Determination of Ipriflavone, 5-Methyl-7-hydroxy-isoflavone and their metabolites in human urine by GC-MS and HPLC-MS

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ABSTRACT

There is an increasing interest in the biological effects of the isoflavonoids, members of the large group of plant phytoestrogens. High-performance liquid chromatography and gas chromatography coupled with mass-spectrometry were used to study isoflavone phytoestrogen substances with potential anabolic activity: ipriflavone and 5-methyl-7-hydroxy-isoflavone. Excreation study showed that ipriflavone has five metabolites besides parent drug: four mono-hydroxylated metabolites and one di-hydroxylated metabolite. The main metabolites of 5-methyl-7-hydroxy-isoflavone are four mono-hydroxylated metabolites, three di-hydroxylated metabolites, one metabolite with reduced C=O bond and one di-hydroxylated metabolite with reduced C=O bond. In our study GC-MS and HPLC-(ESI+)/MS methods were compared. The results of our investigation supposed that the main long-term metabolite for both substances is para-hydroxylated metabolite. The limit of detection for ipriflavone and 5-methyl-7-hydroxy-isoflavone was 50 ng/ml. The method can be applied for toxicological purposes, and for the analysis of sport nutritional supplements. The detection of isoflavones in course of doping control might indirectly indicate possible abuse of banned anabolizing agents.

Keywords: LC-MS; GC-MS; doping analysis; isoflavone; ipriflavone; 5-methyl-7-hydroxy-isoflavone; metabolism

INTRODUCTION

Isoflavones belong to a group of compounds that share a basic structure consisting of two benzyl rings joined by a three-carbon bridge, which may or may not be closed in a pyran ring. The structure is generally simplified as C6-C3-C6 [1,2]. Phytoestrogenes are plant compounds that have a wide range of biological effects, many related to the close structural relation between phytoestrogens and endogenous reproductive hormones such as estrogens [3].

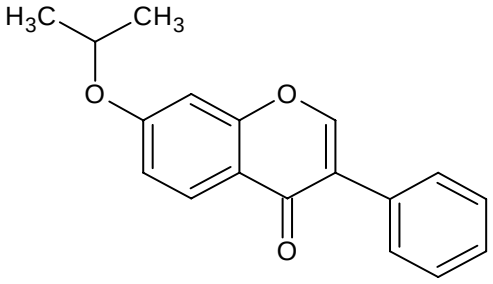
Although ipriflavone and 5-methyl-7-hydroxy-isoflavone are used as dietary supplements, little is known about their metabolism. Moreover, it is well established that the biological activities of isoflavones are dramatically altered with the chemical structure [4]. The hydroxylated products can have other biological properties than those of parent compound. Therefore it seems necessary to completely understand the metabolic pathways of isoflavones and to identify all metabolites, which were formed during this process.

Genistein and daidzein are the most abundant and the best characterized isoflavones, and are particularly abundant in soy including its food product [5,6]. Genistein, daidzein, and daidzein metabolites equol and O-desmethylangolensin (O-DMA) are the compounds found in urine after consumption of foods containing isoflavones [7]. Recently, another metabolite of genistein was identified, 6'-hydroxy-O-DMA [8].

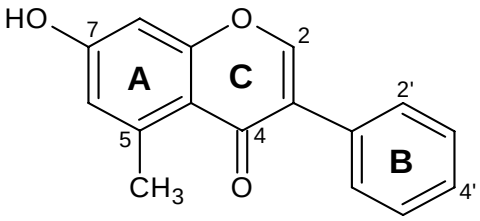
The ipriflavone and 5-methyl-7-hydroxy-isoflavone were selected for this study due to the widespread human exposure to the putative anabolic properties of these compounds. According to the statements of supplement manufacturers, isoflavones have potential anabolic activity, are safe and effective. Reportedly, isoflavones are abused as concomitant drugs at the last stage of anabolic

stacking, and their monitoring as indirect markers might be helpful in doping control routine. In this paper we investigated metabolic transformation of two isoflavones in human urine: ipriflavone and 5-methyl-7-hydroxy-isoflavone (Fig. 1).
The aim of this investigation was to examine the human urinary metabolites of ipriflavone and 5-methyl-7-hydroxy-isoflavone by LC-ESI(+)/MS Ion Trap and GC-MS following oral administration with a view to identify suitable targets for monitoring of these compounds.

Fig. 1: The chemical structure of ipriflavone and 5-methyl-7-hydroxy-isoflavone



Ipriflavone



5-methyl-7-hydroxy-isoflavone

EXPERIMENTAL

1. Reagents and chemical

Ipriflavone, 5-methyl-7-hydroxy-isoflavone and Daidzein were purchased from Promochem (Wesel, Germany). Biologically active supplements as AXIS HT (BSN, USA) and Proxylon (Syntrax, USA) were used to get real urine samples for the analysis. The standard solution of methyltestosterone was prepared as the internal standard for GC-MS analysis (Sigma Aldrich, Germany). Potassium hydroxide was purchased from ChimMed (Boris, Moscow). β-glucuronidase from *Escherichia coli* was used for enzymatic hydrolysis (Fluka, Germany). MSTFA, ammonium iodide and dithiotreitol were purchased from Merck (Darmstadt, Germany). Diethylether (Merck, Germany) with the addition of sodium sulfate (Himmed, Moscow) were used in extraction procedure. HPLC-grade methanol was purchased from Merck (Darmstadt, Germany). Water was purified with a Milli-Q system (Millipore, Molsheim, France). Other chemicals were of analytical reagent grade.

