Pergamon

0960-0760(95)00131-X

J. Steroid Biochem. Molec. Biol. Vol. 54, No. 3/4, pp. 167–184, 1995

0960-0760/95 $9.50 + 0.00

A Urinary Profile Study of Dietary Phytoestrogens. The Identification and Mode of Metabolism of New Isoflavonoids

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The metabolic fate of the dietary isoflavones daidzein and genistein was investigated in human volunteers challenged with soya. Urinary diphenols, isolated by partition chromatography on Sephadex LH-20, were characterized and identified by profile capillary gas chromatography (GC) and electron ionization mass spectrometry (GC–EIMS) analysis of the trimethylsilyl ether (TMS) derivatives. Novel isoflavonic phytoestrogens found in the urine of volunteers were those of tetrahydrodaidzein, dihydrogenistein, 6′-hydroxy-*O*-demethylangolensin and 2-dehydro-*O*-demethylangolensin. Other known diphenols identified were those of equol, dehydrodaidzein, *O*-demethylangolensin, daidzein, genistein, glycitein, and the lignan enterolactone. Two other urinary isomers with a fragmentation pattern closely resembling that of the persilylated TMS ethers of *cis*/*trans*-isomers of tetrahydrodaidzein, were characterized based on the elucidation of fragments associated with the loss of a non-phenolic-OTMS functional group in ring-C. These are fragments presented in the persilylated mass spectra of isoflavan-4-ols and isoflav-3-ene-4-ols, demonstrated here by a combination of simple and tandem mass spectrometry study of the deuterated persilylated TMS ethers of dihydrodaidzein. In a similar study we also present the data on the structural identification and fragment elucidation of the keto/enol tautomers of the TMS ether derivatives of the dihydro derivatives of daidzein and genistein, observed in the urine of volunteers and considered probable products of the derivatization process. Finally, the GC and GC–MS data of two unknown isoflavonoids and that of a lignan-like compound are presented together with those of dihydrodaidzein, dihydrogenistein, tetrahydrodaidzein and 2-dehydro-*O*-demethylangolensin. The latter four were obtained here as products of small scale chemical synthesis in a preliminary study on the tentative identification of urinary isoflavonoids in human volunteers challenged with soya.

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INTRODUCTION

Phytoestrogens are plant chemicals (diphenols), of which isoflavones and lignans are the two principal varieties (see Scheme 1 on next page). The former occur principally in legumes and related food products such as soya. Animal experiments have shown diphenols to have a wide range of biological functions, including an ability to bind to estrogen receptors, to simulate the production of sex hormone binding globulin, and to inhibit enzyme systems such as tyrosine protein kinase and estrogen synthetase [1]. These biological functions are thought in part to be related to the close structural relationship between phytoestrogens and endogenous reproductive hormones such as estrogens.

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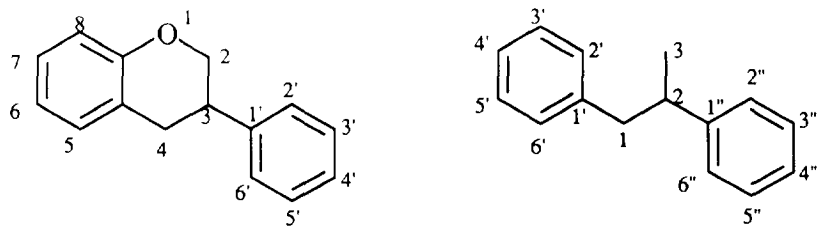
Received 29 Oct. 1994; accepted 27 Mar. 1995.

Abbreviations: daidzein (7,4′-dihydroxyisoflavone); genistein (5,7,4′-trihydroxyisoflavone); glycitein (5-methoxy,7,4′-dihydroxyisoflavone); equol (7,4′-dihydroxyisoflavane); dehydroequol (Intermediate-E, 7,4′-dihydroxyisoflav-3-ene); dihydrodaidzein (Intermediate-*O*, 7,4′-dihydroxyisoflavanone), *Int-O-D*; dihydrogenistein (5,7,4′-trihydroxyisoflavanone); tetrahydrodaidzein (7,4′-dihydroxyisoflavanol), *TetraD*; tetrahydrogenistein (5,7,4′-trihydroxyisoflavanol); *O*-demethylangolensin [1-(2′,4′-dihydroxyphenyl)-2-(4″-hydroxyphenyl)-propan-1-one], *O-Dma*; 6′-hydroxy-*O*-demethylangolensin [1-(2′,4′,6′-trihydroxyphenyl)-2-(4″-hydroxyphenyl)-propan-1-one], 6′-*OH-O-Dma*; 2-dehydro-*O*-demethylangolensin [1-(2′,4′-dihydroxyphenyl)-2-(4″-hydroxyphenyl)-prop-2-en-1-one], 2-*de-O-Dma*.



ISOFLAVAN **ANGOLENSIN**

Scheme 1. Molecular structures of angolensin and isoflavan numbered according to *Chem. Abstracts*.

In the human, in view of the epidemiological evidence linking high dietary isoflavonoid phytoestrogen intake with low prevalence of hormone-dependent diseases, there is an increased interest in the likely role of phytoestrogens as possible hormone modulators and protective agents against coronary heart diseases and cancers of the breast, colon and prostate [1–3]. The finding that phytoestrogens are potent, selective and reversible inhibitors of human alcohol dehydrogenase isozymes [4, 5], that seem to suppress the desire for alcohol in animal experiments, has also led to the suggestion that isoflavonoid phytoestrogens could play a role in the treatment of alcoholism [6].

In spite of the purported significant role of phytoestrogens in human health, the pathways of metabolism of daidzein and genistein, the two principal isoflavones found in legumes and soya, remain unclear. Profile GC and GC–MS analyses are procedures which have provided in the past detailed information on the metabolism and role of biological substances in normal and pathological conditions in humans [7]. However, to the best of our knowledge no such profile analysis of isoflavonoids has been reported before, although a profile study of the diphenol lignans isolated from human urine resulted in the identification of several new lignans [8].

In this investigation, as an extension of an earlier study [9], we present the data of new isoflavonoid phytoestrogens found in the urine of human volunteers challenged with soya. The characterization and identification of the new urinary metabolites by GC and GC–MS profile analysis, shed fresh light on the metabolic pathway of daidzein as proposed by Adlercreutz and colleagues [10], and allow us to propose for the first time a pathway for the metabolism of genistein.

EXPERIMENTAL

Participants

Details of human volunteers and soya challenge have been described previously [9]. In brief, 12 healthy Caucasian men and women aged between 25 and 51 years old, of normal height and weight, and of typical Western European omnivorous dietary habits, were challenged with 40 g of soya flour daily for two consecutive days. The isoflavone content of the single batch of soya used was 98 mg (genistein), 80 mg (daidzein) and 3 mg (glycitein) per 100 g of soya flour. Twenty-four hour urine samples were collected on the day prior to and 3 consecutive days following soya challenge.

The urine was collected in polypropylene containers without preservative. On collection the bottles were kept as cool as possible and delivered to the laboratory daily. The volume of urine was recorded and a 200 ml aliquot was stored at −20°C until ready for analysis. The analysis of samples was commenced within 3 months of collection.

Chemicals

All solvents were of analytical grade and used as received. 10% Palladium on calcium carbonate (10% Pd/Ca_2CO_3), 5% palladium on charcoal (5% Pd/C) and 5% rhodium on alumina (5% Rh/Al_2O_3) were obtained from Fluka Chemie AG, Switzerland. *N,O*-Bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) was obtained from Supelco Inc., Bellefonte, PA, U.S.A., and *N,O*-bis-(d_9-trimethylsilyl)-acetamide (BSA-d_9) from MSD-Isotopes Ltd, Montreal, Canada.

Reference phytoestrogen and steroid standards

Equol, daidzein, genistein, formononetin, biochanin A, were kindly supplied by Dr Lamberton, Division of Organic Chemistry, CSIRO, Australia, and dihydrodaidzein by Professor H. Adlercreutz, Department of Clinical Chemistry, University of Helsinki, Finland. Lignan standards were not commercially available. Glycitein was extracted from soya and used as a reference standard after structural confirmation by NMR and GC–MS [9]. Estradiol and estriol were obtained from Steraloids and [^{3}H]estradiol glucuronide from New England Nuclear.

Enzymatic hydrolysis and extraction of the urinary diphenols

Enzymatic hydrolysis of the urinary isoflavone glucuronides was carried out with β-glucuronidase/aryl sulphatase from *Helix pomatia* (Calbiochem cat. no. 34742). The urine (40 ml) was adjusted to pH 4.6 with 70% acetic acid and incubated at 37°C for 48 h after addition of β-glucuronidase (1000 Fishman Units/ml of urine) at the beginning of the incubation and after 24 h. Cold and radioactive recoveries were

obtained using estriol and [^{3}H]estradiol glucuronide, added to all the samples at the start of the analyses. A urine sample (40 ml) with no detectable levels of equol, daidzein or genistein, was used as a recovery control in every batch of assays. The urine control samples ($n = 11$) were spiked with 60 μg of each of the above phytoestrogens.

Free phytoestrogens, including those released by the enzyme were extracted with diethyl ether (2 × vol.) and evaporated to dryness under nitrogen. The dry residue was dissolved in 1.0 ml of ethanol and stored cold for analysis. Purification of the crude hydrolysis extract was carried out on Sephadex LH-20 on 200 μl aliquots prior to GC and GC–MS analysis.

Isolation of urinary diphenols

Urinary diphenols, free of neutral steroids, were isolated in a straight-phase partition chromatography on Sephadex LH-20 using chloroform–heptane–methanol (10:10:1; v/v) as eluent. A similar system was used for the separation of steroids and simplification of urinary steroid extracts in newborn infants [7]. Sephadex LH-20 (1 gm), suspended in a solution of the eluent, was allowed to settle in a silanized pasteur pipette used as a column. Aliquots (200 μl) of the hydrolysate extract were concentrated to 20 μl in special tapered glass vials and applied on the gel after dilution to 50 μl with eluent.

