Metabolism of Ipriflavone (TC-80) in Rats

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Received June 17, 1985

Metabolic studies of ipriflavone (TC-80) in rats by gas-liquid chromatography-mass spectrometry led to the characterization of the following metabolites: the parent compound, 7-hydroxy-3-phenyl-4H-1-benzopyran-4-one, 7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, 3-(4-hydroxyphenyl)-7-isopropoxy-4H-1-benzopyran-4-one, 2-(3-phenyl-4-oxo-4H-1-benzopyran-7-yl)oxypropionic acid, 2-(3-(4-hydroxyphenyl)-4-oxo-4H-1-benzopyran-7-yl)oxypropionic acid and 2-(3-(3-hydroxyphenyl)-4-oxo-4H-1-benzopyran-7-yl)oxypropionic acid. From the metabolites identified, TC-80 was shown to be metabolized primarily by oxidation. In vitro study using tissue slices of rats indicated that the above metabolic changes occurred exclusively in the liver. It was also demonstrated that the compound did not undergo metabolic conversion by gut flora of rats.

Key Words: metabolism, ipriflavone, rat, gas-liquid chromatography-mass spectrometry, gut flora

1. Introduction

Ipriflavone (TC-80)*, 7-isopropoxyisoflavone, is a synthetic flavonoid possessing the activities to reduce renal excretion of calcium, to enhance calcium-stimulated calcitonin secretion in the presence of estrogen and to improve osteopenia induced by low calcium, low vitamin D diet[^1-3], and is therefore expected to be useful in the treatment of osteoporosis.

The present report describes the metabolism of ¹⁴C-labeled TC-80 ("C–TC-80" in rats, and disposition of the compound in rats and dogs is reported in the following paper[^4].

2. Materials and Methods

2.1 Materials

Two samples of "C–TC-80, with specific radioactivities of 0.492 MBq (13.3 µCi)/mg and 0.540 MBq (14.6 µCi)/mg, were synthesized by the method of Bánfi (personal communication) (Fig. 1). Radiopurity (>99%) and chemical identity of the compounds were verified by thin-layer chromatography (TLC, described below). Nonlabeled TC-80, 7-hydroxy-3-phenyl-4H-1-benzopyran-4-one (I), 7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one (II), 3-(4-hydroxyphenyl)-7-isopropoxy-4H-1-benzopyran-4-one (III), 2-(3-phenyl-4-oxo-4H-1-benzopyran-7-yl)oxypropyl alcohol (IV), 2-(3-phenyl-4-oxo-4H-1-benzopyran-7-yl)oxypropionic acid (V), 2-(3-(4-hydroxyphenyl)-4-oxo-4H-1-benzopyran-7-yl)oxypropionic acid (VI), 2-(3-(3-hydroxyphenyl)-4-oxo-4H-1-benzopyran-7-yl)oxypropionic acid (VII) were synthesized in Chemical Development Laboratories of this Division.

β-Glucuronidase (Type H–1, from Helix pomatia) containing phenol sulfatase was purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and N,O-bis(trimethylsilyl)acetamide (BSA) was from Tokyo Chemical Industry Co. Ltd. (Tokyo). All other reagents used were of analytical grade.

2.2 Animal experiments

¹⁴C–TC-80 diluted appropriately with the nonlabeled compound was suspended in 1
% w/v hydroxypropylcellulose solution for administration to male Jcl:Wistar rats (268 – 291 g; CLEA Japan Inc., Tokyo), fasted overnight. After dosing, urine and feces were collected by using the usual metabolism cages, and the portal blood was from the rat with jejunal loop.

2.3 Measurement of radioactivity

All radioactivity determinations were carried out by using Nonion NS–210 (Nippon Yushi, Ltd., Tokyo) based scintillator (DPO 18 g, bis-MSB 1.2 g, toluene 1200 ml and Nonion NS–210 516 ml) and an Aloka LSC–683 or LSC–903 liquid scintillation counter (Aloka, Co., Ltd., Tokyo).