2. GC-MS and LC-ESI/MSD Ion trap-analysis

GC-MS analyses were carried out using GC-MS Agilent 6890N/5973inert system. Separation was performed on a HP-1 Ultra column (12 m x 0.2 mm x 0.33 μm). The GC-MS operating conditions were as follows: split mode 1:10, injector port temp 280°C; oven temperature program: 190-234°C at 2°C/min then 234-300°C at 12°C/min with a final hold of 4.5 min. Helium was used as carrier gas at a flow-rate of 1 ml min^{-1}. The analyses were performed in the full-scan mode.
HPLC-MS experiments were carried out using a 1100 Series LC/MSD Trap system from Agilent Technologies (Palo Alto, CA, USA) equipped with an autosampler. Chromatographic separations were performed using a Restek Ultra C-18 (100 mm x 2.1 mm x 5 μm) column connected to a guard column cartridge (2.1 × 12.5 mm) filled with the same packing material. Nitrogen gas was produced by nitrogen generator with output pressure of 80 psi, and the ion source (nebulizer) inlet pressure

was set at 30 psi. The drying gas was heated to 350°C at a flow rate of 8 litre per min. Capillary voltage was – 3500 V, skim trap drive and capillary exit were 46.4 and 104.0 V, respectively. The ion accumulation time was 300 ms, scan range 100 to 350 *m/z*.
The mobile phase was a mixture of **A**, 20 *mM* formic acid (pH = 3.0), and **B**, methanol, in a gradient elution mode. The starting mobile phase was 90 % **A** and 15 % **B**, and the linear gradient was run over 15 min to a proportion of 40 % **A** and 60 % **B** then the linear gradient was run over 25 min to a proportion of 15 % **A** and 85 % **B**. The flow rate was 0.25 ml min^{-1}.

3. Sample preparation

3.1. Administration

Two healthy volunteers gave their informed consent to participate in the study. Blank urine was collected before the administration of a single oral dose of nutritional supplements: **Proxylon** (ipriflavone – 250 mg (one capsule), *Syntrax Innovations Inc.*, USA) and **AXIS HT** (5-methyl-7-hydroxy-isoflavone – 200 mg (one capsule), *Bio-Engineering Supplements and Nutrition*, USA) to 2 healthy volunteers (male, 21 years, 55 kg; male, 20 years, 60 kg). The samples urine were collected during 3 days and immediately frozen at -30°C.

3.2. Urine extraction

To 5 ml of urine 30 µl of internal standard (methyl-testosterone) was added. Then 1 ml of phosphate buffer (pH = 7.4) and 30 µl of β-glucuronidase *E. Coli* were added prior to enzymatic hydrolysis. The hydrolysis process was carried out at 55°C for 1 hour. The solution was cooled to room temperature and the pH was adjusted to 9 by adding solid buffer (Na_2CO_3/$NaHCO_3$, 1:1 mixture). Then, *ca*. 2 g of anhydrous sodium sulfate were added, and the mixture was extracted with 5mL of diethyl ether. After shaking (2 min, Vortex) and centrifugation (5 min, 3000 rpm), the organic layer was separated and taken to dryness at 60°C. The dry dried residue was derivatized with 50 µl of MSTFA-NH_4I-DTE (1000:2:4, v/w/w) for 20 min at 60°C. One µl was injected into the GC-MS system. For HPLC-MS analysis the residue was dissolved in 50 µl of methanol, and 5 µl of this solution was injected into the LC-MS ion trap system.

RESULTS AND DISCUSSION

The first step in the work involved the characterisation of the GC-MS and HPLC-MS mass spectra properties of parent drug. The standard solutions of ipriflavone and 5-methyl-7-hydroxy-isoflavone were analysed by HPLC-MS with positive API-ESI, and by GC/EI-MS after trimethylsilylation.
Figure 2 shows the EI mass spectra of ipriflavone, ipriflavone without isopropyl group (loss during the derivatisation step) and 5-methyl-7-hydroxy-isoflavone after derivatisation with MSTFA/NH_4I/DTE. Important fragments are formed via cleavage of C-ring with formation of two diagnostic fragments: *a* and *b*. GC-MS analysis of the TMS derivative of the ipriflavone shows two chromatography peaks with molecular ions at *m/z* 280 (trace) and *m/z* 310 (main peak) (data not presented). The structure of ipriflavone does not contain any center for TMS derivatisation, and the base ion at *m/z* 237 results from the loss of an isopropyl group (Fig. 2A).
The HPLC-API/ESI mass spectrum displays single peak of ipriflavone with protonated molecular ion $[M+H]^+$ at *m/z* 281. Fragmentation of protonated ipriflavone in the ion trap leads to one product ion *m/z* 239 resulting from to the loss of the isopropyl group $[M+H\text{-}iPr]^+$ (Table 1).
The GC-MS analysis of the TMS derivative of 5-methyl-7-hydroxy-isoflavone shows a molecular ion at *m/z* 324 and the characteristic fragment ions: *m/z* 309, 222 and 102 (Fig. 2C). The positive HPLC-API/ESI mass spectrum displays a protonated molecular ion $[M+H]^+$ at *m/z* 253.