Chromatography was commenced with the removal of a septum used as a plug with a further 3 × 50 μl of the eluent applied to the column after rinsing the glass tapered vials each time to ensure complete transfer of the hydrolysate extract. The first 14.0 ml of eluent released the bulk of urinary steroids, while the diphenol fraction (non-methylated) was retained to the column and eluted subsequently with 4.0 ml of methanol. The methanolic fraction was then evaporated under a stream of nitrogen and made to 600 μl in ethanol. Aliquots (200 μl) were used for derivatization and analyses by GC and GC–MS.

Derivatization

To the dry diphenol residue from above, 50 μl of a mixture of BSTFA in dry pyridine (or tetrahydrofuran; THF) (3:1, v/v) was added and heated at 100 C for 1 h. Small aliquots (1–2 μl) of the reaction mixture were applied onto the glass solid injectors for analyses by capillary GC and GC–MS.

d_9-substituted TMS ethers. In the deuterated structural studies of dihydrodaidzein the mono-, di- and tri-substituted deuterated TMS ethers of the keto/enol tautomers, were obtained as follows. *The keto form*: Dihydrodaidzein (100 μg) was dissolved in 50 μl of BSTFA and incubated at 100 C for 1 h. After analysis by GC and GC–MS, confirming its quantitative conversion to the di-TMS ether, the BSTFA reagent was removed gently over a stream of nitrogen at room temperature. To the dry silylated residue, 50 μl of BSA-d_9 were added and incubated at 60 C. Progress of the reaction with separation and identification of the mono- and di-substituted d_9-TMS ethers was achieved by capillary GC and GC–EIMS. *The enol form*: dihydrodaidzein (100 μg) was dissolved in 50 μl of BSTFA in dry pyridine (3:1; v/v) and incubated at 100°C for 1 h. After analysis by GC and GC–MS, confirming its quantitative conversion to the tri-TMS ether, the BSTFA reagent was removed by evaporation as above and rederivatized in d_9-BSA/THF (3:1; v/v).

Capillary GC and GC–MS

Capillary GC was performed on a Hewlett-Packard 5710A gas chromatograph equipped for flame ionization using a 30 m SE 30 × 0.25 mm column from Altech Associates Pty. Ltd. (cat 982530) and helium as carrier gas (flow rate 1 ml/min). The flash heater was set at 250 C and the detector temperature at 300°C. Samples were applied on the column via an all-glass solid injector modified for a syringe-type plunger in place of the cumbersome magnet [11]. Separation of chromatographic peaks was obtained by temperature programming from 197 to 260 C at 1°C/min.

Preliminary identification of urinary diphenols was based on predetermined relative retention indices expressed as methylene units [MU]. These were obtained on a Shimadzu CR4A integrator interfaced with a software program named Eurekasoft, designed by one of us (GEJ), for the computation and identification of unknowns based on a library data search and matching of MU values with those from reference standards. Quantitation of the results was based on relative areas of cholesteryl butyrate co-injected and used as the internal standard.

GC–MS was performed on a Finnigan-Mat TSQ-70 mass spectrometer interfaced with a Varian 3400 gas chromatograph equipped with a capillary column, as that described above, directly connected to the ionizing chamber. Electron ionization mass spectrometry (EIMS) was carried out with repetitive scanning over the mass range of 80–700 Da. Tandem mass spectrometry (MS/MS) was carried out using argon as the collision gas at a pressure of 1.0 mTorr and a collision energy of 10 eV. Chemical ionization mass spectrometry (CIMS) was performed using methane or methane/ammonia as plasma. The temperature of transfer line was set at 280°C and the ion source and analyser temperatures at 150 and 70°C, respectively. The EIMS and MS/MS mass spectrometric measurements were recorded at 70 eV with ionization emission current of 200 mA respectively.

Chemical Synthesis of Isoflavonoids

The conversion of daidzein to equol by simple hydrogenation has been demonstrated in several studies [12, 13]. The aim of this investigation, however, was the small scale syntheses of intermediates of such a conversion from well-precedented chemical reactions. These compounds were required for the matching of

Table 1. The GC and GC–MS data of the trimethylsilyl ethers of isoflavonoids obtained by chemical synthesis together with the mono-, di- and tri-substituted d_9-TMS ethers of the keto/enol tautomers of dihydrodaidzein

Isoflavonoid	Rel. RT MU	EIMS/CIMS mass spectra M^+ ion; m/z (rel int %)
Dihydrodaidzein (keto)	26.66	**eims** = M$^+$ 400 (8); 192 (100); 385 (4); 281 (28); 235 (1); 177 (22); 151 (2). **cims** = M$^+$ ^{1}401 (100); 385 (34); 281 (10); 192 (14); 177 (3).
Dihydrodaidzein (keto) mono-7-d_9-TMS	26.58	**eims** = M$^+$ 409 (8); 192 (100); 290 (26); 177 (20); 151 (4); 117 (2).
Dihydrodaidzein (keto) di-7,4′-d_9-TMS	26.50	**eims** = M$^+$ 418 (4); 201 (100); 299 (30); 183 (22); 157 (2)
Dihydrodaidzein (enol)	27.11	**eims** = M$^+$ 472 (100); 457 (20); 399 (3); 383 (12); 355 (2); 307 (7); 281 (12); 209 (4); 191 (6). **cims** = M$^+$ ^{1}473 (100); 457 (12); 399 (18).
Dihydrodaidzein (enol) mono-4-d_9-TMS	27.06	**eims** = M$^+$ 481 (100); 463 (12); 383 (15); 355 (2); 316 (8); 290 (13); 232 (4); 218 (4); 191 (7).
Dihydrodaidzein (enol) di-4,7-d_9-TMS	27.00	**eims** = M$^+$ 490 (100); 472 (18); 417 (4); 392 (14); 325 (5); 299 (12); 236 (5); 219 (3); 191 (6).
Dihydrodaidzein (enol) tri-4,7,4′-d_9-TMS	26.94	**eims** = M$^+$ 499 (100); 481 (16); 417 (4); 401 (13); 325 (9); 299 (16); 240 (7); 219 (5); 200 (7).
2-de-O-Dma	25.92	**eims** = M$^+$ 472 (15); 281 (100); 457 (25); 267 (9); 209 (6); 191 (5). **cims** = M$^+$ ^{1}473 (100); 457 (15); 407 (5); 338 (3); 212 (3).
Tetrahydrodaidzein	26.05	**eims** = M$^+$ 474 (2); 267 (100); 383 (2); 355 (7); 282 (12); 192 (3); 179(11);
cis/trans-isomers	26.39	163 (3). **cims** = M$^+$ ^{1}475 (3); 385 (100); 460 (2); 413 (3); 267 (2); 179 (7).
Dihydrogenistein (keto)	27.45	**eims** = M$^+$ 488 (1); 296 (100); 473 (12); 369 (11); 229 (5); 192 (14); 177(10) 151 (7). **cims** = M$^+$ ^{1}489 (100); 473 (94); 417 (12); 296 (6); 234 (14); 222 (8).
Dihydrogenistein (enol)	27.98	**eims** = M$^+$ 560 (100); 545 (18); 530 (3); 471 (14); 379 (7); 369 (44); 279 (4); 191 (18)

the GC and GC–MS data with those of unknown urinary metabolites. Table 1 lists the GC and GC–MS data of all the isoflavonoids obtained here as products of chemical synthesis from daidzein and genistein as outlined below. Structural characterization of products, which GC and GC–MS data could not be matched against reference samples, was based on the elucidation of the mass spectral data. This is dealt with in the results and discussion section in comparison with the data obtained from the urinary unknowns.

Chemical reactions

Hydrogenation of daidzein and genistein with Pd/Ca_2CO_3. Preliminary studies on the hydrogenation of daidzein with either Pd/C or Rh/Al_2O_3 catalysts was facile and provided equol as the major product. However, catalytic hydrogenation of daidzein and genistein with Pd/Ca_2CO_3 gave a mixture of hydrogenation adducts. It will be well to emphasize here, therefore, that Pd/Ca_2CO_3 was our choice of catalyst in these reactions. The products were obtained by bubbling hydrogen gas through a solution of a 1.0 mg sample of phytoestrogen standard dissolved in 2.0 ml of ethanol containing 0.5 mg of the catalyst. Aliquots (20 μl) obtained at regular intervals (10 min), were centrifuged and the supernatant derivatized in BSTFA or BSTFA in dry pyridine (3:1; v/v) for analysis by GC and GC–MS.

Major products of hydrogenation obtained on Pd/Ca_2CO_3 from daidzein and genistein were dihydrodaidzein and dihydrogenistein, respectively. Other products were the *cis/trans*-isomers of tetrahydrodaidzein (ratio 3/2) and 2-dehydro-*O*-demethylangolensin obtained as minor products of hydrogenation from daidzein. Dihydrodaidzein was positively identified based on the matching of its GC and GC–MS data with those of its reference sample provided. Structural characterization of tetrahydrodaidzein and 2-dehydro-*O*-demethylangolensin was based on the elucidation of mass spectral data. Further evidence of these latter two products was obtained by sodium borohydride reduction of dihydrodaidzein and catalytic transfer hydrogenation of daidzein, respectively (see below).

Sodium borohydride ($NaBH_4$) reduction of dihydrodaidzein. Tetrahydrodaidzein was obtained here as the sole product of $NaBH_4$ reduction from dihydrodaidzein (*cis/trans*-isomers; 1:3). Dihydrodaidzein (0.5 mg) was dissolved in 100 μl of dioxane and 20 μl of water. $NaBH_4$ (1 mg) was added and let to stand at room temperature for 2 h. Excess $NaBH_4$ was then destroyed with acetic acid and the mixture evaporated to dryness. The residue was extracted with ethyl acetate, washed with water and evaporated to dryness. On derivatization, analysis by GC and GC–MS, identified two isomers as the sole products of reduction from dihydrodaidzein. Based on the mass spectral data these were characterized as the tetrahydrodaidzein isomers, which data were in total agreement with those obtained from above as products of hydrogenation from daidzein. Sodium borohydride reduction, however, seemed to favour the formation of the second isomer. Based on the elution on SE-30, this was considered to be the more planar of the two isomers and was therefore tentatively identified as the *trans*-isomer.

