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Identification of Urinary Metabolites of the Red Clover Isoflavones Formononetin and Biochanin A in Human Subjects

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Dietary supplements manufactured from red clover are widely marketed to provide the beneficial health effects of isoflavones without changing the original diet. In this study the metabolism of formononetin and biochanin A, the principal isoflavones of red clover, was studied in human subjects. Seven women ingested four red clover dietary supplements, and the metabolites of the isoflavones were identified in their urine samples. The structures of trimethylsilyl derivatives of the metabolites were established by GC-MS. New reduced metabolites of formononetin (dihydroformononetin and angolensin) and biochanin A (dihydrobiochanin A and 6′-hydroxyangolensin) were identified in urine samples using authentic reference compounds. Possible metabolic pathways are presented for the red clover isoflavones formononetin and biochanin A.

KEYWORDS: Biochanin A; formononetin; daidzein; genistein; metabolism

INTRODUCTION

Isoflavonoids are phenolic compounds that occur mainly in plants belonging to the Leguminosae family, soy being the main source in the human diet (*1*, *2*). There are several suggested health benefits reported for isoflavones, particularly for daidzein, **1**, and genistein, **2** (**Figure 1**), the principal isoflavones of soy and soy products. Their biological activities include anticancer, antioxidant, anti-inflammatory, cardioprotective, and enzyme inhibitory effects (*1*−*4*). To make use of the proposed beneficial properties of isoflavones, it is possible nowadays to supplement the ordinary diet with tablets manufactured from soy or red clover (*5*, *6*). The dietary supplements are widely sold, although the metabolism of isoflavones, particularly the metabolism of red clover isoflavones, has not been fully established. A possibility exists that these compounds, or their metabolites, may have an adverse effect on human health, by virtue of their estrogenic properties.

The main isoflavones of red clover (*Trifolium pratense*) are formononetin, **3**, and biochanin A, **4**, and their glycosidic conjugates, ononin, **5**, and sissotrin, **6**, respectively (*7*, *8*). Other isoflavones that have been identified in red clover include daidzein, genistein, prunetin, **7**, pratensein, **8**, calycosin, **9**, and pseudobaptigenin, **10** (*7*). Because of the limited occurrence in the human diet, the metabolism of formononetin and biochanin A in humans has not been studied in detail. It is known that once ingested, these isoflavones are readily demethylated by intestinal microflora to daidzein and genistein, respectively (*9*). It has been shown that daidzein can be converted to dihydrodaidzein, **12**, *cis*-4-OH-equol, **18**, *O*-desmethylangolensin, **19**, and equol, **16**, whereas genistein is metabolized to dihydrogenistein, **13**, and 6′-OH-*O*-desmethylangolensin, **20** (*10*, *11*). Isoflavones and their metabolites are absorbed, and they appear in blood plasma mainly as glucuronide and/or sulfate conjugates and to a small extent as unconjugated compounds. The conjugates are excreted in both urine and bile, and they undergo enterohepatic circulation. Recent studies have demonstrated that daidzein and genistein are also converted to oxidized metabolites by cytochrome P450 enzymes of liver microsomes (*12*−*14*). The liver microsomes also catalyze the demethylation, as well as hydroxylation, of intact formononetin and biochanin A (*12*, *15*).

To investigate the metabolism of formononetin and biochanin A in humans, we carried out a feeding study in which seven female participants took four red clover based dietary supplements. The urine samples, collected before and after the isoflavone supplementation, were analyzed by GC-MS, and the structures of isoflavone metabolites were characterized using authentic reference compounds. As far as we are aware, this is the first paper to describe a detailed study on the metabolism of formononetin and biochanin A in human subjects. In addition, the identification of other isoflavones of red clover in human urine after red clover supplementation is presented for the first time.

MATERIALS AND METHODS

Standards and Reagents. Formononetin, biochanin A, daidzein, genistein, 7,8,4′-trihydroxyisoflavone (*16*), dihydroformononetin, dihydrobiochanin A, dihydrodaidzein, dihydrogenistein, 4′-*O*-Me-equol (*17*), angolensin, and 6′-OH-angolensin (*18*) were synthesized in the

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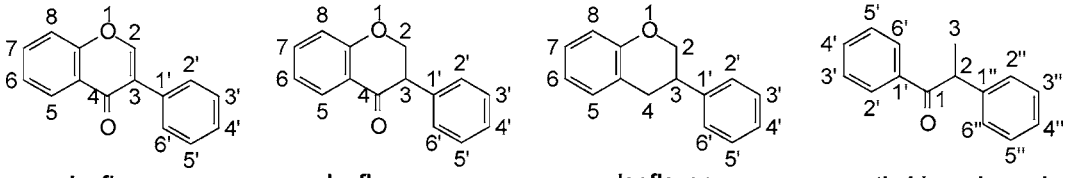

		Substitution	Trivial name
Isoflavone	**1**	7,4′-$(OH)_2$	Daidzein
	2	5,7,4′-$(OH)_3$	Genistein
	3	7-OH, 4′-OCH_3	Formononetin
	4	5,7-$(OH)_2$, 4′-OCH_3	Biochanin A
	5	7-OGlc, 4′-OCH_3	Ononin
	6	5-OH, 7-OGlc, 4′-OCH_3	Sissotrin
	7	5,4′-$(OH)_2$, 7-OCH_3	Prunetin
	8	5,7-$(OH)_2$, 6, 4′-$(OCH_3)_2$	Pratensein
	9	7,3′-$(OH)_2$, 4′-OCH_3	Calycosin
	10	7-OH, 3′-O-CH_2-O-4′	Pseudobaptigenin
	11	7,4′-$(OH)_2$, 6-OCH_3	Glycitein
Isoflavanone	**12**	7,4′-$(OH)_2$	Dihydrodaidzein
	13	5,7,4′-$(OH)_3$	Dihydrogenistein
	14	7-OH, 4′-OCH_3	Dihydroformononetin
	15	5,7-$(OH)_2$, 4′-OCH_3	Dihydrobiochanin A
Isoflavan	**16**	7,4′-$(OH)_2$	Equol
	17	7-OH, 4′-OCH_3	4’-*O*-Me-equol
	18	4, 7,4′-$(OH)_3$	4-OH-equol
α-methyldeoxybenzoin	**19**	2′,4′,4′′-$(OH)_3$	*O*-desmethylangolensin
	20	2′,4′,6′,4′′-$(OH)_4$	6′-OH- *O*-desmethylangolensin
	21	2′,4′-$(OH)_2$, 4′′-OCH_3	Angolensin
	22	2′,4′,6′-$(OH)_3$, 4′′-OCH_3	6’-OH-angolensin

Figure 1. Structures of selected isoflavonoids.