Fragmentation of protonated 5-methyl-7-hydroxy-isoflavone in the ion trap leads to product ions *m/z* 235 $[M+H-H_2O]^+$, 197, 179, 225 and 210 (Table 2).

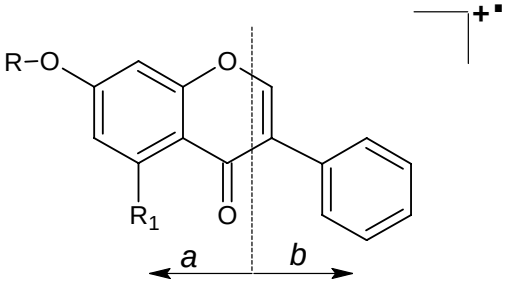
Table 1: Product ions for ipriflavone and its metabolites, protonated molecule $[M+H]^+$, retention time (RT), changes in observed mass for the metabolites (ΔM) and fragmentation results from MS-MS spectrum when were obtained via fragmentation of molecular ions. HPLC-ESI(+)/MS results.

	MW	$[M+H]^+$	RT [min]	ΔM	MS-MS
(1) Ipriflavone	280	281	24.0		281->239 $[M+H-iPr]^+$
Mono-hydroxyl metabolites					
M2	296	297	19.8	+16	297->255, 237
M3	296	297	21.6	+16	297->255, 237
M4	296	297	22.4	+16	297->255, 237
M5	296	297	22.7	+16	297->255, 237
Dihydroxy metabolite					
M6	312	313	16.3	+32	313->271,253,235

Table 2: Product ions for 5-methyl-7-hydroxy-isoflavone and its metabolites, protonated molecule $[M+H]^+$, retention time (RT), changes in observed mass for the metabolites (ΔM) and fragmentation results from MS-MS spectrum when were obtained via fragmentation of molecular ions. HPLC-ESI(+)/MS results.

	MW	$[M+H]^+$	RT [min]	ΔM	MS-MS
(1) 5-methyl-7-hydroxy-isoflavone	252	253	19.7		253->235, 210, 197
Metabolite with reduced C=O bond					
M2	254	255	15.3	+2	255->237, 227, 199, 137
Mono-hydroxyl metabolites					
M3	268	269	17.1	+16	269->251, 223, 213
M4	268	269	18.1	+16	269->251, 223, 213
M5	268	269	19.1	+16	269->251, 223, 213
Dihydroxy metabolite					
M6	284	285	14.1	+32	285->267, 249, 211, 177
M7	284	285	15.0	+32	285->267, 249, 211, 177
M8	284	285	15.5	+32	285->267, 249, 211, 177
Dihydroxy metabolite with reduced C=O bond					
M9	286	287	12.2	+34	287->269, 251

Fig. 2: Mass spectra of ipriflavone (**A**), ipriflavone without isopropyl group (**B**) and 5-methyl-7-hydroxy-isoflavone (**C**). GC-MS method



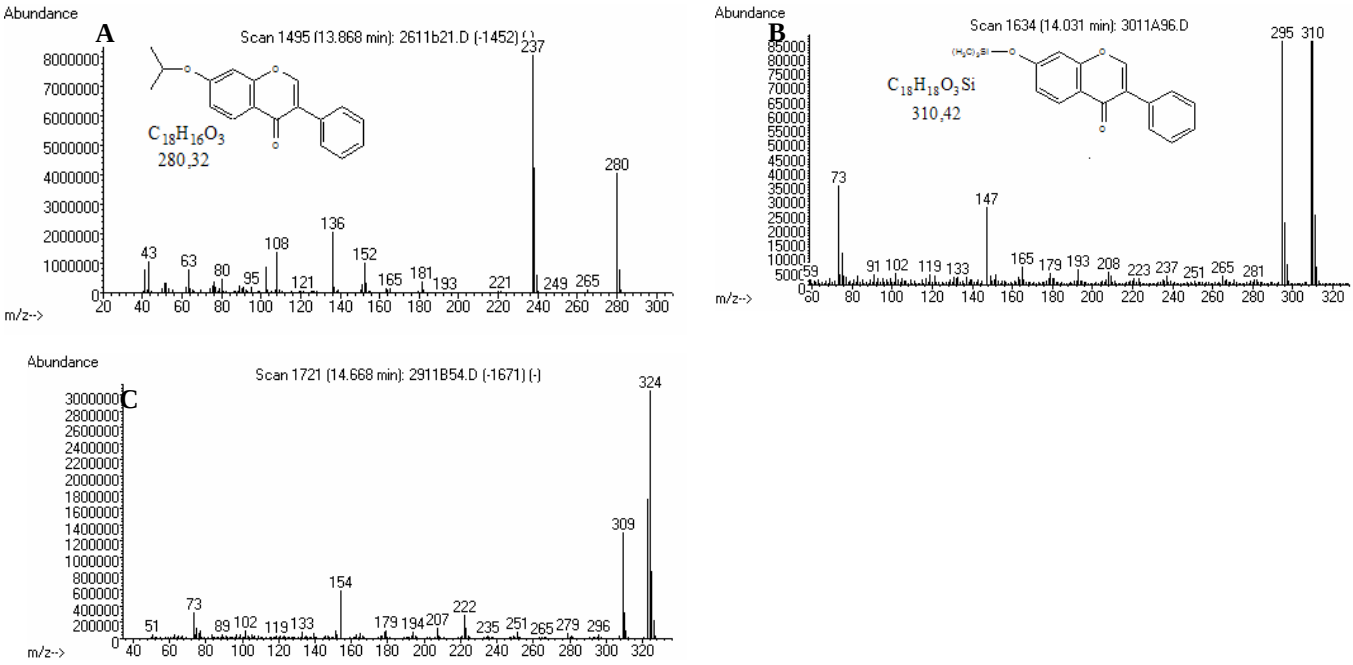
Ipriflavone:

