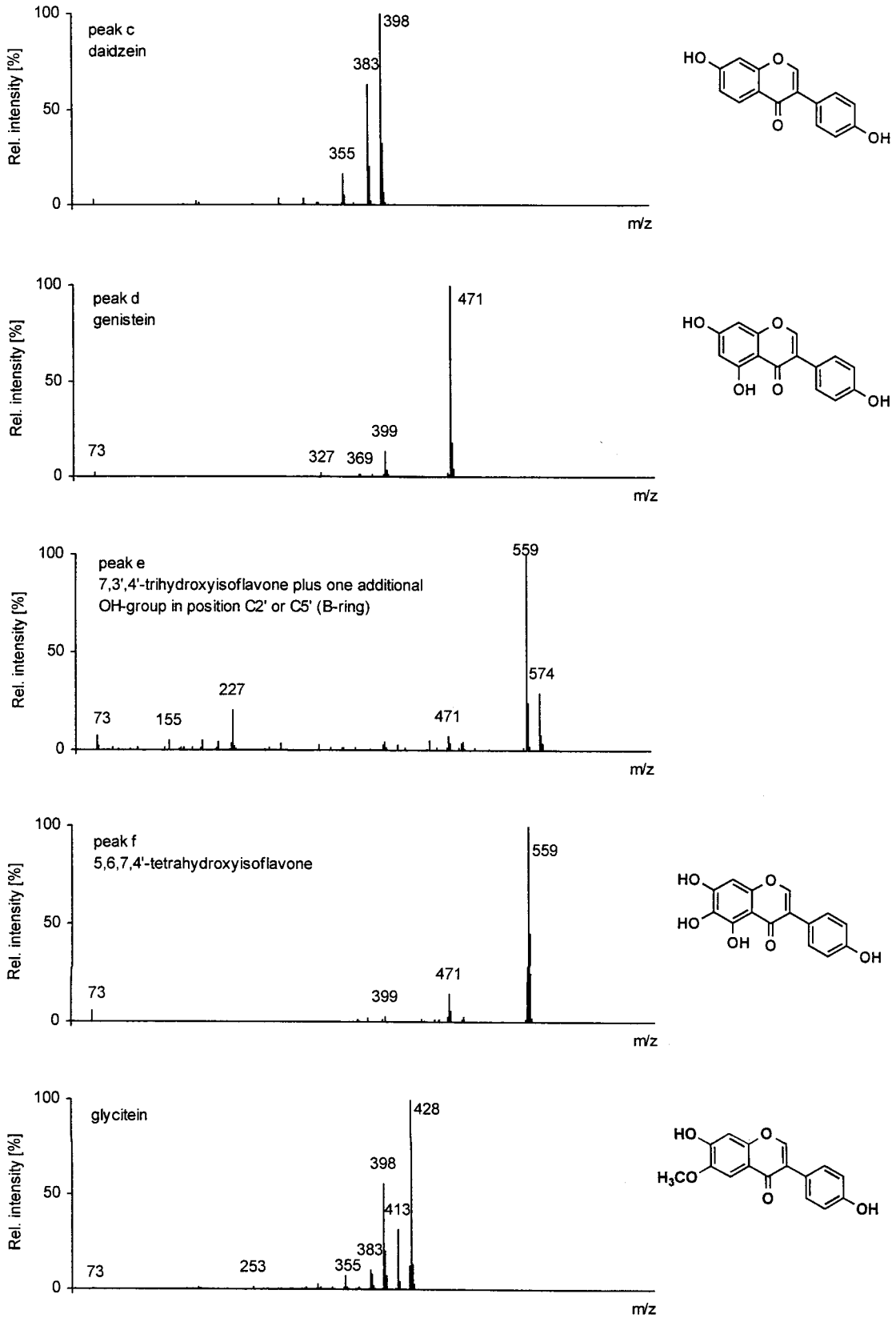
Co-chromatography by GC/MS of the urinary metabolites with the well characterized rat microsomal metabolites of daidzein and genistein (*10*) showed that peak f in Figure 5 is 5,6,7,4′-tetrahydroxyisoflavone, peak g/h contains 7,3′,4′-trihydroxyisoflavone and 5,7,8,4′-tetrahydroxyisoflavone, peak i is 7,8,4′-trihydroxyisoflavone, peak j is 5,7,3′,4′-tetrahydroxyisoflavone, peak k is 6,7,4′-trihydroxyisoflavone, peak m is 5,6,7,3′,4′-pentahydroxyisoflavone, peak n is 5,7,8,3′,4′-pentahydroxyisoflavone, peak o is 7,8,3′,4′-tetrahydroxyisoflavone, and peak q is 6,7,3′,4-tetrahydroxyisoflavone. The mass spectra of these urine metabolites were identical with those of the respective rat microsomal metabolites and are shown in Figure 6. It should be noted that the peak sizes of the oxidative metabolites as depicted in Figure 5 B, C, and D do not exactly reflect their corresponding amounts, as the chromatograms were reconstructed from different ions.