Laboratory of Organic Chemistry, Department of Chemistry, University of Helsinki, Finland. Calycosin, prunetin, pseudobaptigenin, 7,3′,4′- and 6,7,4′-trihydroxyisoflavones, and orobol (5,7,3′,4′-tetrahydroxyisoflavone) were obtained from Apin Chemicals Ltd. All reagents were of pro analysis (pa) grade or higher. Hydrochloric acid, L-(+)-ascorbic acid, chloroform, and diethyl ether were obtained from Merck. Hexane, heptane, and methanol were from Rathburn Chemicals Ltd. *Helix pomatia* juice was purchased from BioSepra SA. Hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) were obtained from Pierce, and pyridine, super-purity solvent, was from Romil Ltd., Cambridge, U.K.

Analysis of Isoflavones in Red Clover Supplements. The dietary supplement (red clover, Novogen) was a commercially available product that was purchased from a local store. For qualitative and quantitative analyses, two tablets were finely ground and an amount of 20 mg of homogenized sample was weighed. The sample was first hydrolyzed with *H. pomatia* and extracted with diethyl ether. The water phase was further hydrolyzed with 2.0 M HCl and extracted again with diethyl ether as described by Mazur et al. (*19*). Further purification with ion exchange chromatography was not needed, because of the high levels of isoflavones in the dietary supplement. Combined ether extracts from both hydrolysates were evaporated to dryness, dissolved in 5 mL of methanol from which a 100 μL aliquot was pipetted, evaporated to dryness, silylated with 100 μL of quick silylation mixture (QSM; pyridine/HMDS/TMCS, 9:3:1), and analyzed by GC-MS. Quantitative analysis of daidzein, genistein, formononetin, and biochanin A was carried out by pipetting a 1 μL aliquot of sample, to which deuterated internal standards were added. Sample was then evaporated to dryness, dissolved to QSM, and analyzed by GC-MS.

Study Design and Collection of Urine Samples. Seven healthy Finnish women, aged 20−60 years, were recruited for this study. Subjects were asked to abstain from foods containing high levels of isoflavones, for example, soy or foods derived from soy, for 1 week (days 1−7) before and during the study (days 8−12). In the morning of the eighth day of the study, subjects took four red clover based dietary supplements with a glass of water. Baseline 24-h urine samples were collected 1 day (day 7) before the isoflavone supplementation, after which the 24-h urine sample collections were continued for five subsequent days (days 8−12). The urine samples were collected in plastic containers, and 1% of ascorbic acid and 0.1% of sodium azide were added as preservatives. The study protocol was approved by the Ethics Committee for Research in Epidemiology and Public Health, Hospital District of Helsinki and Uusimaa, Finland.

Sample Pretreatment. From daily urine samples a $^1/_{300}$ fraction was analyzed each time (average sample volume = 10 mL). Urine samples were extracted with Sep-Pak C18 cartridges followed by hydrolysis with *H. pomatia*, extracted twice with diethyl ether, and chromatographed on Sephadex LH-20 as described in our previous study (*11*). The first fraction was evaporated to dryness under N_2 flow. The samples were dissolved in 0.5 mL of methanol, which was applied on a 0.5 × 5.0 cm QAE Sephadex A-25 (Amersham Biosciences) anion exchange column in the acetate form (QAE-Ac^-), adapted from our isotope dilution GC-MS method for the determination of isoflavones and lignans in human urine (*20*). The first fraction was eluted with 6.5 mL of methanol and the second fraction with 7.0 mL of 0.2 M acetic acid in methanol. All three fractions were evaporated to dryness under nitrogen and silylated with 100 μL of QSM. An amount of 1−3 μL of the sample was injected to GC-MS. The sample pretreatment method was tested with blank urine samples spiked with reference compounds (10−20 μg). The method and the distribution of red clover isoflavones and their metabolites in the different chromatographic fractions are presented in the flow diagram in **Figure 2**.