Catalytic transfer hydrogenation of daidzein with Pd/C. Catalytic transfer hydrogenation was carried out

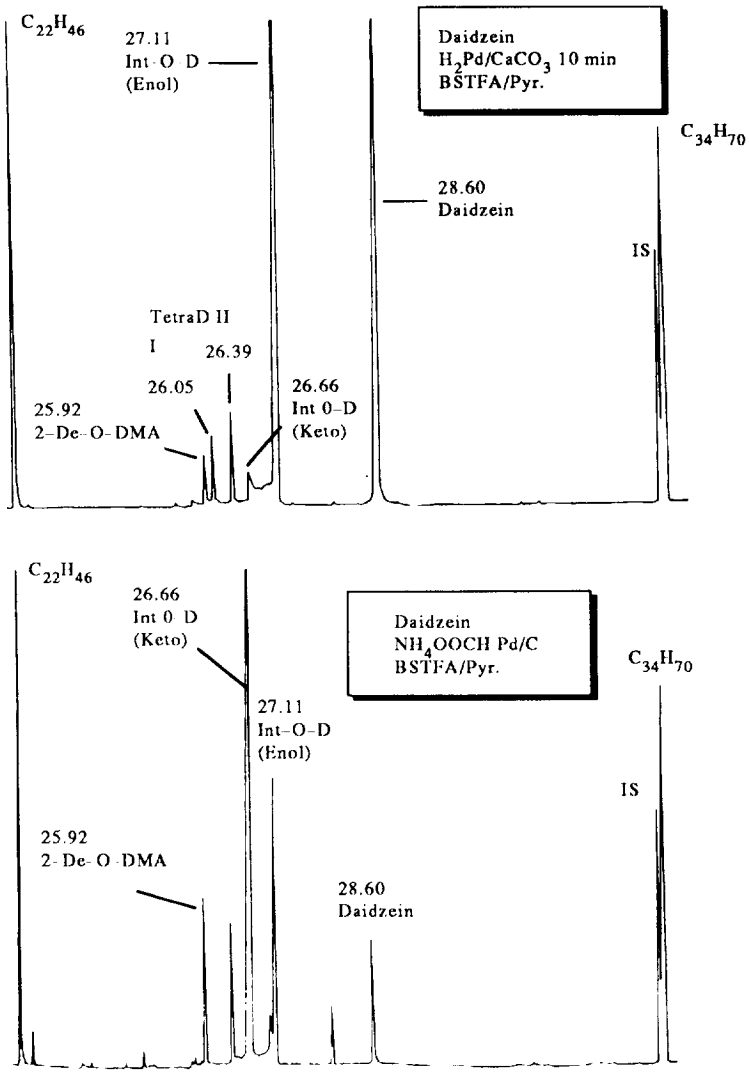


Fig. 1. The GC traces of the TMS ethers of dihydrodaidzein reference sample, obtained by hydrogenation of daidzein using Pd/Ca_2CO_3 and that of a reference sample supplied by Professor H. Adlercreutz and obtained by catalytic hydrogenation with ammonium formate (NH_4OOCH) on Pd/C. Other side products of hydrogenation confirmed by EIMS were those of the 2-dehydro-*O*-demethylangolensin (MU 25.92) and the *cis*/*trans*-isomers of tetrahydrodaidzein (MU 26.05; 26.39).

according to Wähälä *et al.* [14]. In a typical reaction 1.0 mg of daidzein dissolved in 20 ml of methanol and ammonium formate (1.0 mg) in Pd/C (5%, 1.0 mg) was stirred overnight at room temperature. After centrifugation the solution was decanted and evaporated to dryness. Catalytic transfer hydrogenation of daidzein on Pd/C gave the same products as those obtained by hydrogenation on Pd/Ca_2CO_3 but with no evidence of the tetrahydrodaidzein isomers. A finding which was also observed on comparison of the GC and GC–MS data with those obtained from the reference sample of dihydrodaidzein (a product also of catalytic transfer hydrogenation) (Fig. 1).

Keto/enol formation of the dihydro derivatives of daidzein and genistein. Evidence of keto/enol formation of the dihydro derivatives of daidzein and genistein was obtained in this study. Based on GC and GC–MS data, derivatization of extracts obtained by hydrogenation with Pd/Ca_2CO_3 using BSTFA in pyridine or THF, showed a preference for the enol over the keto tautomers. However, when same extracts were treated with an acidified solution of 70% methanol–HCl, prior to derivatization, subsequent derivative formation with BSTFA alone favoured the keto tautomers, while rederivatization with BSTFA in dry pyridine or THF gave a mixture of the two tautomers. Catalytic transfer hydrogenation extracts of dihydrodaidzein using ammonium formate as a catalyst and subsequent derivatization with BSTFA in dry pyridine gave mainly the keto tautomer (cf Fig. 1). The variation in formation of the two tautomers seems therefore to be attributed to the degree of basicity of the medium employed on derivatization. A preference towards the enol tautomer is observed in basic medium and

therefore in aliquots of hydrogenation used in derivatization without prior purification and elimination of excess calcium carbonate.

Structural confirmation of the keto/enol tautomers of the dihydro product of daidzein was achieved in this study based on the fragmentation pattern of the deuterated TMS ethers of dihydrodaidzein obtained with *N,O*-bis(trimethyl-d_9-silyl)acetamide derivative. The results are presented below in respect to fragment and structural elucidations (see section on dihydrodaidzein).

RESULTS AND DISCUSSION

Fractionation of Steroids and Diphenols on Sephadex LH-20

The structural resemblance of isoflavonoids to steroids with similarities in polarity and molecular size makes these two different families of compounds quite inseparable by simple extraction procedures. Direct GC and GC–MS profile analysis of urinary extracts, presented a fortuitous coincidence of retention indices of steroid and diphenol metabolites. To this end, the separation and isolation of urinary diphenols by partition chromatography, facilitated the identification of the new metabolites.

The high affinity of aromatic substances to Sephadex LH-20, was exploited by Setchell *et al.* [8] in a method of separation of lignans from steroids in a combination of straight-phase chromatography and anion exchange chromatography on Sephadex LH-20 and its TEAP derivative. In this investigation, the high reproducibility characterized in partition chromatography, permitted the isolation and separation of diphenols from the bulk of the urinary steroids on Sephadex LH-20 using chloroform–heptane–methanol (10:10:1; v/v) as eluent.

The phenomenon of unusually large retention volumes of aromatic substances in straight-phase chromatography on Sephadex LH-20 is explained by the high aromaticity and interaction of π-electrons with the ether linkages of the gel matrix. In partition chromatography, where two or more polar solvents may be used, the mechanism of separation is more complex since, in addition to the aromaticity, carbonyl and hydroxyl functional groups may influence the retention of compounds more than expected when chromatographed on Sephadex LH-20 in chloroform. The separation of urinary steroids based on functional groups was demonstrated on the above-mentioned partition chromatographic system in an earlier study [7] where elution of the bulk of the steroids was achieved between 2–14 ml of eluent. In this same system it was found that diphenols were eluted between fractions 20–58 ml; with equol (80%) recovered mainly in fraction 22–27 ml, daidzein (88%) in fraction 28–35 ml and genistein (74%) in fraction 25–56 ml. Methylated diphenols were eluted with the steroid fraction as established from reference standards. Formononetin (100%) and 17-dihydroequiline (93%) were recovered in fraction 0–7 ml, whereas biochanin-A (88%) in fraction 8–14 ml.

Consequently the unusually large retention volumes of diphenols observed here in partition chromatography on Sephadex LH-20 due to their high aromaticity and group functionality, allowed the separation of diphenols from the bulk of urinary steroids. Unlike similar methods employed this was achieved on a single lipophilic gel system. The small volume of reagents used makes this method inexpensive and convenient for application to routine analysis.

GC and GC–MS analysis of the fractionated diphenol extracts showed no evidence of interfering urinary steroids and required no further purification. Analysis of the steroid fraction appeared free of any major methylated diphenols, possibly due to the limited contribution from soya ingestion. Control urine samples spiked with equol, daidzein and genistein showed good reproducibility and recoveries after isolation by chromatography on Sephadex LH-20. The mean percentage recovery was 87% (range 82–93%; $n = 11$) as calculated from data obtained by GC and GC–MS analyses. The combined mean recoveries of the control samples and test samples following enzyme hydrolysis, extraction and fractionation by LH-20 chromatography was the subject of a separate publication on the quantitation of the urinary diphenols [9].

Urinary Diphenols

A number of urinary diphenol metabolites have been identified in recent years, both of isoflavonoid [10, 15–20] and lignan in origin [8, 16, 21–23]. Based on the identification of the urinary isoflavonoid metabolites, *viz.* equol, *O*-demethylangolensin and dihydrodaidzein, a pathway of metabolism of daidzein has been proposed [10]. However, no pathway of metabolism of genistein has been defined as yet as this awaits the identification of comparable metabolites.

The results of the urinary isoflavonoids identified here are presented below as products of metabolism of daidzein and genistein. It is well understood that a deuterated metabolic study would need to be conducted to confirm the origin of the isoflavonoid metabolites as current information is based on the structural similarities of the parent isoflavone ingested. The urinary diphenol profiles of volunteers challenged with soya flour were presented earlier [9]. Below, we present the GC and GC–MS data of all urinary diphenols identified in this study including those unknowns still awaiting characterization and identification.

Urinary metabolites of daidzein

Dihydrodaidzein and dehydroequol have been suggested as the intermediary products of metabolism of daidzein, referred to as Intermediate-O and Intermediate-E, respectively [10]. As products of intestinal

microbial transformations, in the proposed pathways of daidzein metabolism put forward by the same authors, dihydrodaidzein and dehydroequol are further metabolized to give *O-Dma* and equol, respectively. To the best of our knowledge no GC or GC–MS data for dihydrodaidzein and dehydroequol have been cited in the literature in spite of the fact that these have been tentatively identified in human urine.

Equol [MU 25.45]

The identification of equol (4′,7-dihydroxyisoflavan) [15] and that of an isomer (3′,7-dihydroxyisoflavan) [19] has been reported in human urine. The latter was also found in cow's milk which was suggested as a possible source of occurrence in human urine [19]. The mass spectra of equol and its isomer have been cited in literature demonstrating the close similarities and the distinct difference in the ratio of *m/z* 267 and *m/z* 206 [19]. It was also shown then that separation of the two isomers was accomplished on a 12.5 m fused silica SE-30 capillary column with the 3′,7-isomer eluting before equol. In this study the GC and GC–MS data of the TMS ether derivative of a urinary diphenol observed in the urine of some of the subjects were identical with those obtained from the reference sample of equol and in agreement with the published data. However, repeated attempts to identify the 3′,7-isomer of equol in the urine of volunteers failed to provide any evidence of its occurrence in human urine.