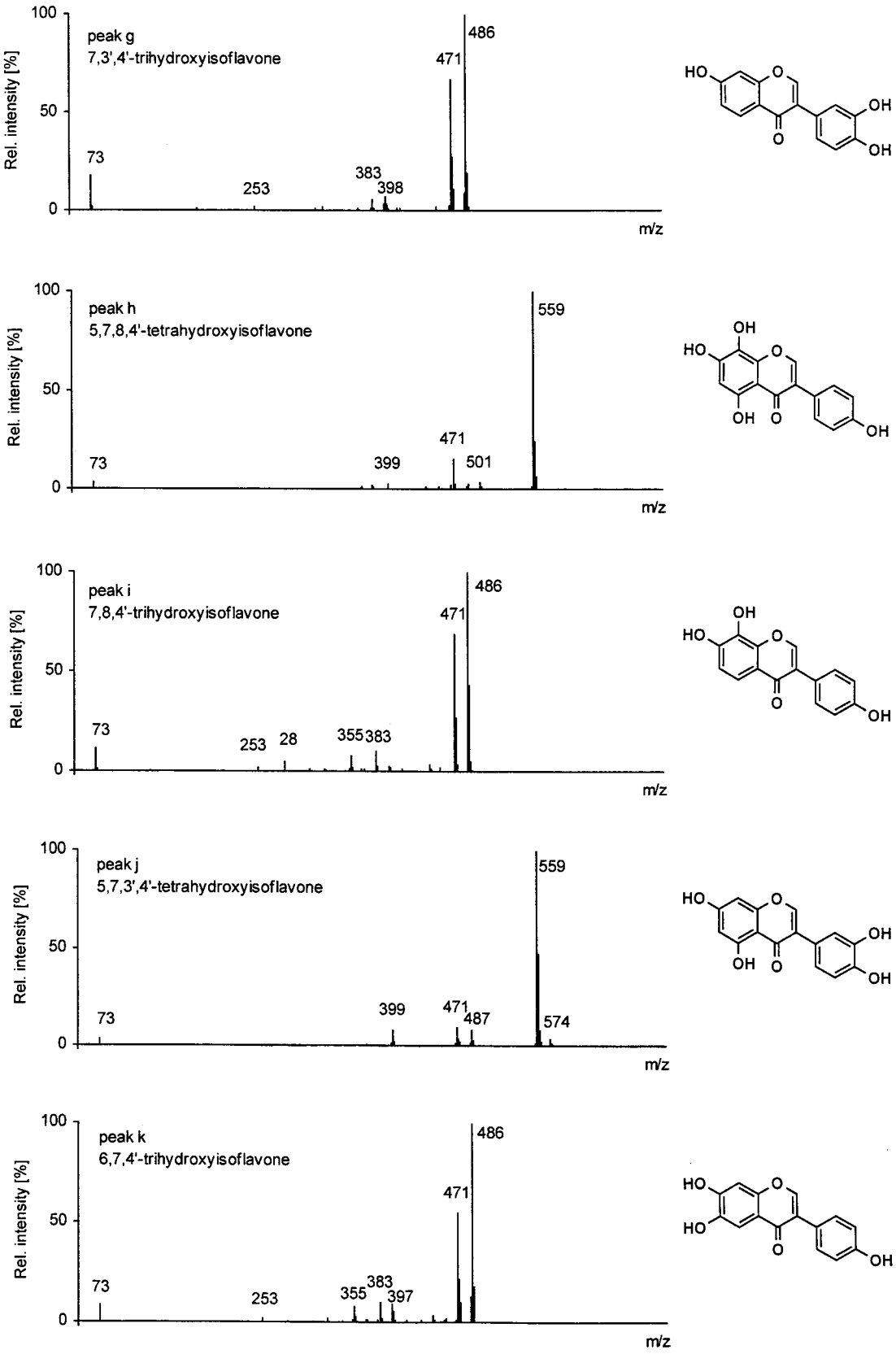
One metabolite with the GC retention time of 28.5 min (peak e, Figure 5) was not identified. This metabolite is derived from 7,3′,4′-trihydroxyisoflavone as shown in a previous study (*10*). The mass spectrum of the TMS derivative showed a molecular ion at *m*/*z* 574, indicating the structure of 7,3′,4′-trihydroxyisoflavone plus one additional hydroxy group. It can be assumed that this hydroxy group is located in the C-2′ or C-5′ position of the B-ring (Figure 1), because hydroxylation in the possible positions of the A-ring (C-5, C-6, C-8) would result in metabolites which have already been assigned.