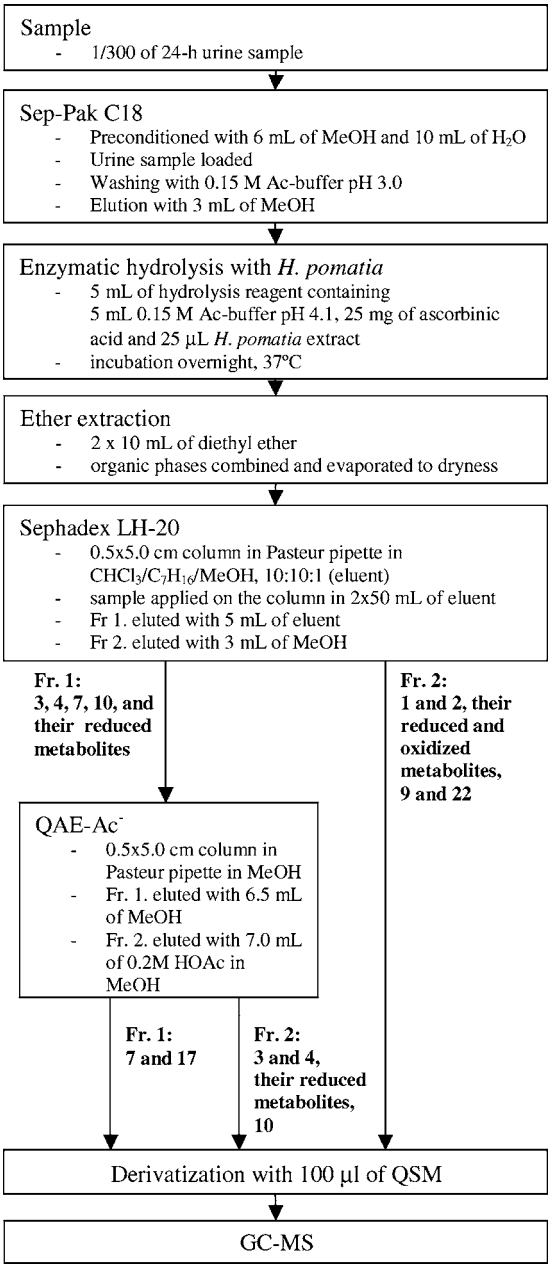

Figure 2. Pretreatment method used for urine samples and distribution of isoflavones of red clover and their metabolites in different chromatographic fractions.

Analytical Conditions. The capillary GC-MS instrument consisted of a Fisons GC 8000 gas chromatograph coupled to a Fisons Instrument MD 1000 quadrupole mass spectrometer. The column was a 12.5 m × 0.22 mm i.d., 0.25 μm, BP-1 (SGE). Helium was used as carrier gas at a flow rate of 1 mL/min. The column temperature was kept at 150 °C for 1 min and raised at 50 °C/min to 220 °C and then to 260 °C at 2 °C/min. The temperature of the injector was set at 280 °C, and the ion source and interface temperatures were 200 and 250 °C, respectively. Mass range was scanned from *m*/*z* 100 to 700 using 70 eV of electron energy.

Identification of Urinary Metabolites. After the isoflavone supplementation, the red clover isoflavones and their metabolites appeared in the mass spectrometric chromatograms of urine sample extracts. The metabolites could be distinguished from other urinary compounds by comparing the chromatograms of urine samples before the isoflavone challenge with the chromatograms after the isoflavone challenge. The isoflavones and their metabolites were identified using synthetic reference compounds by comparing the retention times and the mass spectra of the metabolites and the authentic reference compounds. The criterion that was used to positively identify a metabolite in urine sample was that the eight most abundant fragment ions of the mass spectrum of the trimethylsilyl derivative of the metabolite had the correct relative ion ratios when compared to the mass spectrum of the trimethylsilyl derivative of the reference compound.

RESULTS

Figure 3 presents the mass spectrometric total ion chromatogram from an extract of the red clover dietary supplement showing the isoflavones identified using authentic reference compounds. The main isoflavones of the tablet were formononetin and biochanin A. The tablet contained also small amounts of daidzein, genistein, pseudobaptigenin, prunetin, and calycosin (**Figure 1**). The ingested amounts of isoflavones were 51.7 mg of formononetin, 84.0 mg of biochanin A, 3.2 mg of daidzein, and 5.2 mg of genistein.

The ingested isoflavones and their urinary metabolites were distributed in three different chromatographic fractions obtained during sample pretreatment, as shown in **Figure 2**. Representative mass spectrometric chromatograms of the three fractions of urine sample extracts of one person on the first day after isoflavone supplementation are shown in **Figure 4**. The isoflavan, equol, was excreted by three of the seven participants. **Figure 5** presents the three fractions of urine sample extracts from one of the so-called equol producers. Other metabolites having an isoflavan structure were identified only in these three urine sample extracts.

Some unknown urinary compounds coeluted with the analytes as seen in **Figure 5**. These compounds were present in both pre- and postsupplementation samples and did not originate from the red clover supplement. In some samples the levels of these compounds were higher than levels of metabolites and thus disturbed the identification of the metabolites. In these cases the mass spectra of the analytes were refined by subtracting the background, that is, the ions of the coeluting compounds. The refined mass spectra were then compared to those of reference compounds, and the criteria of identification of the metabolite described under Materials and Methods were applied. As an example, the identification of dihydrobiochanin A in the urine sample of an equol producer using refined mass spectra is presented in **Figure 6**.

The least polar compounds, including neutral steroids, were eluted in the first fraction of the QAE-Ac$^-$ column (**Figures 4A** and **5A**). Prunetin, at the retention time of 14.19 min, was identified in urine sample extracts of all participants in this chromatographic fraction. An isoflavan, 4′-*O*-Me-equol, at the retention time of 7.13 min (**Figure 5A**) was present in urine samples of the three equol producers only.

The second fraction of the QAE-Ac$^-$ column (**Figures 4B** and **5B**) contained the ingested red clover isoflavones, formononetin and biochanin A, at retention times of 12.16 and 13.81 min, respectively. In addition, angolensin, dihydroformononetin, and dihydrobiochanin A were identified in this fraction at retention times of 6.71, 8.99, and 10.78 min, respectively. All of the isoflavonoids were detected in urine sample extracts of both non-equol and equol producers.