Dehydroequol

The tentative identification of dehydroequol has been proposed in human urine with the structural configuration of 7,4′-dihydroxy-isoflav-2-ene [10]. The existence of an isomer with the more stable conjugated structure of 7,4′-dihydroxy-isoflav-3-ene must not be excluded. In the absence of a reference sample a search

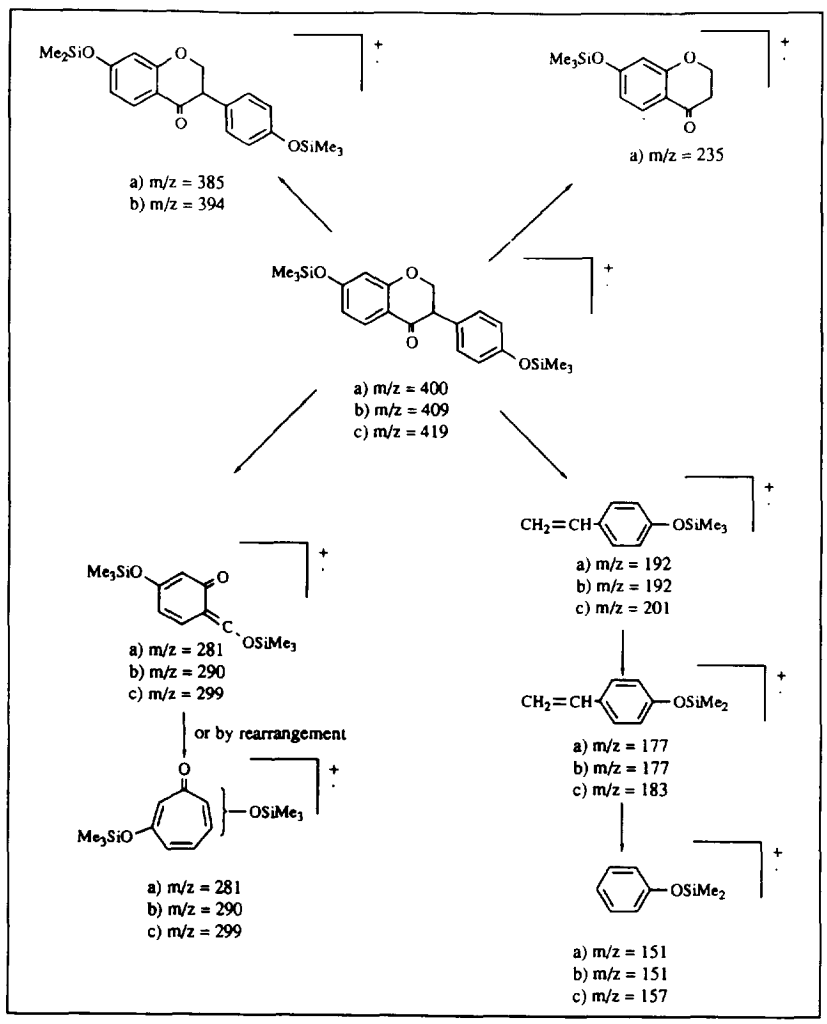


Fig. 2. Structural elucidation of fragments presented in the EIMS spectra of the TMS ethers of (a) the dihydrodaidzein keto tautomer [4′-TMS,7-TMS-isoflavan-4-one (MU 26.66)] and its (b) mono-[4′-TMS,7-d_9-TMS-isoflavan-4-one (MU 26.58)] and (c) di-[4′-d_9-TMS,7-d_9-TMS-isoflavan-4-one (MU 26.50)]; d_9-TMS substituted products, respectively.

for the identification of dehydroequol was conducted based on the parent fragment ion *m/z* 384 and fragments *m/z* 267, *m/z* 207 and *m/z* 192. These are common fragments observed in the mass spectra of isoflavans [15, 19]. However, this investigation failed to find evidence of the existence of dehydroequol in the urine of any of the volunteers.

Dihydrodaidzein [MU 26.66 (keto) and MU 27.11 (enol)]

On derivatization of urinary extracts with BSTFA in dry pyridine two urinary metabolites were observed at MU 26.66 and MU 27.11. These were identified as the keto (MU 26.66; M^+ 400) and enol (MU 27.11; M^+ 472) tautomers of dihydrodaidzein based on the matching of the GC and GC–MS data with authentic samples of dihydrodaidzein obtained by chemical synthesis and from the reference standard provided. Final structural confirmation of these two tautomers was achieved in a study of the fragmentation pattern of the trimethyl-d_9-silyl ethers of dihydrodaidzein by mass spectrometry. The results of the fragment and structural elucidations are presented below.

The d_9-TMS order of substitution of the keto and enol TMS ethers of dehydrodaidzein. On rederivatization of the 4′,7-bis-trimethylsilyl ethers (keto form; MU 26.66) and 4′,4,7-tris-trimethylsilyl ethers (enol form; MU 27.11) of dihydrodaidzein with d_9-BSA/THF (4:1; v/v), GC–EIMS identified the mono-, di- and tri-substituted d_9-TMS ethers of dihydrodaidzein tautomers which were readily separated by capillary GC: namely: 4′-TMS,7-d_9-TMS-isoflavan-4-one (mono-keto; MU 26.58); 4′-d_9-TMS,7-d_9-TMS-isoflavan-4-one (di-keto; MU 26.50); 4′-TMS,4-d_9-TMS,7-TMS-isoflav-3-ene (mono-enol; MU 27.06); 4′-TMS,4-d_9-TMS,7d_9-TMS-isoflav-3-ene (di-enol; MU 27.00) and 4′-d_9-TMS,4-d_9-TMS,7-d_9-TMS-isoflav-3-ene (tri-enol; MU 26.94). Table 1 lists the GC

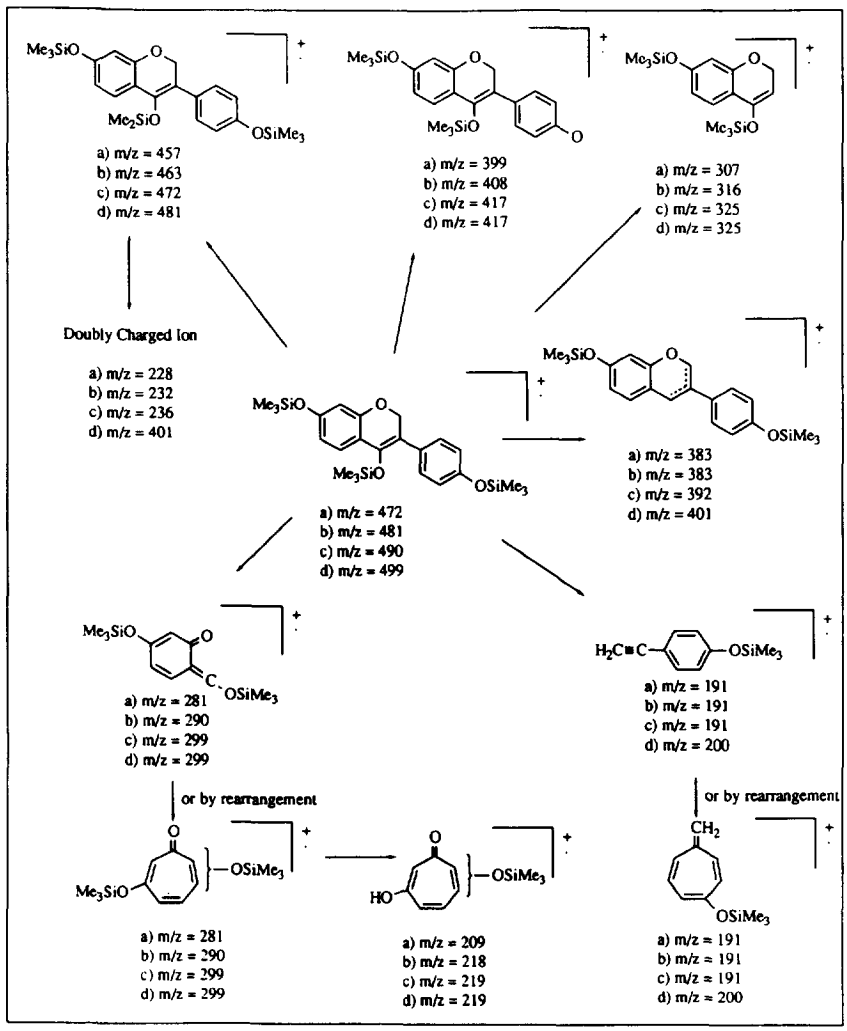


Fig. 3. Structural elucidation of fragments presented in the EIMS spectra of the persilylated TMS ethers of (a) dihydrodaidzein enol tautomer [4′-TMS,4-TMS,7-TMS-isoflav-3-ene (MU 27.11)] and its (b) mono-[4′-TMS,4-d_9-TMS,7-TMS-isoflav-3-ene (MU 27.06)]; (c) di-[4′-TMS,4-d_9-TMS,7d_9-TMS-isoflav-3-ene (MU 27.00)] and (d) tri-[4′-d_9-TMS,4-d_9-TMS,7-d_9-TMS-isoflav-3-ene (MU 26.94)]; d_9-TMS substituted products, respectively.

and GC–MS data of all the d_9-TMS substituted products of dihydrodaidzein. The order of substitution was achieved by fragment elucidation, summarized in Figs 2 and 3, respectively. In brief, fragments *m/z* 281 and *m/z* 192; and *m/z* 383, *m/z* 281 and *m/z* 191 in the spectra of the trimethylsilyl ethers of the keto and enol tautomers, provided evidence to show that the enolic ether at C_4 was the first to be substituted followed by the phenolic ethers at C_7 and C_4' in ring-A and ring-B respectively.