2.4 Identification studies

2.4.1 Isolation of metabolites

The one hour portal blood was collected from one rat with jejunal loop after instillation of 14C-TC-80 (20 mg/kg) into the loop. The 24 h samples of urine and feces were obtained from six rats given po dose of the labeled compound (100 mg/kg) and pooled. The portal blood, urine and feces contained 1.6, 29.0 and 61.1% of the dosed 14C, respectively. Unaltered TC-80 was isolated from the portal blood, I, II, V, VI and VII from the urine, and II from the feces as follows. The urine was treated with an excess of Helix pomatia preparation for 16 h at 37°C. The aqueous homogenate of feces was extracted with acetone (5 vol. x 1) and the extract, after evaporation, dissolved in water. The portal blood, urine and the extract of feces were then adjusted to pH 2 to 3 with HCl and extracted with ethyl acetate (2 vol. x 3). 14C-TC-80 and its metabolites in the ethyl acetate extracts were separated by TLC using precoated silica gel 60F254 plates (0.25 mm thick; E. Merck, Darmstadt, FR Germany) and a solvent mixture of toluene–ethyl acetate–acetic acid (100:100:1, v/v/v; system A) (Fig. 2). The silical gel sections corresponding to the unaltered compound and metabolites were scraped off the plates, suspended in 0.1 N HCl and extracted with ethyl acetate (2 vol. x 3). Metabolites I, V, VI and VII obtained were further purified by TLC with a solvent mixture of chloroform–methanol–acetic acid (100:100:1, v/v/v; system B) (Fig. 2).

2.4.2 Derivatization

Isolated metabolites were derivatized by treating with BSA for more than 10 min at 25°C or by treating with diazomethane in diethyl ether for 10 min at 25°C and subsequently with BSA as above.

2.4.3 Instrumental analysis

Gas-liquid chromatography–mass spectrometry
metry (GC–MS) was carried out by using a Shimadzu LKB 9000B or GCMS 9020DF spectrometer (Shimadzu Corp., Kyoto) equipped with a computer system under the following conditions: 50 cm coiled glass column (3 mm ID) packed with 1% OV–17 on 60–80 mesh Gas Chrom Q; carrier gas, helium (30 ml/min); column temperature, 160 to 240°C using a temperature programmer at a rate of 4°C/min; ionization potential, 70 eV; trap current, 60 μA; accelerating voltage, 3 or 3.5 kV. After the injection of the samples into the GC–MS instrument, mass spectra were taken at the top of the GC peaks by the electron ionization method.

2.5 in vitro metabolism
Rats were killed by exsanguination from the abdominal aorta, and the brain, heart, lung, liver, kidney and duodenum were excised, placed in ice-cold physiological saline and sliced by hand. Tissue slices (about 200 mg) and blood (0.2 ml) were incubated with 14C-TC-80 (4 pg) in Krebs–Ringer bicarbonate buffer (1 ml) for 15 to 60 min at 37°C under O₂–CO₂ (95:5). The metabolism of 14C-TC-80 by gut flora was studied by the method of Bakke6). The incubation medium consisted of 0.5% yeast extract (Daigo Nutritive Chemicals, Ltd., Osaka), 0.5% polypeptone (Daigo Nutritive Chemicals, Ltd.) and 0.5% glucose in 0.1M sodium phosphate buffer, pH 7.4. The cecal contents of rats (200 mg) were suspended in the medium (1 ml) containing the labeled compound (4 μg) and incubated for 30 min at 37°C anaerobically under nitrogen. After the incubation, the reaction mixtures were homogenized and extracted with acetone (5 vol. x 1) for quantitative determination of unchanged TC-80 and metabolites by TLC using the system A (Fig. 2).