Most of the metabolites of red clover isoflavones eluted in the second fraction in the Sephadex LH-20 chromatography. Representative total ion current (TIC) chromatograms are shown in **Figures 4C** and **5C**. This fraction contained daidzein and genistein, the principal metabolites of formononetin and biochanin A, respectively. Additionally, a number of recently identified reduced and/or oxidized metabolites of daidzein and genistein were also detected. The compounds are listed in

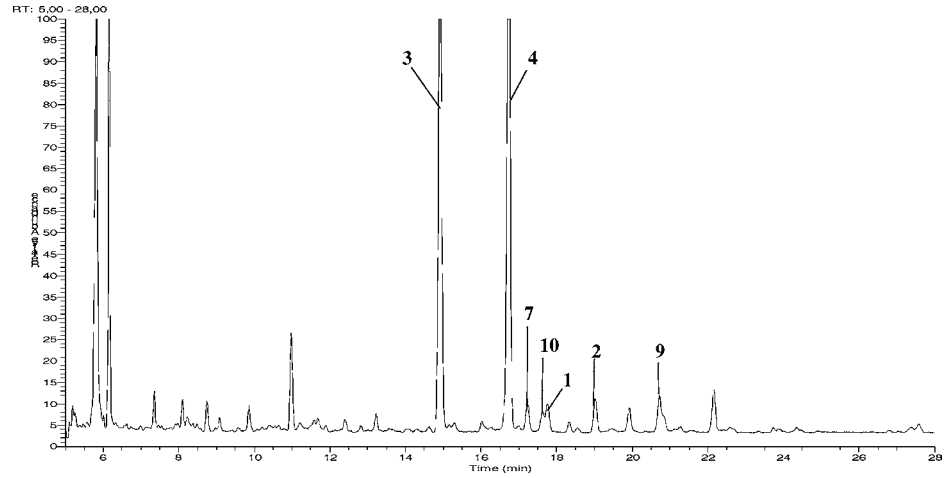

Figure 3. Total ion current (TIC) chromatogram of red clover tablet extract showing the isoflavones that were identified.

Figures 4C and **5C**. The full identification and the mass spectra of these compounds are presented elsewhere (*21*). Two new isoflavonoid metabolites originating from red clover were identified in this chromatographic fraction. The compound at the retention time of 7.01 min was identified as 6′-OH-angolensin by comparison with an authentic reference compound. This compound, as well as calycosin, one of the red clover isoflavones, at the retention time 17.33 min, was detected in all urine sample extracts analyzed.

The mass spectra of red clover isoflavones and the new metabolites of formononetin and biochanin A, identified using authentic reference compounds, are listed in **Table 1**. The interpretation of the spectra is described below in more detail. Fragmentation and interpretation of the spectra of trimethylsilyl derivatives of isoflavonoids has been described and discussed in a previous study of the metabolism of soy isoflavones (*21*).

4′-*O*-Methylequol. The metabolites having an isoflavan structure were present in urine samples of so-called equol producers only. The presence of 4′-*O*-methylequol (t_R 7.13 min, M^+ 328, base peak of the TMS ether *m*/*z* 134) in the urine sample extracts was confirmed using an authentic reference compound. The ion at *m*/*z* 313 is formed from the molecular ion, *m*/*z* 328, by the loss of one methyl radical. The base peak of the spectrum at *m*/*z* 134 is a retro-Diels−Alder (rDA) fragment of ring B. Other peaks of the spectrum are of low intensity.

Prunetin. The mass spectrum of prunetin (t_R 14.19 min) is identical with that of biochanin A, its isomer. The molecular ion of the TMS ether at *m*/*z* 428 has a very low intensity, and the base peak of the spectrum is at *m*/*z* 413, an ion formed by the loss of one methyl radical from the molecular ion. The rDA fragments at *m*/*z* 190 and 238 are absent.

Formononetin. The molecular ion at *m*/*z* 340 is the base peak in the mass spectrum of the trimethylsilyl ether of formononetin (t_R 12.16 min). A loss of one methyl radical from the trimethylsilyl group yields the rather intensive peak at *m*/*z* 325. The other intense peaks of the spectrum, at *m*/*z* 208 and 132, are formed by the rDA reaction from phenolic rings A and B, respectively.

Biochanin A. The molecular ion of biochanin A (t_R 13.81 min) is of a very low intensity (<1%) due to the loss of one methyl radical from the trimethylsilyl group attached to the oxygen at C5, to give the more stable M − 15 ion, base peak at *m*/*z* 413. The origin of the rather intense peaks at *m*/*z* 177 and 199 has not been elucidated. The rDA fragments of rings A and B occur at *m*/*z* 296 and 132, respectively.

Pseudobaptigenin. Trace amounts of pseudobaptigenin (t_R 14.59 min) were identified in all urine sample extracts. The molecular ion, which is the base peak of the mass spectrum, of pseudobaptigenin TMS ether is at *m*/*z* 354. The loss of one methyl radical from a trimethylsilyl group produces the ion at *m*/*z* 339. The ring B rDA fragment is a rather abundant ion at *m*/*z* 146. The ion at *m*/*z* 193 is most probably formed from the ring A rDA fragment by the loss of one methyl radical. Other intense peaks of the spectrum are at *m*/*z* 169 and 209. The structures of the ions have not been elucidated.

Dihydroformononetin. Dihydroformononetin (t_R 8.99 min, M^+ of the TMS ether 342, base peak *m*/*z* 134) was identified as a minor metabolite of formononetin in all urine samples analyzed. The loss of one methyl group from a trimethylsilyl group yields the ion at *m*/*z* 327. The base peak of the spectrum, *m*/*z* 134, is a ring B rDA fragment. The ion at *m*/*z* 119 is formed by the loss of a methyl group from the ring B rDA fragment. The ring A fragment of the rearrangement reaction is at *m*/*z* 208.

Angolensin. Angolensin, 2′,4′-dihydroxy-4″-methoxy-α-methyldeoxybenzoin, eluted at a retention time of 6.71 min. The molecular ion of the spectrum of the TMS derivative is at *m*/*z* 416, from which the loss of a methyl radical yields a more abundant peak at *m*/*z* 401. The base peak of the spectrum at *m*/*z* 281 is formed by α-cleavage of the bond between C1 and C2.

Dihydrobiochanin A. The metabolite at a retention time of 10.78 min was identified as dihydrobiochanin A (M^+ 430, base peak of the TMS ether *m*/*z* 296). The molecular ion, *m*/*z* 430, is low in abundance. The loss of a methyl radical produces the ion at *m*/*z* 415. The base peak of the spectrum, *m*/*z* 296, is a ring A rDA fragment. The losses of a methyl group and carbon monoxide from the ring A rDA fragment yield ions at *m*/*z* 281 and 268, respectively. The ring B rDA fragment occurs at *m*/*z* 134, from which the loss of a methyl group from the methoxy substituent produces the ion at *m*/*z* 119.