Fragment and structural elucidations. The most abundant fragments in the spectra of isoflavans are those which have come about as a result of retro-Diels-Alder (rDA) reaction with or without hydrogen rearrangement. Other common fragments are those associated with the elimination of methyl or TMS groups. The mechanism of formation of these fragments has been presented in the deuterated and high resolution mass spectra studies of the trimethylsilyl derivatives of equol [15, 19]. Fragment *m/z* 267 has been proposed to be a rearrangement ion resulting by cleavage of the ether linkage ($O–C_2$) and the carbon bridge ($C_3–C_4$) and transfer of a silyl group to the aromatic oxygen atom.

In this investigation the deuterated mass spectral studies of the dihydrodaidzein tautomers showed a similar fragmentation pattern, with the fragments of rDA reaction (*m/z* 191 or *m/z* 192 and *m/z* 281) being the most abundant. The order of substitution of the d_9-TMS ethers in the molecule of these two tautomers provided a means of pin-pointing the elimination or transfer of specific functional groups.

In the proposed structure of the keto tautomer of dihydrodaidzein the deuterated studies established that fragment *m/z* 281 is a rearrangement ion which contains two TMS ether groups formed by cleavage of the ether linkage ($O–C_2$) and the carbon bridge ($C_3–C_4$) with transfer of the C_4' silyl group to the aromatic oxygen at C_4. The analogy between *m/z* 267 proposed earlier and *m/z* 281 in the keto tautomer of dihydrodaidzein, is that they are both A-ring fragments with a C_4' silyl ether transfer. However, in the enol tautomer where the carbonyl oxygen at C_4 is replaced with a silyl ether, formation of fragment *m/z* 281 is not by rearrangement as there is no evidence of such a transfer.

In the proposed enolic structure of dihydrodaidzein, fragment *m/z* 383 showed no increase in 9 Da in its mono-substituted product suggesting that the loss of *O*-d_9-TMS ether from the parent ion [M^+481] to give *m/z* 383 [M-89]$^+$ is due to the loss of the enolic ether at C_4. Tandem mass spectrometry confirmed that fragment *m/z* 355 is the daughter ion of *m/z* 383, the probable loss of CO as a result of the opening of the pyran ring. Further evidence of the loss of the enolic ether was obtained when subsequent substitutions of d_9-TMS at C_7 and C_4' in the di- and tri-substituted products, *m/z* 383 increased by 9 and 18 Da to give *m/z* 392 and *m/z* 401 with daughter ions at *m/z* 364 and 373, respectively. The probable loss of –CO from *m/z* 383 was also proposed in the mass spectrum of daidzein [24]. In this latter case the parent ion of *m/z* 383 is *m/z* 398, the loss of CH_3 from *m/z* 398 the M^+ ion. Based on the elucidation and origin of fragments *m/z* 383 and

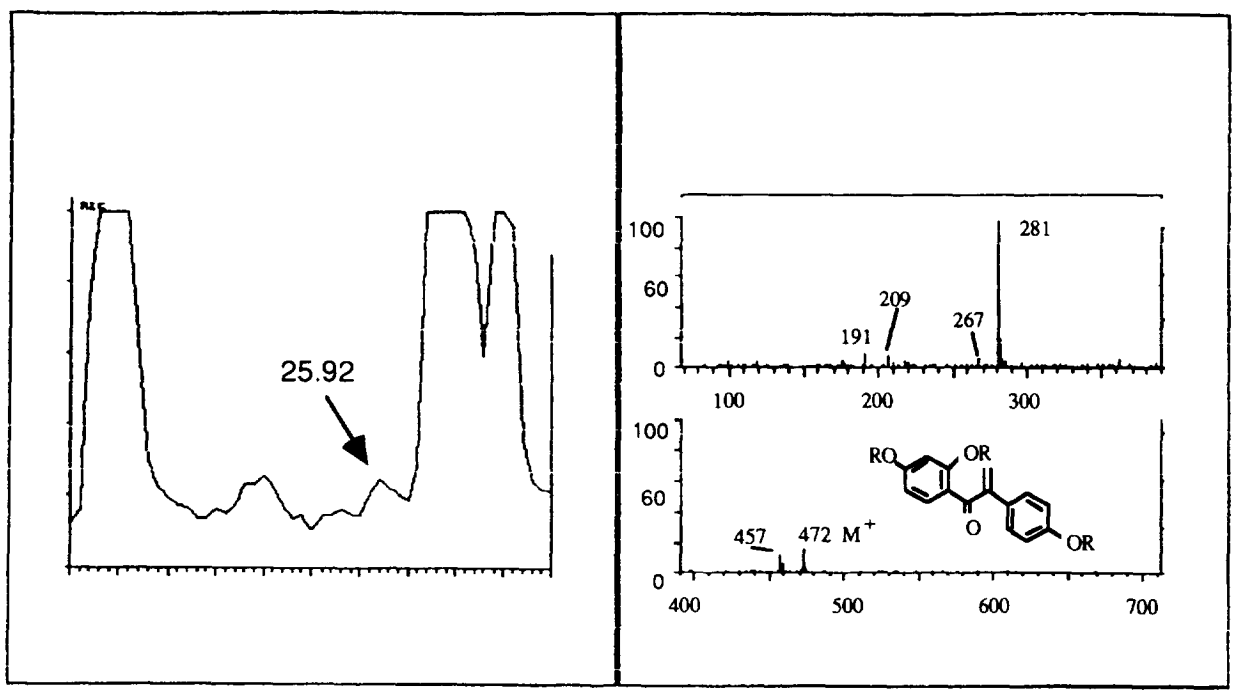


Fig. 4. The total ion chromatogram (TIC) and EIMS data of the TMS ether of the 2-dehydro-*O*-demethylangolensin (2-*de*-*O*-*Dma*; MU 25.92) as obtained from the urine of human volunteers and proposed structure.

m/z 355, the deuterated studies confirmed the proposed enol structure and order of substitution with the enolic ether to be the first to be substituted followed by the phenolic ethers at C_7 and C_4' respectively. The stability of the phenolic OTMS ethers over the enolic or neutral is demonstrated in that diphenols favour the loss of $(CH_3)_2Si{=}CH_2$ with or without hydrogen substitution as compared with neutral steroids which favour the loss of OTMS *m/z* $[M\text{-}90]^+$ from the parent ion.

Other fragments of interest and which the deuterated studies have shed some light into the structural elucidation of the keto and enol tautomers were those of *m/z* 399 and *m/z* 209 in the enol tautomer, associated with the loss of TMS groups; *m/z* 177 (keto) and *m/z* 457 (enol), associated with the loss of CH_3 group; whereas *m/z* 235 (keto) and *m/z* 307 (enol), associated with the loss of ring-B after cleavage at C_3. The deuterated studies confirmed the loss of TMS in *m/z* 399 and *m/z* 209 is from the C_4' and C-, whereas the loss of CH_3 in *m/z* 177 and *m/z* 457 is from the silyl ethers at C_4' and C_4, respectively.

O-Dma [MU 24.85; M$^+$ 474] and 2-de-O-Dma [MU 25.92; M$^+$ 472]

The EI mass spectral data and fragment interpretation of the TMS ether derivative of *O-Dma* have already been cited in the literature [17]. In this investigation a metabolite (MU 24.85), observed in the urine of all volunteers, was identified as the *O-Dma* based on the total agreement of its silyl ether EI mass spectra reported in literature with the characteristic very low intensity M^- ion at *m/z* 474. CIMS confirmed the $[M + 1]^+$ molecular ion at *m/z* 475, which was also the base peak.

A second urinary metabolite (MU 25.92), found in the urine of volunteers (GK, SK, RM and JJ), with a fragmentation pattern characteristic of an α-phenylketone, was tentatively identified on the basis of its mass spectral data as the C_2-dehydro derivative of *O-Dma*. Unlike its *O-Dma* analogue, this gave an intense M^+ ion at *m/z* 472 (18%) in EIMS and an $[M + 1]^+$ ion at *m/z* 473 in CIMS, which was also its base peak. The splitting of the molecule between the carbonyl and methyl groups would result in the loss of the benzoyl fragment containing two OTMS ether groups to give *m/z* 281. However, in the proposed structure of *2-de-O-Dma* the second fragment representing the phenyl group and containing one OTMS ether and the side chain of carbon units C_2–C_3 gave *m/z* 191 as compared with *m/z* 193 for *O-Dma*. The loss of two protons is most likely to come from the side chain to give the proposed fragment $CH_2{=}CH\text{–}Ph\text{–}OTMS$ with retention of charge at *m/z* 191.

An intermediary product similar to that proposed for *2-de-O-Dma* has been suggested in the catalytic hydrogenation of isoflavanones in basic medium [25]. Evidence of such an intermediate was also obtained here as a product of hydrogenation from daidzein with Pd/Ca_2CO_3 or by catalytic transfer hydrogenation with ammonium formate. The GC and GC–MS data of this product were identical to those obtained from the urinary metabolite (cf Figs 4 and 5). The consideration

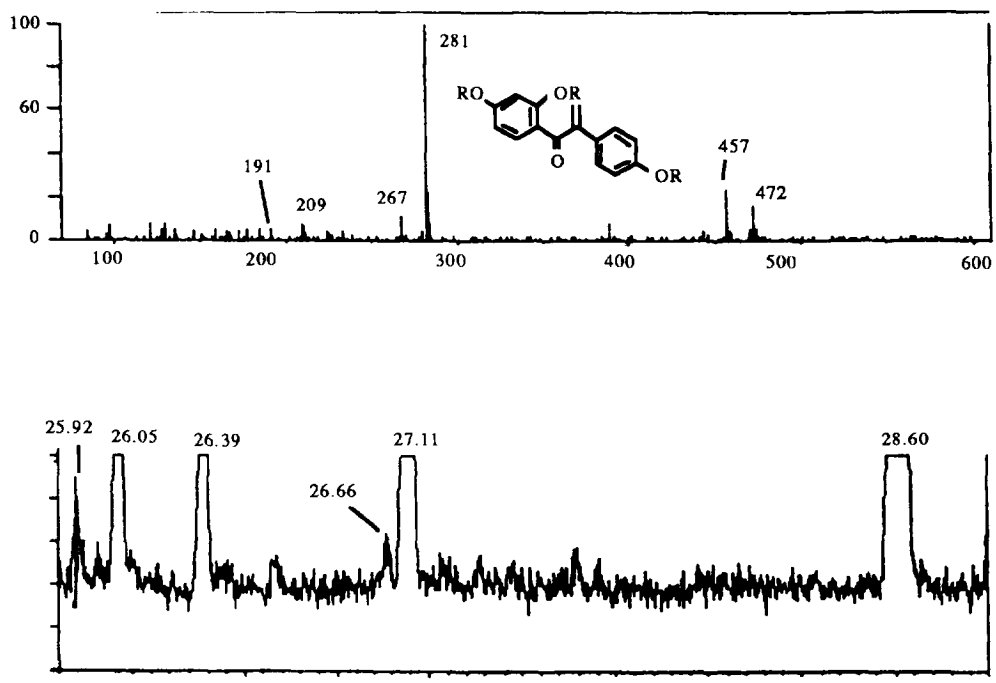


Fig. 5. The TIC and EIMS data of the TMS ether of the 2-dehydro-*O*-demethylangolensin (*2-de-O-Dma*; MU 25.92) obtained as a product of hydrogenation from daidzein. Other products of hydrogenation were the *cis/trans*-isomers of tetrahydrodaidzein (MU 26.05 & MU 26.39) and the keto/enol tautomers of dihydrodaidzein (MU 26.66 and MU 27.11) with unconverted daidzein at MU 28.60.