3. Results and Discussion
3.1 Identification of metabolites
Preliminary studies using TLC and GC–MS demonstrated that the parent compound, I, II, III, IV, V, VI and VII were present in biological specimens of rats treated with 14C–TC–80. The fractions corresponding to unaltered TC–80 and metabolites were then isolated from the portal blood, urine or feces of rats, derivatized and injected into the GC–MS instrument as described in "Materials and Methods". The structures of the compounds in these fractions were estimated by analysis of the mass spectral data and confirmed by comparing their chromatographic and mass spectral characteristics with those of authentic compounds (Tables 1 and 2).

3.1.1 Parent compound
The mass spectrum of the parent compound gave a peak at m/z 280 for the molecular ion (M⁺), together with two diagnostic peaks at m/z 237 (M⁺–C₃H₇) and 43 (C₃H₇⁺) (Table 2). These mass spectral data and GC retention time (Table 1) showed complete identity with those of authentic TC-80.

3.1.2 Metabolite I
The M⁺ at m/z 398 in the mass spectrum of II as its TMS derivative suggested the hydroxylation of the molecule of I (Table 2), Two fragment peaks were observed at m/z 383
Table 2 Mass spectra of TC-80 and its metabolites isolated from the portal blood, urine or feces of rats given 14C-TC-80

<table>
<thead>
<tr>
<th>Compound</th>
<th>Substituent</th>
<th>M⁺</th>
<th>Diagnostic fragment ion at m/z (relative intensity, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC-80</td>
<td>H₃C·CHO</td>
<td>H</td>
<td>280(59), 237(100), 437(7)</td>
</tr>
<tr>
<td>I</td>
<td>TMSO</td>
<td>H</td>
<td>310(92), 295(100), 237(4)</td>
</tr>
<tr>
<td>II</td>
<td>TMSO</td>
<td>OTMS</td>
<td>398(100), 383(65), 325(3)</td>
</tr>
<tr>
<td>III</td>
<td>H₃C·CHO</td>
<td>OTMS</td>
<td>368(100), 353(12), 326(45), 311(66), 43(20)</td>
</tr>
<tr>
<td>V</td>
<td>TMSOOC·CHO</td>
<td>H</td>
<td>382(58), 367(10), 323(66), 265(42), 237(9), 73(100)</td>
</tr>
<tr>
<td>VI</td>
<td>TMSOOC·CHO</td>
<td>OTMS</td>
<td>470(100), 455(12), 411(13), 353(15), 325(4)</td>
</tr>
<tr>
<td>VII</td>
<td>H₃C·CHO</td>
<td>OTMS</td>
<td>470(100), 455(48), 411(21), 353(20), 325(6)</td>
</tr>
</tbody>
</table>

(M⁺−CH₃) and 325 (M⁺−TMS). From these fragmentation patterns, the location of the additional hydroxy group was not clear. The mass spectral properties, Rf values in TLC (Fig. 2) and GC data (Table 1) of this metabolite were, however, in good agreement with those of authentic 7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one.

3.1.4 Metabolite II

The mass spectrum of II as its TMS derivative gave the M⁺ at m/z 368, suggesting the hydroxylation of the parent compound (Table 2). Four fragment peaks were observed at m/z 353 (M⁺−CH₃), 326 (M⁺−CH₂), 311 (M⁺−CH₂•CH₂) and 43 (CH₃⁺). The peaks at m/z 326 and 43 indicated that the additional hydroxy group was located in the isoflavone ring. This metabolite was identified as 3-(4-hydroxyphenyl)-7-isopropoxy-4H-1-benzopyran-4-one by comparing the mass spectral data and chromatographic characteristics (Fig. 2, Table 1) with those of the authentic compound.