A) R = -iPr, R_1 = H, m/z 280; *b* - m/z 102

B) R = -Si(CH_3)$_3$, R_1 = H, m/z 310; *a* - m/z 208, *b* - m/z 102

5-methyl-7-hydroxy-isoflavone:

C) R = -Si(CH_3)$_3$, R_1 = -CH_3, m/z 324; *a* - m/z 222, *b* - m/z 102



Ipriflavone Metabolite Identification

The GC-MS and positive HPLC-API/ESI-MS methods were applied to the analysis and confirmation testing of both isoflavones and their metabolites in human urine. Possible positions for the hydroxylation of ipriflavone are C-5, C-6 and C-8 in the aromatic A-Ring, C-2′, C-3′ (equivalent to C-6′ and C-5′, respectively) and C-4′ in the B-Ring, and C-2 in the C-Ring. We have not established direct correlation between peaks of metabolites which were found by HPLC-MS and GC-MS analyses. Therefore, we assigned numerical order as **M2**, **M3** to the metabolites found by HPLC-MS, and the similar but with «′» mark was done for the metabolites found by GC-MS: **M2′**, **M3′** etc.

Analysis by LC-ESI(+)/MS of extracts from enzymatic fraction urine gave the chromatogram of urine sample that shows a number of products appearing at retention times shorter than those of the parent drug i.e. more polar compounds. These results have shown that besides the unchanged parent drug (**1**) the following five metabolites were detected: four isomers of monohydroxy-ipriflavone **M2**, **M3**, **M4**, **M5** and one dihydroxy-ipriflavone **M6** (Table 1).

Fig. 3: Structural assignments for Ipriflavone metabolites.

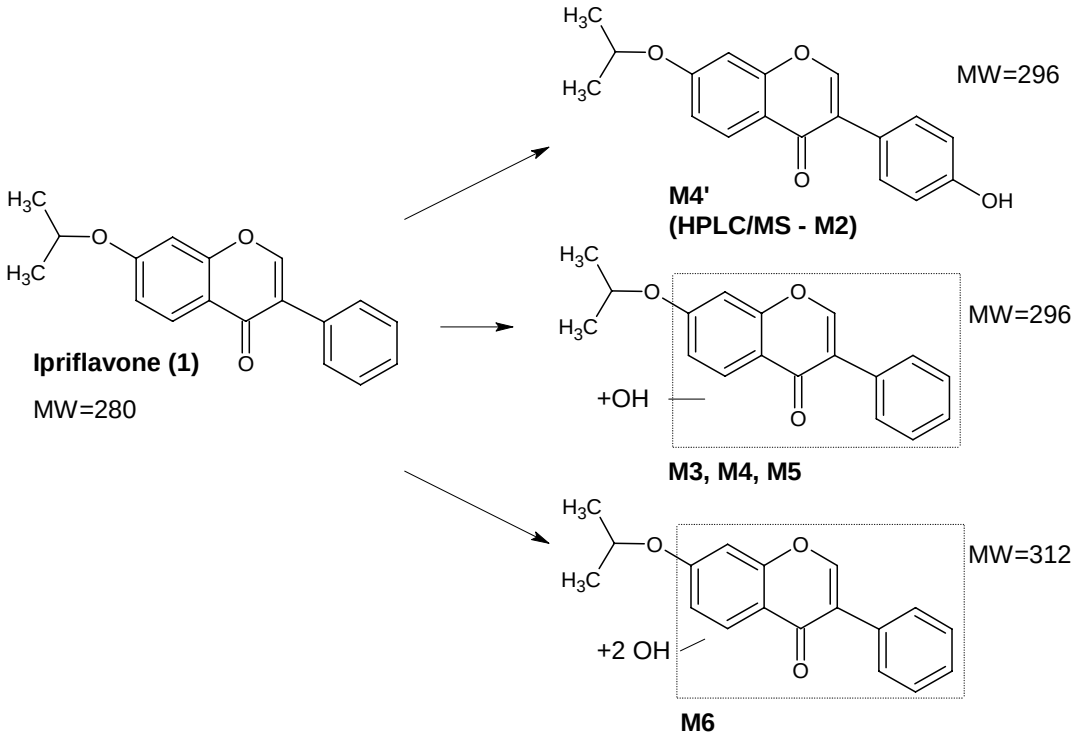


Fig. 4: Electron impact mass spectra of the TMS derivatives of ipriflavone metabolites.