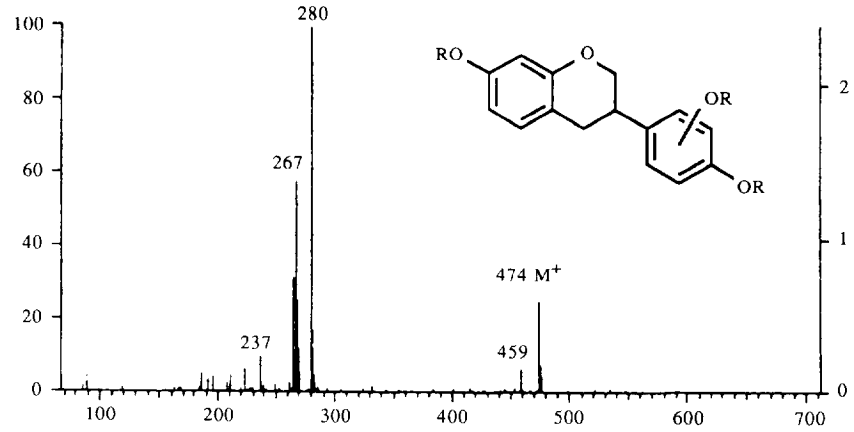


Fig. 6. The EIMS spectrum of the persilylated TMS ether of the urinary structural isomer of tetrahydrodaidzein (MU 26.77) and proposed structure.

that *2-de-O-Dma* is a product of basicity and therefore its existence in the urine a direct result of the derivatization process cannot be excluded. However, repeated derivatization of dihydrodaidzein (enol or keto forms) with BSTFA in pyridine failed to give any evidence of this compound. As such, *2-de-O-Dma*, observed in the urine of volunteers studied here, is considered a probable product of daidzein catabolism rather than a product of basicity or thermal rearrangement.

Tetrahydrodaidzein isomers

The GC and EI–MS data of the *cis/trans*-isomers of tetrahydrodaidzein (MU 26.05 and MU 26.39; M$^{\cdot}$ 474), obtained here as products of hydrogenation and sodium borohydride reduction were closely matched with those obtained from a minor urinary metabolite (MU 26.38), observed in the profiles of two of the subjects (GK and RM) with a ratio of *O-Dma*/equol of 10:1 and 2:1, respectively. Although its EIMS spectra were indistinguishable from those of the two isomers of tetrahydrodaidzein, the MU value agreed only with that of the second isomer (MU 26.39). Based on these data, the urinary metabolite (MU 26.38) was identified as the probable *trans*-isomer of tetrahydrodaidzein. The low levels of the tetrahydrodaidzein urinary metabolite and the fortuitous coincidence of the retention index of the first isomer of tetrahydrodaidzein (MU 26.05) with that of a major urinary isoflavonoid metabolite namely, *6-OH-O-Dma* (MU 26.05) did not permit the identification of the urinary *cis*-isomer of tetrahydrodaidzein.

From the EI mass spectra, the two ions of structural interest for the characterization of the tetrahydrodaidzein isomers were those at *m/z* 355 (8%) and *m/z* 383 (2%). These are fragments also observed in the TMS ethers of the enol tautomer of dihydrodaidzein and which deuterated experiments established that the origin of formation of *m/z* 383 is the loss of the enolic OTMS groups from the parent ion, whereas *m/z* 355 is the daughter ion of *m/z* 383. It is proposed here that *m/z* 383 $[M-91]^{+}$ and *m/z* 355, in the mass spectra of the tetrahydrodaidzein isomers, is also as a direct result of the elimination of the non-phenolic ether at C_4. However, whilst tetrahydrodaidzein favoured the formation of *m/z* 355 over *m/z* 383 (ratio 1/4), dihydrodaidzein (enol) favoured the formation of *m/z* 383 over *m/z* 355 (ratio 7/1).

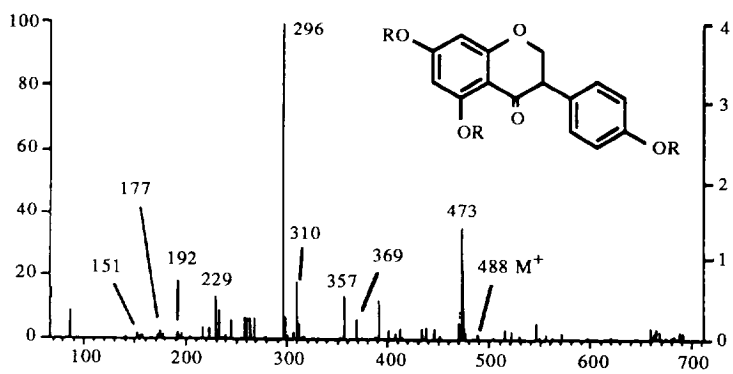


Fig. 7. The EIMS spectra of the trimethylsilyl ether of the urinary metabolite MU 27.45, identified as the dihydrogenistein.

Structural isomers of tetrahydrodaidzein (MU 26.77 and MU 27.15)

Two other metabolites observed in the urine of volunteer (RA) at MU 26.77 and MU 27.15, EIMS established these to be isomeric with a fragmentation pattern which resembled that of the tetrahydrodaidzein isomers (Fig. 6). However, the absence of *m/z* 355 and *m/z* 383 from the EI mass spectra of these latest isomers (fragments which have been demonstrated above to be indicative of non-phenolic ethers), first suggested that the extra –OTMS must be phenolic. As probable metabolites of genistein, the addition of a hydroxy group in ring-A should result in fragments *m/z* 369 and *m/z* 355, the equivalent fragments to *m/z* 281 and *m/z* 267 observed in the mass spectra of isoflavanones or isoflavans, respectively. However, the presence of *m/z* 267 and absence of *m/z* 369 and *m/z* 355 confirmed the exclusion of an extra TMS ether is ring-A. The second fragment of an rDA cleavage at *m/z* 280 on the other hand is a fragment equivalent to *m/z* 191 with the addition of an extra OTMS in ring-B. Based on these findings these two structural isomers of tetrahydrodaidzein were assigned a structure containing one hydroxy group in ring A and two in ring-B. Although these two isomers may be considered possible hydroxylation products of daidzein, the natural occurrence of 2′,3′,7-dihydroxy-4′-methoxy-isoflav-3-ene

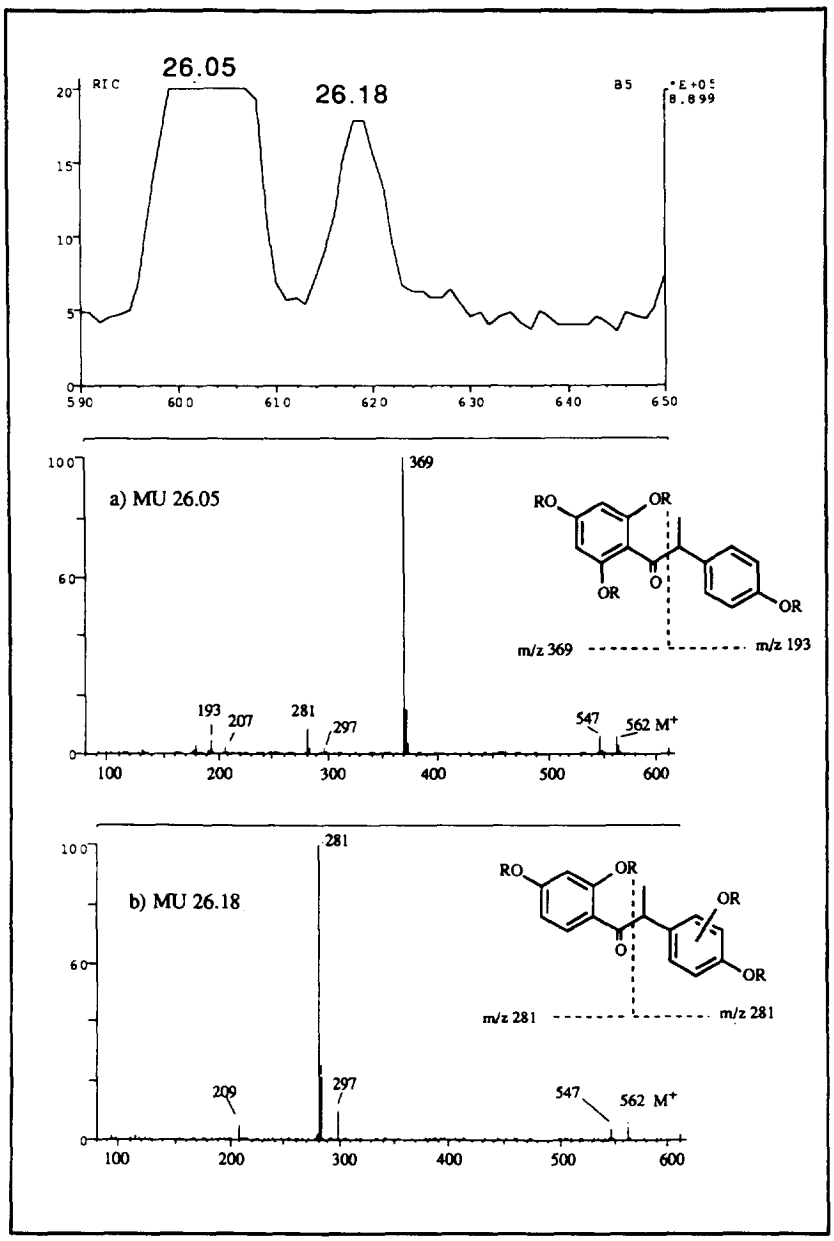


Fig. 8. The TIC and EIMS data of the trimethylsilyl ethers of the urinary metabolites MU 26.05 and MU 26.18 characterized as the 6-hydroxy-*O*-demethylangolensin and one of its structural isomers with proposed structures.