The hydroxylated metabolites of daidzein and genistein could already be detected in the first urine samples, which were collected 6 to 8 h after the first soy intake. The pattern of the hydroxylated metabolites did not change during the observed time period (afternoon of day 1 until morning of day 3); the main metabolites being the three monohydroxylated daidzein products, 7,3′,4′-, 7,8,4′- and 6,7,4′-trihydroxyisoflavone, and the three monohydroxylated genistein products 5,6,7,4′-, 5,7,8,4′-, and 5,7,3′,4′-tetrahydroxyisoflavone. These six metabolites were detected in every urine sample, regardless of the collection time. The dihydroxylated daidzein and genistein metabolites were found only in small amounts or were not detected at all. A correlation between the formation of oxidative metabolites and the gender of the subjects was not observed. It should be mentioned that some of the hydroxylated metabolites could perhaps also be derived from glycitein by demethylation and further hydroxylation of so-formed 6,7,4′-trihydroxyisoflavone. To date, very little is known about the metabolism of glycitein and it is not clear if demethylation occurs in vivo and,if so, to what extent it does. No attempt has been made so far to quantitate exactly the oxidative metabolites. Assuming identical recovery during the cleanup procedure of the urine it can be estimated, on the basis of the peak areas in HPLC and GC/MS analysis, that the oxidative metabolites account for $\leq$ 10% of the total urinary isoflavones.

Because each of the aromatic hydroxylation products of daidzein and genistein contains a catechol or pyrogallol moiety, it can be assumed that these metabolites undergo further biotransformation by catechol-*O*-methyltransferase (COMT) in vivo. To generate reference compounds for these methylated catechols, we have incubated several isolated metabolites (7,3′,4′- and 6,7,4′-trihydroxyisoflavone, and 6,7,3′,4′-, 5,7,3′,4′-, and 5,6,7,4′-tetrahydroxyisoflavone) with COMT and *S*-adenosyl-L-methionine as methyl group donator. All metabolites were converted by COMT to methylated catechol products. As an example, 6,7,4′-trihydroxyisoflavone was converted to both the 6-methoxy-derivative (glycitein) and the 7-methoxy-derivative, whereas 6,7,3′,4′-tetrahydroxy-isoflavone, which has two catechol moieties in the molecule, was transformed to at least three clearly detectable monomethylated and three clearly detectable dimethylated catechols. The GC and MS data are summarized in Table 2.

When the typical ions for monomethylated (*m*/*z* 428/398 and *m*/*z* 516/501) and for dimethylated (*m*/*z* 458/428) catechol metabolites of daidzein and genistein were searched for in the GC/MS runs of the urine extracts, four such products with retention times of 30.4 min (M^+ 428), 37.6 min (M^+ 458), 38.3 min (M^+ 516), and 38.5 min (M^+ 516) were detected, in addition to glycitein. Comparing the GC retention times and the mass spectra with those of the generated methylated catechols listed


peak c
daidzein
Rel. intensity [%]
m/z
398
383
355
peak d
genistein
471
399
327
369
73
peak e
7,3',4'-trihydroxyisoflavone plus one additional
OH-group in position C2' or C5' (B-ring)
559
574
227
155
471
peak f
5,6,7,4'-tetrahydroxyisoflavone
glycitein
428
413
253
HO
O
OH
H3CO