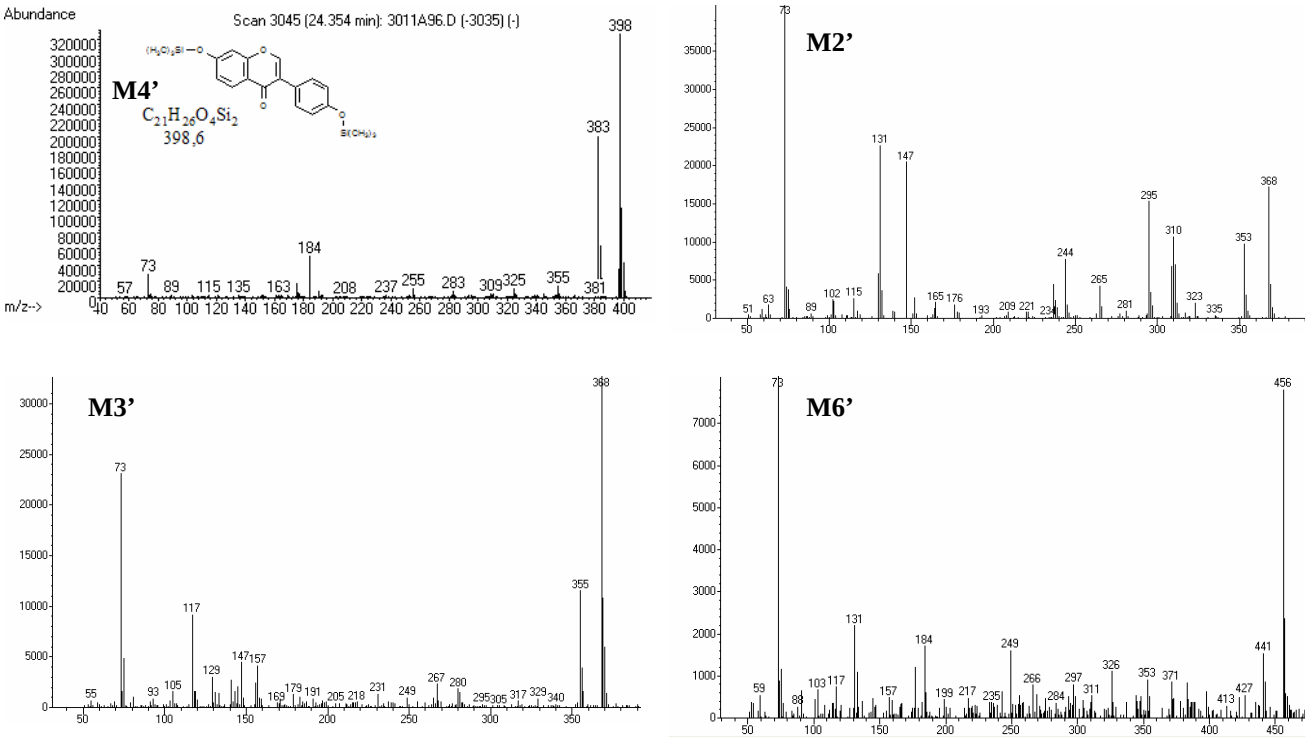


Fig. 5: Structural assignments for 5-methyl-7-hydroxy-isoflavone metabolites.