(epiol) and 7,2′-dihydroxy-3′,4′,-dimcthoxyisoflavan (isomucronulatol) isolated from Leguminosae have been reported previously [26, 27] which may lend some support of the existence of 2′,3′-hydroxylated derivatives of isoflavonoids in soya flour.

Urinary Metabolites of Genistein

Dihydrogenistein [MU 27.45 (keto)]

A minor urinary metabolite (MU 27.45), observed in the urine of volunteer (JJ), was first characterized here by mass spectrometry as the dihydro reduced derivative of genistein. EIMS gave an M^+ ion at m/z 488 and a base peak at m/z 296 (Fig. 7). Other major fragments were those associated with rDA rearrangement and cleavage of the O–C and C_3–C_4 bonds and their associated fragments to give mainly m/z 296 $[M-192]^+$ and m/z 369. Fragments associated with the B-ring and which are comparable to those observed in the keto tautomer of dihydrodaidzein are those of m/z 192, m/z 177 and m/z 151, whereas m/z 369 is the equivalent fragment of m/z 281 with an additional OTMS in ring-A as expected of its genistein analogue (Fig. 2). The matching of its GC and GC–MS data with those of the keto tautomer of dihydrogenistein, obtained here as a product of hydrogenation from genistein, identified this as the trimethylsilyl ether of 5,7,4′-trihydroxy-isoflavo-3-one. Its structural relationship to one of the two isoflavones ingested, suggests this to be the probable catabolic metabolite of genistein.

6-OH-O-Dma and structural isomers

A major metabolite (MU 26.05) observed in the urine of all volunteers with a molecular ion at m/z 562, gave a fragmentation pattern typical of an α-phenyl-ketone. Two other structural isomers of this were also observed at MU 25.05 and MU 26.18. Figure 8 gives the EI mass spectra and proposed structures for compounds MU 26.05 and MU 26.18. The mass spectral data of compound MU 25.05 were almost identical to those of compound at MU 26.05 with a very low intensity M^+ ion at m/z 562 ($<1\%$) and a base peak at m/z 369 with other major fragments at m/z 547 (5%), m/z 400 (12%), m/z 385 (8%), m/z 354 (3%), m/z 281 (38%), m/z 209 (18%), m/z 193 (6%) and m/z 177 (2%). Based on the mass spectral data these were characterized as probable analogues of *O-Dma*.

In the structures proposed in Fig. 8, the splitting of the molecule would result in the elimination of the benzoyl fragment containing three OTMS groups in the first structural isomer (MU 26.05) to give m/z 369 as compared with two –OTMS in the second structural isomer (MU 26.18) to give m/z 281 as the base peaks, as is also the case with *O-Dma*. The second fragment with the retention of charge is that at m/z 193 containing one silyl ether (for MU 26.05) as compared with the equivalent fragment at m/z 281 containing two silyl ethers in the phenolic ring (for MU 26.18).

The base peaks of m/z 369 and m/z 281 in the mass spectra of MU 26.05 and MU 26.18, respectively, and the presence of m/z 193 in the first but not in the second structural isomer is evidently in support of the proposed structures given. Based on the GC and GC–MS data, we propose that MU 26.05 is the 6-hydroxy analogue of *O-Dma*. The structural relationship to genistein lends support to the proposed structure as the probable catabolic product of metabolism of genistein ingested. Evidently MU 25.05 and MU 26.05 are isomeric compounds based on the EIMS data, however the exceedingly low retention index of the former poses questions as to its structural identity. Positive identification of all three proposed analogues of *O-Dma* awaits the synthesis of reference samples for the matching of the GC and GC–MS data.

Other diphenols

In this investigation the only lignan identified in the urine of all volunteers was that of enterolactone (MU 27.75, M^+ 442), which GC and GC–MS data were in total agreement with those cited in the literature [23, 28]. The monitoring of the intense fragment m/z 180, a rearrangement ion characteristic of lignans with a phenolic hydroxy group at the *meta* position, identified a lignan-like compound with an exceedingly low retention index (MU 25.74). This was found in the urine of one of the volunteers (RA; day 0 and day 3) and was the fourth major metabolite after equol, enterolactone and *6′-OH-O-Dma* respectively at day 3. The urinary profiles of this volunteer at days 0 and 3 of ingestion with soya have already been presented [9] and referred to as subject 12. From these profiles it is evident that the levels of the lignan-like compound, eluted between equol and *6′-OH-O-Dma*, increased substantially after ingestion of soya flour while the levels of enterolactone remained unchanged. The EIMS and CIMS mass spectra gave an M^+ ion at m/z 532 (3%) and an $[M+1]^+$ ion at m/z 533 (4%), respectively. Other major fragments of EIMS were those at m/z 180 (100%); m/z 488 $[M-44]^+$ (3%); m/z 398 $[M-90-44]^+$ (2%); m/z 292 (1%); m/z 219 (7%); m/z 205 (6%); m/z 182 (6%); m/z 181 (20%); m/z 165 (4%) and of CIMS at m/z 399 $[M-90-44]^+$ (100%); m/z 517 $[M-15]^+$ (7%); m/z 489 $[M-44]^+$ (15%); m/z 473 $[M-44-15]^+$ (87%); m/z 427 $[M-90-15]^+$ (20%); m/z 233 (6%); m/z 219 (31%); m/z 180 (28%). Fragment m/z $[M-44]^+$, and its associated fragments, is indicative of loss of CO_2 from the lactone ring, whereas m/z $[M-90]^+$ and its associated fragments suggests the presence of a non-phenolic OTMS ether. The absence of m/z 103 precluded the presence of a primary OTMS ether which may arise by the opening of the lactone ring and rearrangement with a phenolic hydroxy functional group. The M^+ ion at m/z 532, although indicative of a molecular structure with an extra hydroxy TMS ether, as compared with that of enterolactone (m/z 442), its exceedingly low methylene unit value

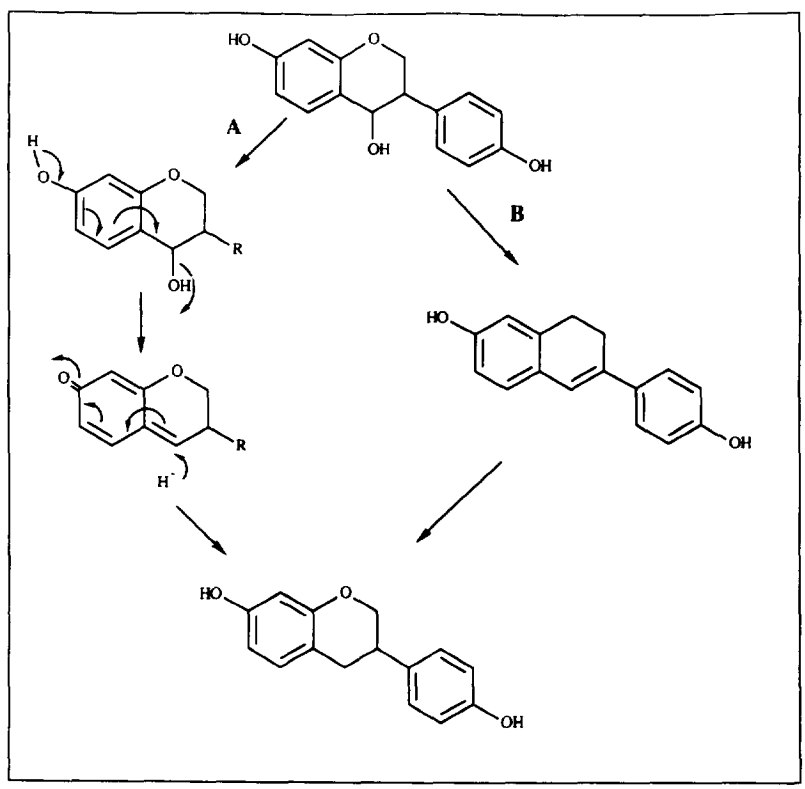
poses a question as to its structural identity since enterolactone and all other lignans identified so far have a much higher retention index value.

Finally, glycitein (MU 29.15; M^+ 486), was identified in the urine of all volunteers. This was achieved by matching of the GC and GC–MS data of its trimethylsilyl ether with those of a reference standard obtained from soya hypocotyl. Structural confirmation of its reference standard was based on comparison of the NMR and mass spectral data of its diacetate derivative with those reported previously [29]. The melting point of the pure compound and that of its diacetate derivative have been presented by us previously together with the GC and GC–MS data of the diacetate and TMS ether derivatives [9]. To the best of our knowledge the metabolism of glycitein in humans has not been presented before.

Proposed metabolism of isoflavonoids

From the above it can be surmised that following the soya challenge, urinary GC and GC–MS profile analysis identified: equol, dihydrodaidzein, dihydrogenistein, *O-Dma*, 2-*de*-*O*-*Dma*, 6′-*OH*-*O*-*Dma*, daidzein, genistein, glycitein and the lignan enterolactone. Of these, 6′-*OH*-*O*-*Dma*, 2-*de*-*O*-*Dma*, dihydrogenistein and tetrahydrodaidzein have not been reported before. The latter three were only minor metabolites, identification of which was made possible through the simplification of urinary extracts and isolation of diphenols by partition chromatography on Sephadex LH-20. Our earlier report on the quantitation of these metabolites [9], has lent support to the proposed conversion of daidzein to *O-Dma* and equol [10] and pointed out an apparent individual variability in the mode of metabolism with preference to equol excretion in some individuals and *O-Dma* in others, irrespective of gender.