Calycosin. Unlike the mass spectra of TMS derivatives of biochanin A and prunetin, which are isomers of calycosin (retention time of 17.20), the molecular ion in the mass spectrum of calycosin TMS ether at *m*/*z* 428 is rather intense. The loss of one methyl radical from a trimethylsilyl group of ring B yields an ion at *m*/*z* 413 of moderate intensity (11%). The base peak at *m*/*z* 398 is formed by loss of ethane from the molecular ion, forming a stable radical cation. Other important peaks of the

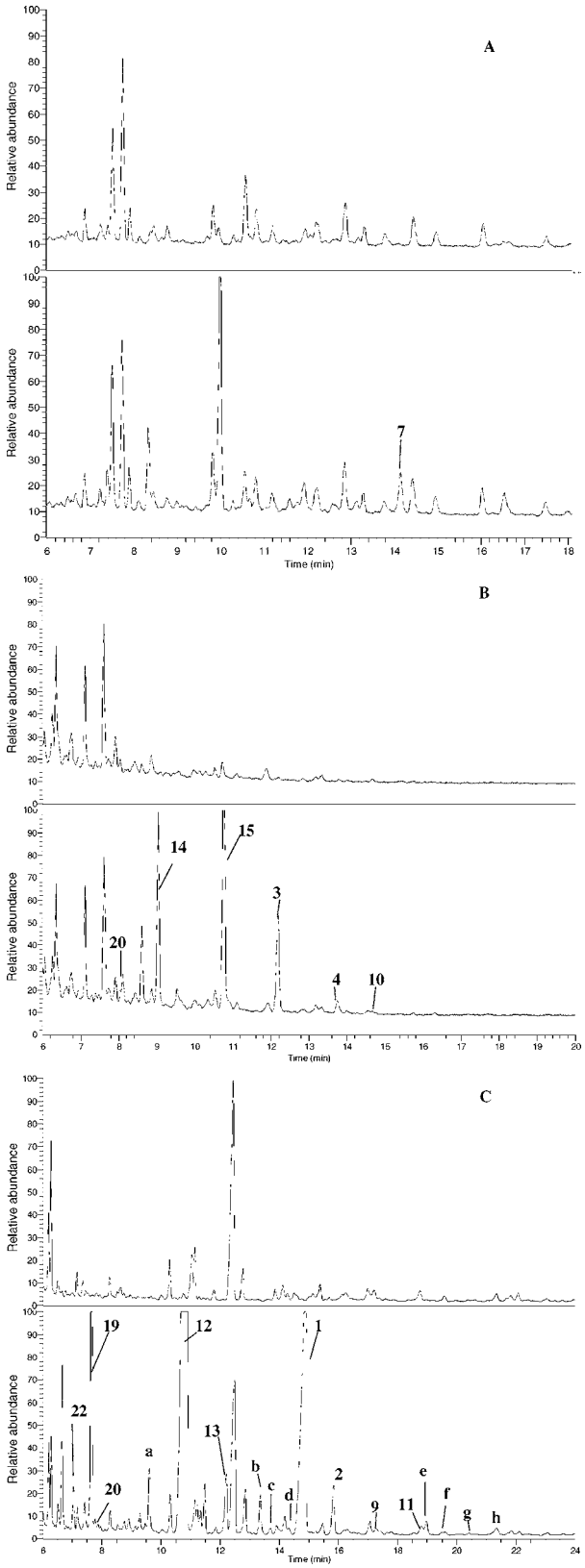

Figure 4. TIC chromatograms of urine sample extracts of non-equol producer, before (upper chromatogram) and after (lower chromatogram) the isoflavone supplementation: (**A**) first fraction and (**B**) second fraction after QAE-Ac$^-$ chromatography; (**C**) second fraction after Sephadex LH-20 chromatography. Numbers refer to structures presented in **Figure 1**. Compounds **a**–**h** are previously identified isoflavonoid metabolites (*21*): **a**, 3′′-OH-*O*-desmethylangolensin; **b**, 3′-OH-dihydrodaidzein; **c**, 8-OH-dihydrodaidzein; **d**, 6-OH-dihydrodaidzein; **e**, 3′-OH-daidzein; **f**, 8-OH-daidzein; **g**, 3′-OH-genistein; **h**, 6-OH-daidzein.