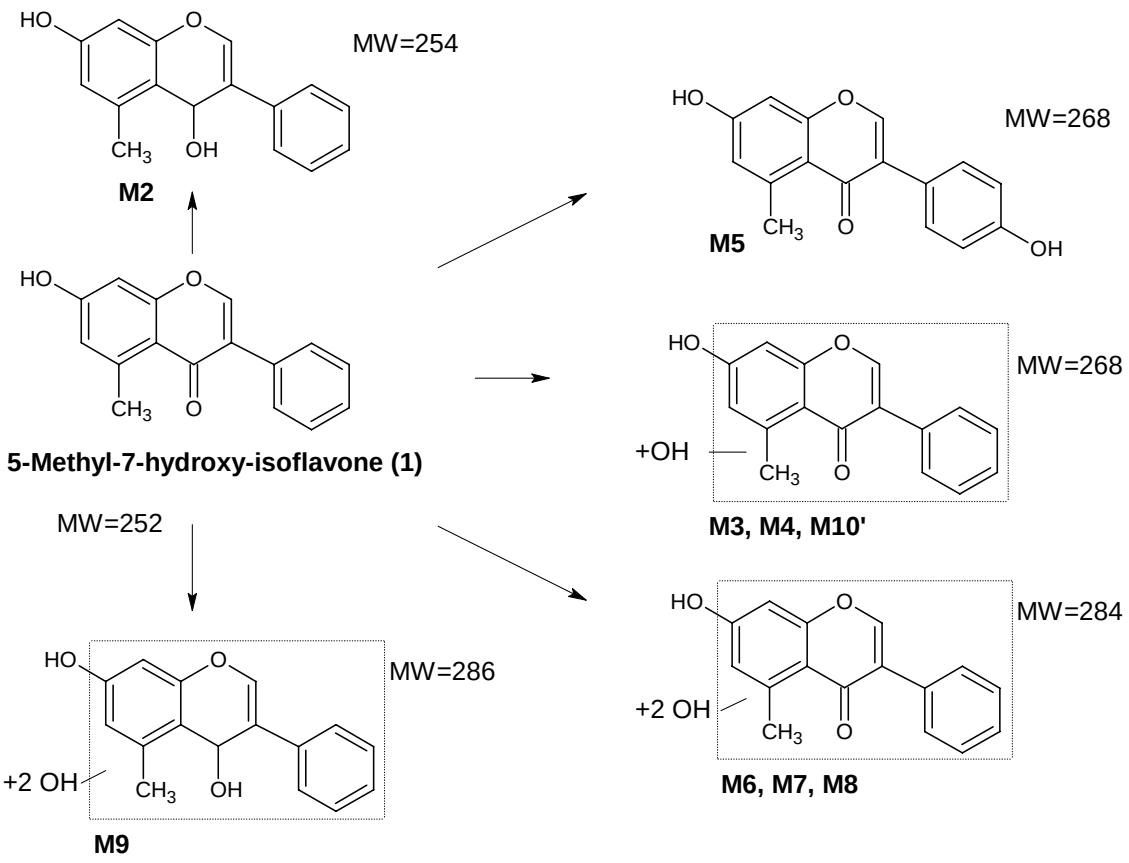


Fig. 6: Electron impact mass spectra of the TMS derivatives of mono-hydroxylated 5-methyl-7-hydroxy-isoflavone metabolites.

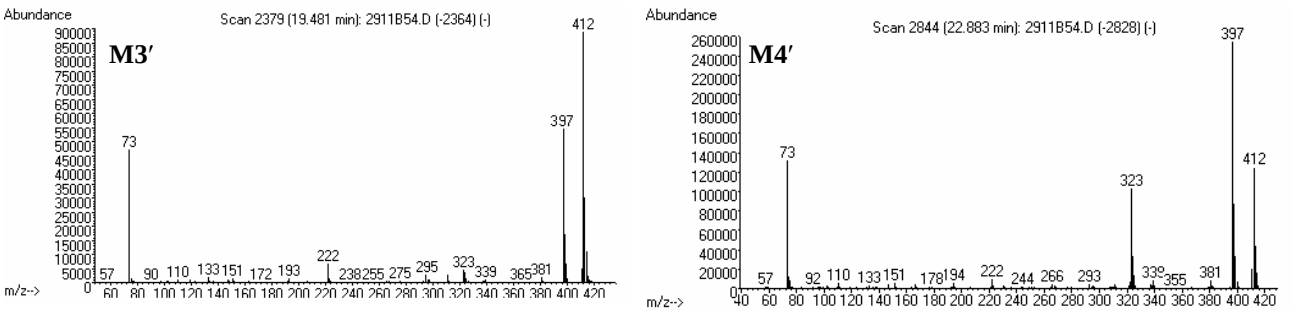


Fig. 7: Electron impact mass spectra of the TMS derivatives of mono-hydroxylated 5-methyl-7-hydroxy-isoflavone metabolites.

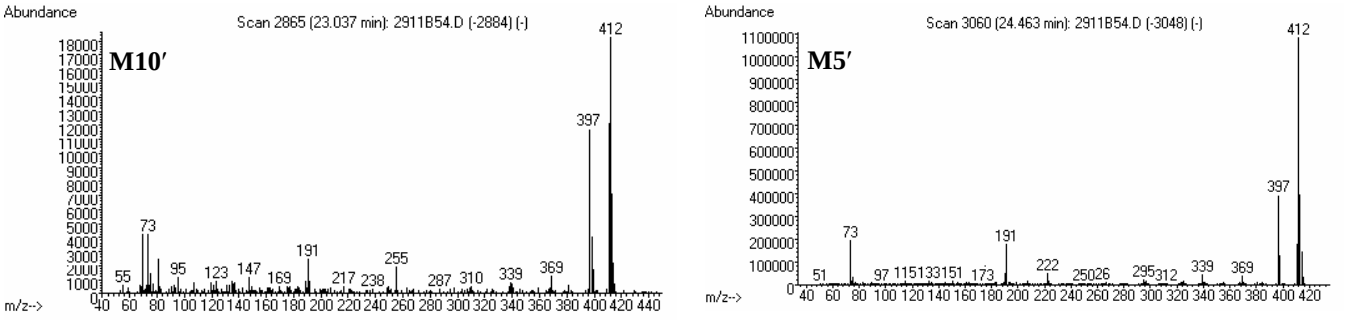
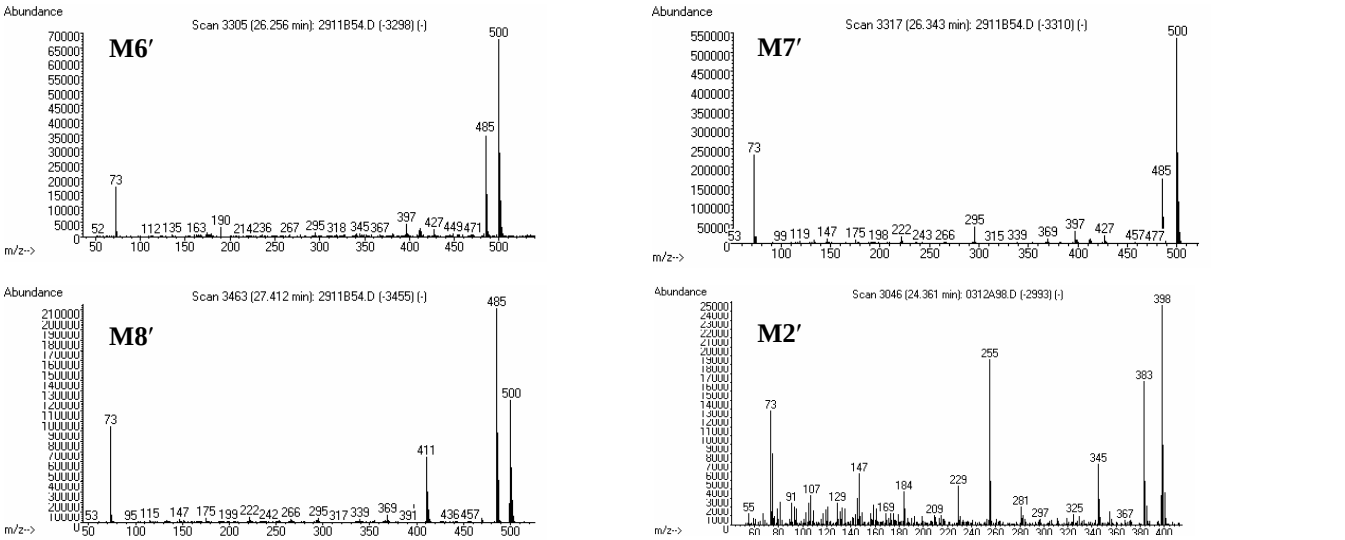