Current knowledge of isoflavone metabolism stems largely from the C_{14}-labelled studies in sheep which confirmed the conversion of daidzein, originating from food, to equol (~70%) and *O-Dma* (5–20%) respectively [30, 31]. The identification of equol [17], *O-Dma* [18], dihydrodaidzein and dehydroequol in human urine first lent support to the alternative pathways of catabolism of daidzein to equol and *O-Dma* via dehydroequol and dihydrodaidzein, respectively [10]. The proposed conversion of daidzein to equol via dehydroequol receives some support in that isoflavenes are highly reactive intermediates occurring naturally in plants and which may therefore play a central role in the biosynthesis of isoflavans and other types of isoflavonoids as it was first suggested in the case of Leguminosae [27]. The successful synthesis of pterocarpin from an isoflavene intermediate by acid hydrolysis lends further support to this hypothesis [32]. However, in humans, the definitive identification of dehydroequol, the proposed isoflavene precursor of

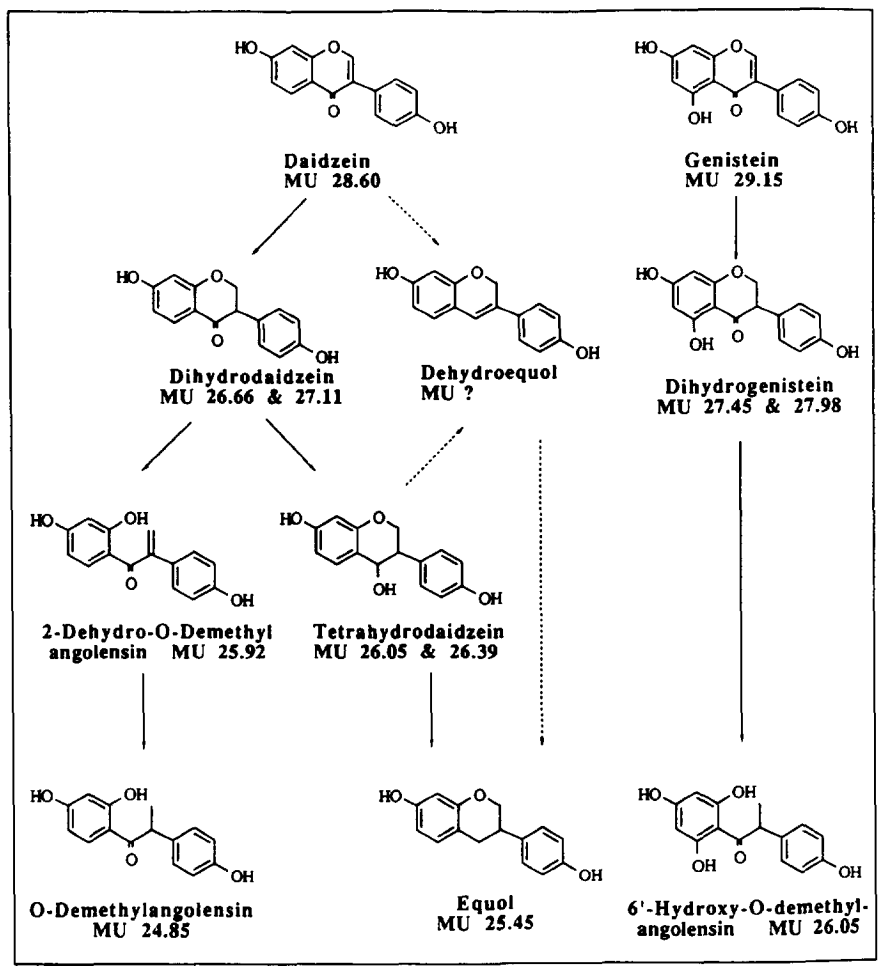


Scheme 2. Proposed pathways of formation of equol from tetrahydrodaidzein by (A) rearrangement or (B) by dehydration and subsequent reduction.

the isoflavan equol, still awaits confirmation and publication of its GC and GC–MS data.

In this investigation no dehydroequol was found in any of the volunteers and in particular those predisposed to equol excretion. Instead, dihydrodaidzein was found in the urine of most individuals including those predisposed towards the formation of equol. This latest finding led us to propose that dihydrodaidzein and not dehydroequol, as first suggested, is the probable precursor of equol. The identification of tetrahydrodaidzein, and 2-*de-O-Dma* in the urine of some volunteers may have also provided some evidence to suggest that these latest metabolites are the most probable intermediates of equol and *O-Dma*, respectively. Conversion of daidzein to equol via dihydrodaidzein and tetrahydrodaidzein may be achieved by either rearrangement or by dehydration and subsequent reduction as depicted in Scheme 2. It is interesting to note that reduction of dihydrodaidzein with sodium borohydride resulted in the synthesis of tetrahydrodaidzein isomers with no evidence of other intermediary products or products of degradation due to instability. Therefore, the relatively low levels of tetrahydrodaidzein observed in the urine of volunteers and its low incidence (observed in only two subjects) may be due to the rate of formation of equol from tetrahydrodaidzein and not due to methodological losses or instability. The fortuitous coincidence of retention indices of one of the isomers of tetrahydrodaidzein with that of *6′-OH-O-Dma*, a major urinary metabolite found in the urine of all volunteers, may indeed have hindered the identification of tetrahydrodaidzein.

Unlike daidzein, genistein metabolism in humans has not been demonstrated before. In sheep this compound is known to be metabolized to give mainly *p*-ethylphenol. In this investigation the identification of dihydrogenistein and characterization of *6′-OH-O-Dma* suggests for the first time that these are the probable products of catabolism of genistein. The close analogy of these metabolites to those proposed for daidzein in the pathway leading to *O-Dma* provides evidence to show that genistein metabolism parallels that of daidzein. The remaining metabolites of genistein in the



Scheme 3. Proposed metabolic pathways of catabolism of daidzein and genistein based on the urinary isoflavonoid metabolites found so far in human urine. Pathway through dehydroequol remains to be substantiated.

pathway leading to tetrahydrogenistein and its equol analogue, still awaits identification in human urine.

Based on the above findings and as a result of the identification of new isoflavonoid metabolites the following pathways are proposed to explain the metabolic fate of daidzein and genistein in humans as depicted in Scheme 3. The individual variability in the mode of metabolism of daidzein for preference in the pathway leading to equol or *O-Dma* excretion, as demonstrated in our studies, raises important issues in regard to the endogenous hormonal and prophylactic effects these may have upon the human health.

It has been demonstrated that of all the estrogen-like substances derived from plants and found in human urine (lignans or isoflavones), isoflavonoids have the strongest estrogenic effect. Genistein, daidzein and equol have relatively strong affinities for estrogen receptors while *O-Dma* and glycitein have much weaker affinities and appear to be non-estrogenic [32–36]. The affinity of equol, and to a much lesser extent of other isoflavonoids, for estrogen receptors failing to stimulate protein synthesis to the same degree as normal estrogens [37], characterizes these compounds as anti-estrogens. Consequently, isoflavonoids found in high concentrations in legumes have been pronounced natural food agents which may be capable of eliciting anti-estrogenic effects comparable to tamoxifen used in the palliative treatment of breast cancer [2]. The finding that the urinary levels of isoflavonoids in individuals consuming soya is greatly in excess of the classical estrogens [9], thus compensating for their much weaker estrogenicity relative to estradiol, points to a potentially significant *in vivo* effect these may have upon the endogenous estrogen metabolism. However, the capacity to metabolize plant estrogens is eclipsed by an apparent individual variability in the mode of metabolism resulting in equol or *O-Dma* producers [9]. As such the individual variability in the preferential pathways proposed here may also point to an individual vulnerability to Western-type diseases in those individuals not capable of producing the most active isoflavonoids. This variability may prove a useful indicator of the susceptibility to hormone-dependent diseases should either of the two pathways be established as the most beneficial to health. It is interesting to note here that the excretion of *O-Dma* in breast cancer patients is particularly low in comparison with normal populations of different dietary habits [38a]. The reason for this individual variation or apparent inability to produce equol or *O-Dma* is not yet known. It is fair to suggest therefore, that prophylactic treatment of hormone-dependent diseases with isoflavones may be of little consequence for "non-responders" to equol and/or *O-Dma* production. Instead ingestion of the active ingredient(s) may provide an alternative isoflavonic supplement to combat excessive endogenous estrogens in humans, should intestinal microflora or enzymatic conditions more conducive to the production of the active isoflavonoids fail to be induced. However, the metabolic fate of pure isoflavonoid metabolites ingested and the effect these have upon the endogenous steroids in humans has not been demonstrated.

Phytoestrogens exert their effect upon the human endogenous hormones by mimicking the action of estrogens. In humans, phytoestrogens have been generally accepted to have a beneficial rather than a deleterious effect. The low incidence of Western-type diseases such as cancers of the breast, colon and prostate and coronary heart diseases in Oriental populations and the observation that unlike their Occidental counterparts whose diet is rich in fat and protein and low in carbohydrates, theirs is a traditionally low-fat vegetarian diet rich in unrefined carbohydrates and soy products, first gave support to the concept of phytoestrogens as cancer protective agents [1–3, 10, 13, 38b, 39]. Animal and tissue culture experiments seem to support these findings where it has been demonstrated that there is a dose-related response for these substances upon the inhibition of growth cells in some cancers [40–43]. Paradoxically, man-made chemicals with similar estrogenic actions in the environment have been suggested as the possible cause for the higher incidence of breast cancer and endometriosis in W. Europe and the U.S.A. [44]. The epidemiological studies on the effects of phytoestrogens upon the uterus and in endometriosis in adult humans still await investigation, even though the ill-effects of isoflavonoids upon the reproductive system in animals have been well demonstrated [45–49]. Similarly the effect upon the health of human newborn infants exposed to isoflavonoids through soya milk or breast feeding and cow's milk has received little attention and warrants an investigation before the overall beneficial effects of these substances is established. The variability in humans to metabolize isoflavonoid phytoestrogens poses further questions as to the role these may have upon different individuals as demonstrated by the preferential mode of metabolism of daidzein in the proposed pathways.

Acknowledgements—We are grateful to Dr Lamberton and Professors H. Adlercreutz and T. Hase, University of Helsinki, Finland, for the gifts of isoflavonoid reference samples and to all volunteers for their cooperation and devotion to this project.

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