peak g
7,3',4'-trihydroxyisoflavone
peak h
5,7,8,4'-tetrahydroxyisoflavone
peak i
7,8,4'-trihydroxyisoflavone
peak j
5,7,3',4'-tetrahydroxyisoflavone
peak k
6,7,4'-trihydroxyisoflavone
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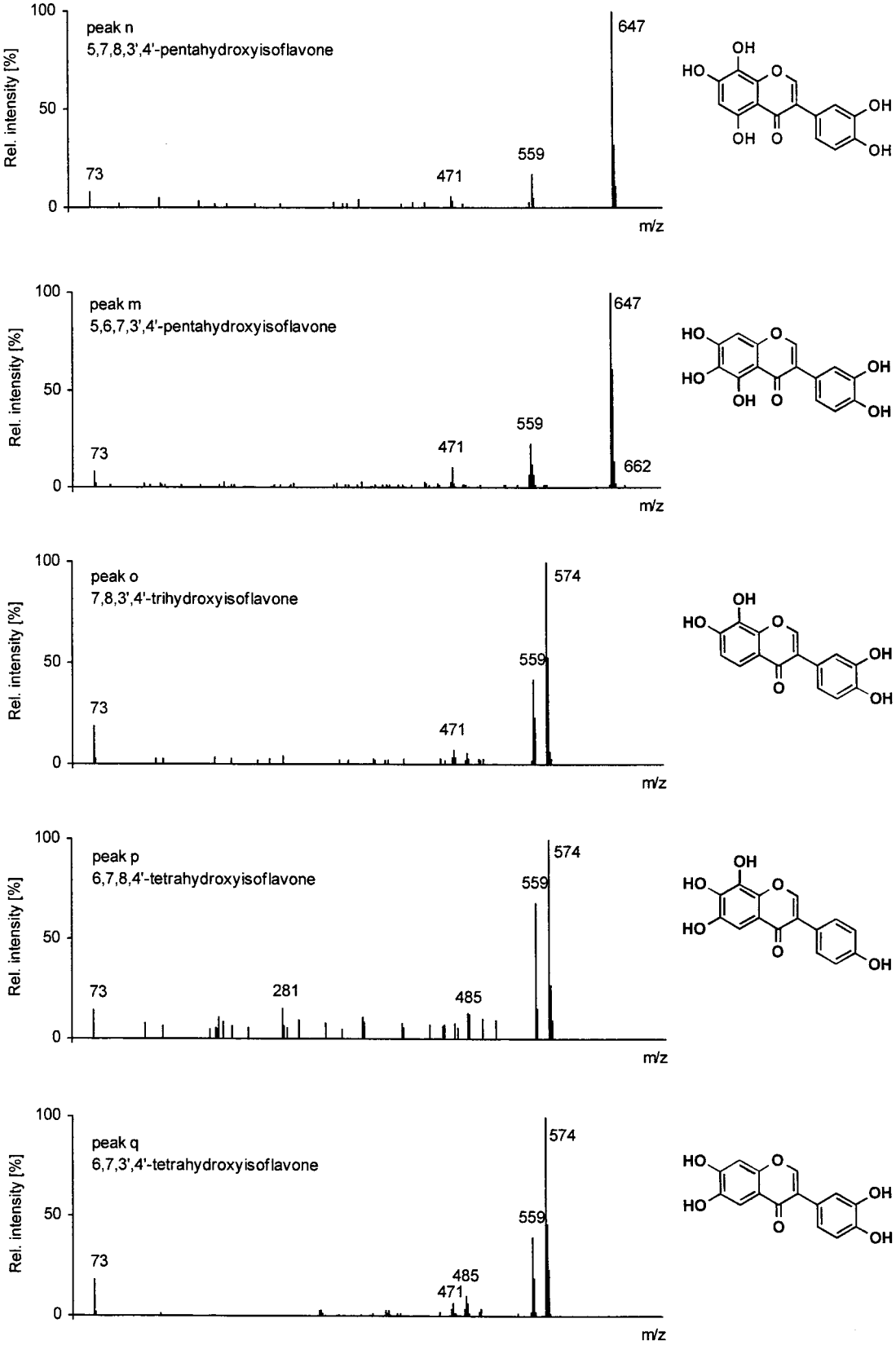