3.1.5 Metabolites VI and VII

VI and VII were separated by GC (Table 1) and the mass spectra of the TMS derivatives of these metabolites gave the similar fragmentation patterns to each other (Table 2). The M⁺ of these metabolites at m/z 470 suggested the hydroxylation of V. Four diagnostic peaks at m/z 455 (M⁺−CH₂), 411 (m/z 353+TMS−CH₃), 353 (M⁺−COOTMS) and 325 were considered to be formed by the same fragmentation as in the case of V (Table 2). These two metabolites were, therefore, postulated to be position isomers with respect to the additional hydroxy group which was located on the isoflavone ring. By comparing the mass spectral data and GC retention times (Table 1) with those of the authentic compounds, VI was identified as 2-(3-(3-hydroxyphenyl)-4-oxo-4H-1-benzopyran-7-yl)oxypropionic acid and VII as 2-(3-(3-hydroxyphenyl)-4-oxo-4H-1-benzopyran-7-yl)oxypropionic acid.

Six metabolites as well as unaltered TC-80
were thus identified. Of the metabolites characterized here, I and II were already isolated from dog urine and identified by MS (Uemura, unpublished). Although we failed to identify VII in rats, this metabolite was isolated from the human urine and characterized as 2-(3-phenyl-4-oxo-4H-1-benzopyran-7-yl)oxypropyl alcohol by GC-MS (Uemura, unpublished). From these results, it is obvious that in rats TC-80 is metabolized primarily by oxidation: elimination of the isopropyl function, oxidation of the isopropyl group and hydroxylation of the B (phenyl) ring (Fig. 3). As will be described in our next paper, all of the above metabolites are also detected in dogs. In addition, conjugation with glucuronic and/or sulfuric acid of I, II, III, IV, VI and VII occurs in both rats and dogs.

3.2 in vitro metabolism

In order to determine the site of biotransformation of TC-80, metabolic conversion of the 14C-labeled compound was studied in vitro using blood and slices of brain, heart, lung, liver, kidney and duodenum of rats. Among the tissues tested the compound was found to be metabolized only in the liver at a rate of 43.4±4.5 µg/h per g tissue (mean with the standard deviation for three experiments).

Griffiths and Smith reported that flavonoids possessing a free 5-hydroxyl group readily underwent ring fission by gut flora of rats. Such metabolic degradation does not occur with TC-80, since no significant amount of the compound was metabolized in the incubation with the cecal contents of rats, thus excluding the participation of gut flora in biotransformation of TC-80.

In conclusion, TC-80 is metabolized exclusively in the liver by elimination of the isopropyl group, oxidation of the isopropyl function and hydroxylation in the B ring followed by conjugation with glucuronic and/or sulfuric acid.

References
3) Yamazaki, I.: Program of the 7th International Congress of Endocrinology, p. 1628, Quebecm Canada (1984)
要 旨

イブリフラボン（TC-80）のラットにおける代謝

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イブリフラボン（TC-80）のラットにおける代謝をガスクロマトグラフ-質量分析法により検討し、未変化体のほか、代謝物として7-ヒドロキシ-3-フェニル-4H-1-ベンゾピラゾン-4-オン、7-ヒドロキシ-3-(4-ヒドロキシフェニル)-4H-1-ペンゾピラゾン-4-オン、3-(4-ヒドロキシフェニル)-7-イゾプロピオキシ-4H-1-ペンゾピラゾン-4-オン、2-(3-フェニル-4-オキソ-4H-1-ペンゾピラゾン-7-イール)オキシプロピオ онл酸、2-[3-(4-ヒドロキシフェニル)-4-オキソ-4H-1-ペンゾピラゾン-7-イール]オキシプロピオ онл酸および2-[3-(3-ヒドロキシフェニル)-4-オキソ-4H-1-ペンゾピラゾン-7-イール]オキシプロピオ онл酸を同定した。これらの代謝物から、TC-80が主として酸化によって代謝されることが示された。ラットの組織切片を用いたin vitroの実験により、上記の代謝反応が肝臓において行われることも明らかにされた。さらに、この化合物はラットの腸内細菌によっては代謝変換をうけないことも分かった。

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