Fig. 8: Electron impact mass spectra of the TMS derivatives of dihydroxylated 5-methyl-7-hydroxy-isoflavone (M6′, M7′ and M8′) metabolites and metabolite with reduced C=O bond (M2′).



The unchanged ipriflavone (**1**) was eluted at 24.0 min in the ipriflavone positive urine. The protonated molecular ion of ipriflavone is *m/z* 281 $[M+H]^+$. MS-MS mass spectra of ipriflavone show one product ion *m/z* 239 resulting from the loss of the isopropyl group $[M+H\text{-}iPr]^+$.

The molecular ion *m/z* 296 of metabolites **M2**, **M3**, **M4** and **M5** was shifted by 16 Da compare to that of the unchanged ipriflavone *m/z* 281, indicating mono-hydroxylation of ipriflavone. MS-MS mass spectra of these metabolites are not informative, further fragment ions are *m/z* 255 and 237, which originate from the loss of isopropyl group and H_2O, respectively.

The metabolite **M6** was eluted at 16.3 min. The molecular ion *m/z* 313 of metabolites **M6** was increased by 32 Da compared to that of the unchanged Ipriflavone, confirming dihydroxylation of ipriflavone (Table 1).
GC-MS data of the ipriflavone positive urine showed ambiguous results (Fig 4). The ipriflavone without isopropyl group was eluted at 14.06 min. The isopropyl group was lost during the derivatisation step. Unchanged ipriflavone was not detected. GC-MS analysis of ipriflavone urine showed four compounds: two isomeres of monohydroxy-ipriflavone **M2′** and **M3′** with retained isopropyl group, one monohydroxy-ipriflavone without isopropyl group **M4′** and one dihydroxy-ipriflavone without isopropyl group **M6′** (Fig 4). The GC-MS analysis of the TMS derivative of the major metabolite **M4′** showe a molecular ion at *m/z* 398 (di-TMS), a fragment ion at *m/z* 208, implaying an unchanged A-Ring, and a fragment ion at *m/z* 190 (102 ->190, adding –OTMS group) indicating a mono-hydroxylated ipriflavone structure in the B-Ring. The characteristic ions are *m/z* 383, 208, 184, 190. The retention time and EI mass spectrum of **M4′** were same with those of Daidzein (4',7-dihydroxyisoflavone) standard. Therefore, the metabolite **M4′** of ipriflavone without isopropyl group corresponds with the structure with a hydroxyl group at C-4′ position in the B-Ring (p-hydroxylated metabolite of ipriflavone).