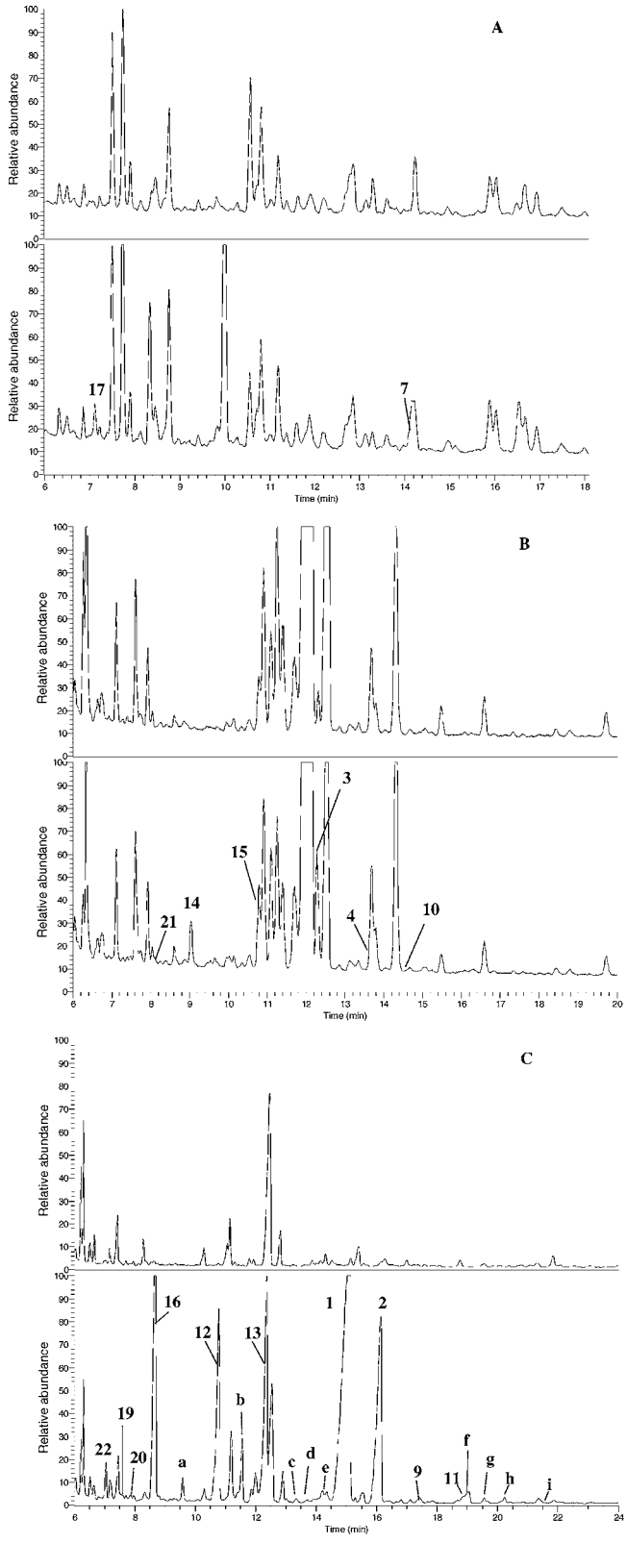

Figure 5. TIC chromatograms of urine sample extracts of an equol producer, before (upper chromatogram) and after (lower chromatogram) isoflavone supplementation: (**A**) first fraction and (**B**) second fraction after QAE-Ac$^-$ chromatography; (**C**) second fraction after Sephadex LH-20 chromatography. Numbers refer to structures presented in **Figure 1**. Compounds **a**–**i** are previously identified isoflavonoid metabolites (*21*): **a**, 3′′-OH-*O*-desmethylangolensin; **b**, 3′-OH-equol; **c**, 3′-OH-dihydrodaidzein; **d**, 8-OH-dihydrodaidzein; **e**, 6-OH-dihydrodaidzein; **f**, 3′-OH-daidzein; **g**, 8-OH-daidzein; **h**, 3′-OH-genistein; **i**, 6-OH-daidzein.

Table 1. Mass Spectrometric Data of Isoflavonoids Identified in Chromatographic Fractions of Urine Samples Collected after Red Clover Supplementation

compound	M^+	t_R (min)	ion (*m*/*z*, abundance in parentheses)
4′-*O*-Me-equol[a]	328	7.13	119 (19), 121 (19), 134 (100), 191 (5), 206 (9), 207 (11), 298 (3), 328 (30)
prunetin[a]	428	14.19	175 (2), 177 (11), 184 (3), 191 (3), 199 (29), 207 (2), 312 (2), 341 (4), 370 (8), 413 (100)
angolensin[b]	416	6.71	105 (3), 135 (7), 209 (9), 223 (2), 281 (100), 401 (3)
dihydroformononetin[b]	342	8.99	119 (9), 134 (100), 193 (2), 208 (3), 327 (1), 342 (5)
dihydrobiochanin A[b]	430	10.78	119 (13), 121 (26), 134 (35), 147 (10), 193 (2), 207 (2), 222 (2), 239 (6), 267 (2), 279 (3), 281 (9), 296 (100), 415 (12)
pseudopabtigenin[b]	354	14.59	145 (33), 146 (71), 147 (40), 169 (41), 193 (11), 207 (12), 208 (7), 209 (13), 339 (33), 353 (33), 354 (100)
formononetin[b]	340	12.16	117 (16), 119 (7), 121 (6), 132 (57), 137 (6), 141 (16), 152 (9), 162(32), 165 (6), 208 (14), 253 (3), 269 (6), 282 (4), 310 (2), 325 (36), 339 (39), 340 (100)
biochanin A[b]	428	13.81	132 (2), 177 (7), 199 (19), 207 (2), 312 (2), 340 (2), 341 (2), 370 (7), 398 (3), 413 (100)
6′-OH-angolensin[c]	504	7.01	135 (12), 147 (9), 281 (4), 297 (3), 335 (4), 369 (100), 489 (5)
calycosin[c]	428	17.33	119 (2), 160 (4), 169 (2), 175 (10), 184 (20), 190 (26), 191 (37), 199 (5), 207 (2), 209 (3), 253 (2), 325 (2), 355 (3), 383 (4), 398 (100), 413 (11), 428 (68)

[a] QAE-Ac$^-$ fraction 1. [b] QAE-Ac$^-$ fraction 2. [c] Sephadex LH-20 fraction 2.