Figure 6. Mass spectra of the hydroxylation products of daidzein and genistein (trimethylsilyl derivatives) and their chemical structures. The GC peak labels refer to Figure 5.

in Table 2, the four in vivo metabolites were tentatively identified: the first metabolite (RT 30.4 min) is most likely the 3′- or 4′-methyl-7,3′,4′-trihydroxyisoflavone. The three products remaining, one dimethylated (M^+ 428) and two monomethylated (M^+ 516) catechols, are most likely derived from 6,7,3′,4′-tetra-hydroxyisoflavone. It should be noted that one monomethylated catechol (7,3′,4′-tri-hydroxy-6-methoxy-isoflavone) derived from 6,7,3′,4′-tetrahydroxyisoflavone could not only be formed by the reaction of COMT, but could also be formed by hydroxylation of glycitein (7,4′-dihydroxy-6-methoxy-isoflavone) at C-3′ in the C-ring of the

Table 2. Reactions of Various Isoflavone Catechol Metabolites with Catechol-*O*-methyltransferase and *S*-Adenosyl-L-methionine[a]

substrate (catechol metabolite)	methylated products: RT in GC (min)	M^+ (TMS)	ratio of formation (in %)
7,3′,4′-trihydroxyisoflavone	30.0	428	33
M^+ (TMS) 486; 31.4 min (GC)	30.4	428	67
6,7,4′-trihydroxyisoflavone	32.1	428	96
M^+ (TMS) 486; 34.2 min (GC)	33.0	428	4
6,7,3′,4′-tetrahydroxyisoflavone	38.1	516	0.3
M^+ (TMS) 574; 39.4 min (GC)	38.3	516	97.7
	38.5	516	2
	36.5	458	13
	37.1	458	1
	37.6	458	86
5,7,3′,4′-tetrahydroxyisoflavone	31.0	516	54
M^+ (TMS) 574; 32.5 min (GC)	31.2	516	46
5,6,7,4′-tetrahydroxyisoflavone	28.5	516	100
M^+ (TMS) 574; 31.0 min (GC)			

[a] Incubations and GC analyses were as described under Materials and Methods. Relative quantification (ratio of formation) is based on the intensities of typical ions in the mass spectra.

molecule (Figure 1), which is probably the reaction of greater importance. Each of the methylated catechols was found only in very small amounts, which suggests that methylation by COMT does not play a major role in the biotransformation of isoflavones in vivo. This may be of importance because catechol metabolites of steroidal estrogens are rapidly methylated by COMT, which is thought to be a detoxification step in the metabolism, because catechols can be easily oxidized via redox cycling to reactive intermediates such as semiquinones and quinones. It is generally believed that semiquinones and/or quinones play a critical role in the carcinogenicity of estrogens (*16*, *17*). Therefore, the formation of hydroxylated metabolites of daidzein and genistein may represent a metabolic activation process in some cases.

This study has for the first time demonstrated that the formation of hydroxylated metabolites of daidzein and genistein, generated by the action of hepatic cytochrome P450 enzymes, also occurs in vivo. It is well established that the biological activity of isoflavones is dramatically altered with the chemical structure. For example, formononetin is a less estrogenic and antioxidant isoflavone, whereas the intestinal metabolites daidzein and equol are more potent estrogens and antioxidants (*18*, *19*). Moreover, genistein is reported to be genotoxic in several in vitro systems, whereas daidzein was without any effect in the same test systems (*20*, *21*). On the basis of these findings, it can be assumed that the hydroxylated metabolites of daidzein and genistein which were found in human urine samples may also have biological activities other than those of their parent compounds. Therefore the hormonal and antioxidant/prooxidant, as well as the genotoxic, potential of these oxidative metabolites should be investigated.

ABBREVIATIONS USED

BSTFA, *N*,*O*-bis(trimethyl-silyl)trifluoroacetamide; COMT, catechol-*O*-methyltransferase; DMSO, dimethyl sulfoxide; EI, electron impact; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced form; RP, reversed phase; SAM, *S*-adenosyl-L-methionine; SIM, single ion monitoring; RT, retention time, TIC, total ion current, TMS, trimethylsilyl; U, units.

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