5-Methyl-7-hydroxy-isoflavone Metabolite Identification

Possible positions for the hydroxylation of 5-methyl-7-hydroxy-isoflavone are C-6 and C-8 in the aromatic A-Ring, C-2′, C-3′ (equivalent to C-6′ and C-5′, respectively) and C-4′ in the B-Ring, and C-2 in the C-Ring. LC-ESI/MS Analysis gave the chromatogram that shows a number of products appearing at retention times shorter than those of the parent drug (i.e. more polar compounds).
These results confirmed the presence of the metabolite with reduced C=O bond, monohydroxylated, dihydroxylated metabolites and dihydroxylated- metabolie with reduced C=O bond in human urine (Fig. 5).
GC-MS analysis: The metabolites **M3′** and **M4′** were eluted at 19.48 min and 22.89 min, respectively. The spectra of these two metabolites only differ in the intensities of the fragment ions (Fig. 6). The molecular ion of metabolites **M3′** and **M4′** was increased by 16 Da compared to that of the unchanged 5-methyl-7-hydroxy-isoflavone. The molecular ion of these metabolites (di-TMS) is *m/z* 412. The characteristic ions are *m/z* 397, *m/z* 323, *m/z* 222. The ion *m/z* 222 of these metabolites was the same as the fragment ion of the parent drug (Fig 2). Therefore, this indicates that the C- or B-Ring of parent drug was hydroxylated.
The metabolites **M10′** and **M5′** were eluted at 23.04 min and 24.46 min, respectively. The spectra of these two metabolites only differ in the intensities of the fragment ions (Fig. 7). The molecular ion of metabolites **M10′** and **M5′** was increased by 16 Da compared to that of the unchanged 5-methyl-7-hydroxy-isoflavone. The molecular ion of these metabolites (di-TMS) is *m/z* 412, the characteristic ions are *m/z* 397, 222, 191. The ion *m/z* 191 of these metabolites was increased by 89 Da (102->191, added –OTMS group) compared to that of the unchanged 5-methyl-7-hydroxy-isoflavon. The ion *m/z* 222 of these metabolites was the same as the fragment ion of the parent drug (Fig 2). Therefore, this indicates that the B-Ring of parent drug was hydroxylated. Possible positions of 5-methyl-7-hydroxy-isoflavone for the hydroxylation at the B-Ring are C-2′ (C-6′), C-3′ (C-5′) and C-4′. The metabolites **M10′** and **M5′** are mono-hydroxy metabolites of 5-methyl-7-hydroxy-isoflavone.
The metabolite **M6′** was eluted at 26.25 min. The molecular ion of metabolite **M6′** was increased by 32 Da compared to that of the unchanged 5-methyl-7-hydroxy-isoflavone. The molecular ion of these metabolites (tri-TMS) is *m/z* 500 (Fig. 8). The characteristic ions are *m/z* 485, 397, 190. The ion *m/z* 190 of thise metabolite was increased by 88 Da (102->190, added –OTMS group) compared to that of the unchanged 5-methyl-7-hydroxy-isoflavone. The ion at *m/z* 222 was not detected into mass spectrum (Fig 2). Therefore, this indicates that the A-Ring and B-Ring of parent drug were

hydroxylated. Hence the metabolite **M6′** is di-hydroxy metabolite of 5-methyl-7-hydroxy-isoflavone, which is formed by the introduction of a hydroxyl group in the A- or B-Ring.
The metabolites **M7′** and **M8′** were eluted at 26.44 min and 27.41 min, respectively. The spectra of these two metabolites only differ in the intensities of the fragment ions (Fig. 8). The molecular ion of metabolites **M7′** and **M8′** was increased by 32 Da compared to that of the unchanged 5-methyl-7-hydroxy-isoflavone. The molecular ion of these metabolites (di-TMS) is *m/z* 500. The characteristic ions are *m/z* 485, 411, 222, 147. The ion at *m/z* 222 of these metabolites was same as the fragment ion of parent drug (Fig 2). The ion at *m/z* 147 indicates that hydroxylation has taken place in the *meta* position relative to the second hydroxy group. Therefore, we suppose that the B-Ring of parent drug was dihydroxylated. Possible positions of 5-methyl-7-hydroxy-isoflavone for the dihydroxylation at the B-Ring are C-2′ (C-6′), C-3′ (C-5′) and C-4′. Hence the metabolites **M7′** and **M8′** are dihydroxy- metabolites of 5-methyl-7-hydroxy-isoflavone.
Metabolites **M2** and **M9** are quite minor. According to the API-ES mass spectra they correspond to metabolite with reduced C=O bond and dihydroxylated metabolite with reduced C=O bond, respectively. The structure of metabolite **M9** has not been identified.
The LC-ESI/MS and GC-MS results have shown that besides the unchanged parent drug (**1**) the following nine metabolites were detected: one metabolite with reduced C=O bond **M2**, four isomeres of monohydroxylated **M3**, **M4**, **M10′**, **M5**, three isomeres of dihydroxylated **M6**, **M7**, **M8** and one dihydroxylated metabolite with reduced C=O bond **M9** (Fig. 5).
Ipriflavone and 5-methyl-7-hydroxy-isoflavone urine were analysed by routine doping control procedure for anabolic steroids and was found that dihydroxylated metabolite **M8′** (Fig. 8) of 5-methyl-7-hydroxy-isoflavone has the same retention time a canrenone. Moreover, this metabolite has characteristic ions (*m/z* 412 and *m/z* 397) in the mass spectrum which are also ions for canrenone. Therefore, the 5-methyl-7-hydroxy-isoflavone may be a potential masking agent of canrenone in human urine.

CONCLUSIONS

High-performance liquid chromatography and gas chromatography coupled with mass-spectrometry were used to study the phytoestrogens: ipriflavone and 5-methyl-7-hydroxy-isoflavone. In humans ipriflavone is metabolized as follows: parent drug ipriflavone, four monohydroxylated metabolites and one dihydroxylated metabolite. The main metabolites for 5-methyl-7-hydroxy-isoflavone are four monohydroxylated metabolites, three dihydroxylated metabolites, one metabolite with reduced C=O bond and one dihydroxylated metabolite with reduced C=O bond. The HPLC-MS and GC-MS procedures allows for the identification of the urinary metabolites of these compounds and precise and sensitive detection of parent drug and/or its metabolites in urine up to 60 hrs after ingestion of a 200-250 mg dose.
Identification and detection of unchanged drug and new metabolites in this study may give especially useful new information for the monitoring of ipriflavone and 5-methyl-7-hydroxy-isoflavone use or abuse.

Acknowledgements

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