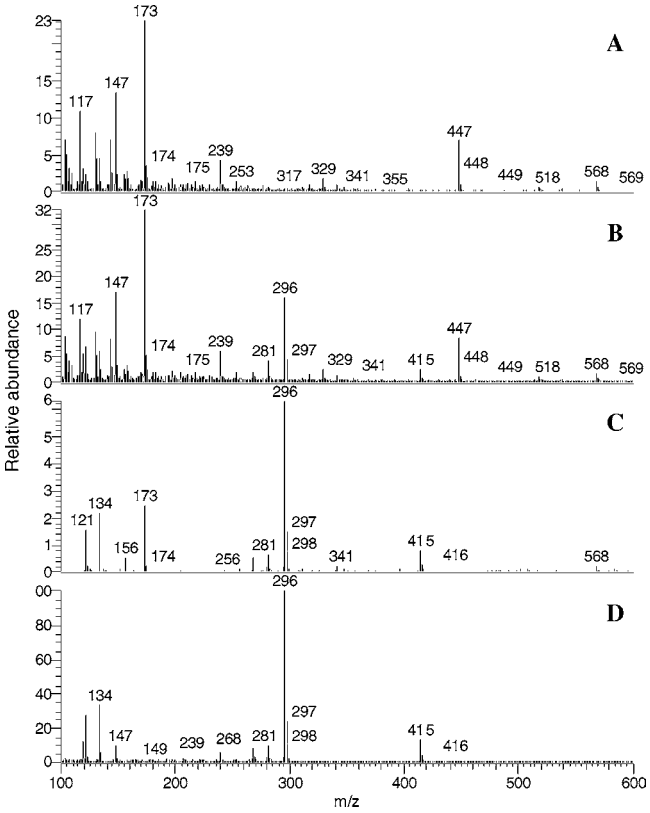

Figure 6. Identification of dihydrobiochanin A in urine sample of an equol producer using refined mass spectra. Mass spectra were obtained at the retention time of dihydrobiochanin A in urine sample collected (**A**) before and (**B**) after isoflavone supplementation; (**C**) is the refined mass spectrum of (**B**), where the background ions have been subtracted, (D) is the mass spectrum of an authentic reference compound.

spectrum are ions at *m*/*z* 190, formed from the rDA fragment of ring B by loss of ethane, and an ion at *m*/*z* 191 of unknown structure.

6′-OH-angolensin. 6′-OH-angolensin TMS ether eluted at the retention time of 7.01 min. The molecular ion at *m*/*z* 504 was absent from the mass spectrum, due to the easy loss of a methyl radical from the trimethylsilyl group at the 2′-O-position, producing an M − 15 peak at *m*/*z* 489. The base peak of the spectrum was at *m*/*z* 369, which is a ring A fragment originating from α-cleavage of the bond between C1 and C2. The ring B fragment is at *m*/*z* 135. Other peaks of the spectrum were of low intensity.

DISCUSSION

In the present study, we have investigated the metabolism of two naturally occurring isoflavonoids, formononetin and biochanin A, in red clover supplement by GC-MS and identified new metabolites, angolensin, 6′-OH-angolensin, dihydroformononetin, and dihydrobiochanin A, and previously tentatively identified 4′-*O*-Me-equol (*22*) in human urine. The ingested red clover tablets contained also calycosin, prunetin, and pseudobaptigenin, the identification of which is reported for the first time in human urine.

The metabolic pathways of formononetin and biochanin A are summarized in **Figures 7** and **8**, respectively. The main reaction is the demethylation of the methoxy group at the 4′-position, yielding high urinary as well as plasma levels of daidzein and genistein (*23*). Minor amounts of reduced metabolites of formononetin and biochanin A, still bearing the methoxy group at the 4′-position, were identified in urine for the first time. The levels of these metabolites were low in all urines examined, suggesting that demethylation of the methoxy group at the 4′-position occurs easily and almost quantitatively.

When the levels of the isomeric compounds prunetin and biochanin A in human urine are compared (**Figure 4A**,**B**), it can be clearly seen that the extent of demethylation of the methoxy substituents of isoflavones depends on its position in the isoflavone structure. Even though the red clover tablet contained higher levels of biochanin A compared to levels of prunetin, the levels of the two isoflavones in human urine are almost equal (**Figure 3**). Thus, the demethylation of the methoxy group at ring B occurs more easily than the demethylation of a methoxy at ring A. Similar observations have been made for flavonoids, where the demethylation was observed to occur more easily at C6 than at C4′ (*24*).

Daidzein and genistein, the main demethylated metabolites formed from formononetin and biochanin A, are reduced to the corresponding isoflavanones and α-methyldeoxybenzoins (i.e., the so-called dihydroisoflavones and *O*-desmethylangolensins), and the levels of metabolites were considerably higher than those of reduced formononetin and biochanin A metabolites. It has been found that only about one-third of the human population is capable of producing higher amounts of equol, the reduced metabolite of daidzein having an isoflavan structure (*10*, *25*−*27*). For genistein or other isoflavones having a hydroxy group at the 5-position no metabolites having an isoflavan structure were identified, although reference compounds were available. It has been suggested that hydrogen bonding between the

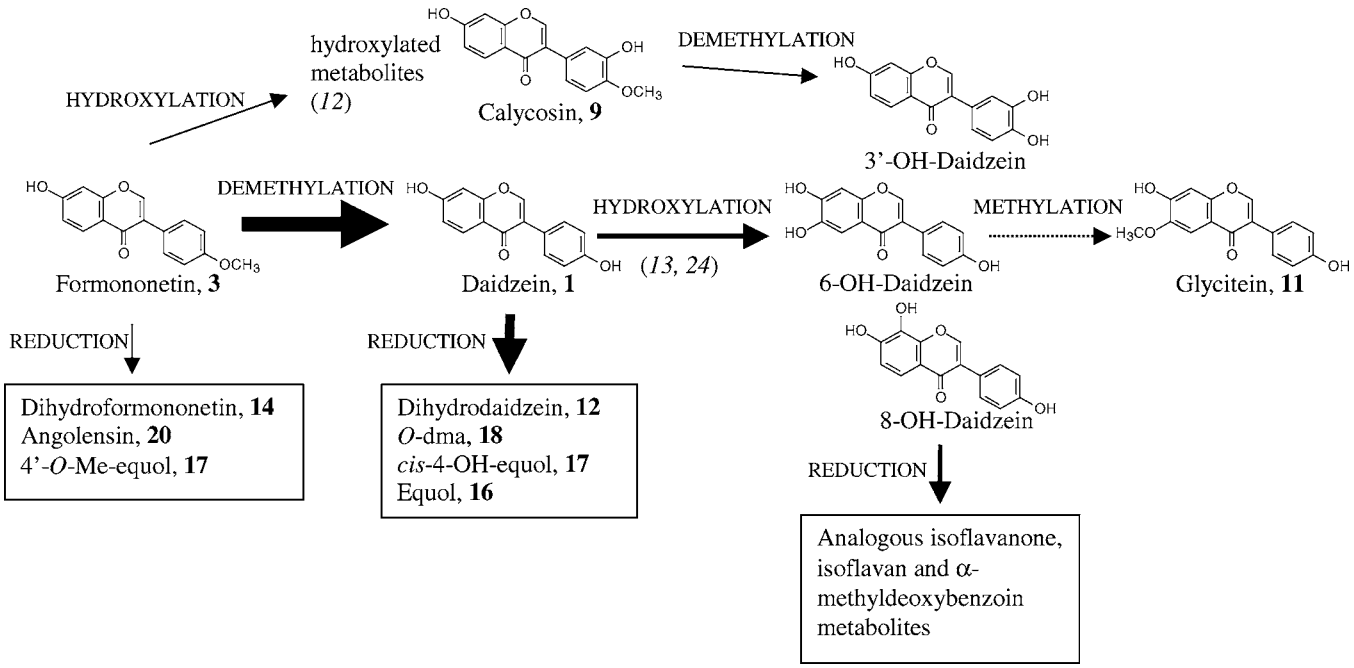

Figure 7. Metabolic pathway of formononetin, including daidzein and calycosin. The thickness of the arrow represents the extent of conversion: the thicker the arrow, the more extensive the conversion.

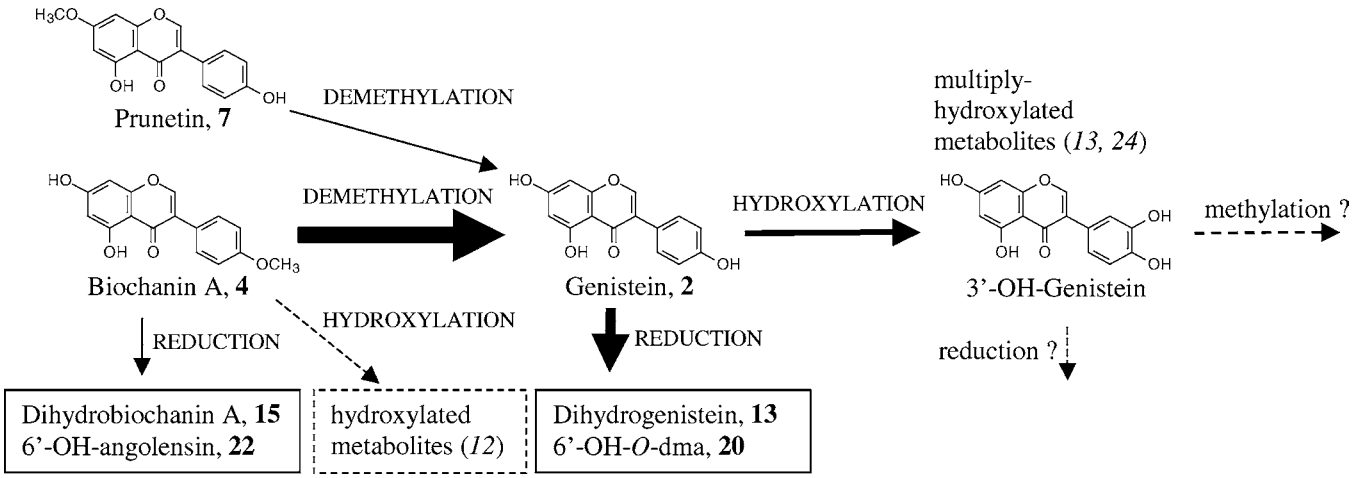

Figure 8. Metabolic pathway of biochanin A, including genistein and prunetin. The thickness of the arrow represents the extent of conversion: the thicker the arrow, the more extensive the conversion. So far, hydroxylated metabolites of biochanin A (dotted line) have not been identified in human urine.

hydroxy group and carbonyl oxygen prevents the reduction of the C4 carbonyl group (*27*).

The oxidative metabolism and methylation of so-formed vicinal hydroxy groups seem to be minor metabolic reactions of isoflavones, and the levels of these metabolites are generally low. An interesting finding in our study was that trace amounts of glycitein, which was not detected in ingested red clover tablets, appeared in urine samples after red clover supplementation. Most probably glycitein is formed by the methylation of 6-OH-daidzein, the oxidized metabolite of daidzein, by catechol-*O*-methyl transferase (COMT), as Kulling et al. (*28*) have demonstrated in vitro.

Furthermore, in addition to glycitein, calycosin, biochanin A, and prunetin, two other isomeric compounds having the same molecular ion at *m*/*z* 428 were detected in urine samples after red clover supplementation at retention times of 16.81 and 17.86 min (data not shown). There are two alternative origins for these metabolites: either they are formed by methylation of hydroxylated metabolites of daidzein, in the same way as glycitein was suggested to be formed by the methylation of 6-OH-daidzein, or the compounds are hydroxylated metabolites of formononetin. Recently, the oxidative in vitro metabolism of both formononetin and biochanin A by human liver microsomes has been studied (*12*, *15*). The results suggest that the liver microsomes mainly catalyze the demethylation of the 4′-methoxy group, the formation of hydroxylated formononetin and biochanin A metabolites being a minor metabolic reaction. Some formation of hydroxylated metabolites does occur, and three hydroxylated metabolites of both formononetin and biochanin A, with additional hydroxy groups at the 3′-, 6-, and 8-positions, have been identified in liver microsomal extracts. The surprisingly high levels of calycosin, that is, 3′-hydroxyformononetin, would be explained by the formation of hydroxylated metabolites of formononetin.

In conclusion, the metabolism of isoflavones in humans is complex, and several metabolites are formed after isoflavone supplementation. However, the levels of newly identified metabolites are low when compared to levels of daidzein and genistein that are either derived directly from soy or formed from formononetin and biochanin A by demethylation. Even

though the levels of the newly identified metabolites are low, further studies are needed to find out whether some of these might have beneficial biological activities.

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