Taylor & Francis
Taylor & Francis Group

Xenobiotica
the fate of foreign compounds in biological systems

ISSN: 0049-8254 (Print) 1366-5928 (Online) Journal homepage: http://www.tandfonline.com/loi/ixen20

Identification of urinary metabolites of ecabapide in rat

Y. Fujimaki, T. Hosokami & K. Ono

To cite this article: Y. Fujimaki, T. Hosokami & K. Ono (1995) Identification of urinary metabolites of ecabapide in rat, Xenobiotica, 25:5, 501-510

To link to this article: http://dx.doi.org/10.3109/00498259509061869

XENOBIOTICA, 1995, VOL. 25, NO. 5, 501–510

Identification of urinary metabolites of ecabapide in rat

Y. FUJIMAKI*, T. HOSOKAMI† and K. ONO‡

Drug Metabolism and Analytical Chemistry Research Center, and
† Exploratory Research Laboratories III, Daiichi Pharmaceutical Co., Ltd,
1-16-13, Kitakasai, Edogawa-ku, Tokyo 134, Japan

Received 1 November 1994

1. ^{14}C-Ecabapide, 3-[[[2-(3,4-dimethoxyphenyl)ethyl]carbamoyl]methyl]amino-*N*-methyl[^{14}C]benzamide, was dosed orally to rat (100 mg/kg). Within 48 h after dosing, 36·7 ± 5·4 and 55·7 ± 11·8% of the administered radioactivity was recovered from urine and faeces respectively.

2. The unchanged drug was the major compound excreted in the urine and accounted for 37% of the urinary radioactivity. Seven urinary metabolites were purified by preparative hplc and their structures were elucidated by mass and ^{1}H-nmr spectrometry.

3. The major metabolic pathway of ecabapide was found to be the formation of 3-amino-*N*-methylbenzamide produced by *N*-dealkylation of the secondary amine at the 3-position of the benzamide moiety followed by acetylation.

4. Further metabolic pathways of the *N*-methylbenzamide moiety were *N*-demethylation via the carbinolamine derivatives, and/or aromatic hydroxylation followed by glucuronidation.

Introduction

Ecabapide (3-[[[2-(3,4-dimethoxyphenyl)ethyl]carbamoyl]methyl]amino-*N*-methylbenzamide (DQ-2511); figure 1) has antiulcer activities in various experimental gastric and duodenal ulcers at oral doses of 30–300 mg/kg to rat. This compound inhibited gastric acid secretion and increased the gastric mucosal blood flow in the conscious restrained rat (Asano *et al.* 1990).

A previous study on the metabolic fate of ecabapide in the rat using ecabapide labelled with ^{14}C at the C-1 position of the ethylamine moiety has demonstrated that 42% of the radioactivity was excreted in the urine and 47% was recovered in the faeces within 48 h after oral dosing. Four major metabolites were purified from the urine and identified as phenylethylamine derivatives with the loss of the benzamide moiety (Tsumura *et al.* 1988). These data suggested that ecabapide was metabolized mainly via the C-N cleavage of the secondary amine at the 3-position of the 3-amino-*N*-methylbenzamide moiety prior to the excretion in urine.

Characterization of the metabolites of ecabapide with respect to the benzamide moiety is the subject of this report. We have investigated the metabolic disposition of ecabapide using ecabapide labelled with ^{14}C in the benzene ring of the benzamide moiety, and identified the structures of the urinary metabolites. In addition, we determined the metabolite profiles in rat plasma, urine and faeces.

* Author for correspondence.
‡ Present address: Experimental Technology Research Center, Daiichi Pharmaceutical Co., Ltd.

0049–8254/95 $10·00

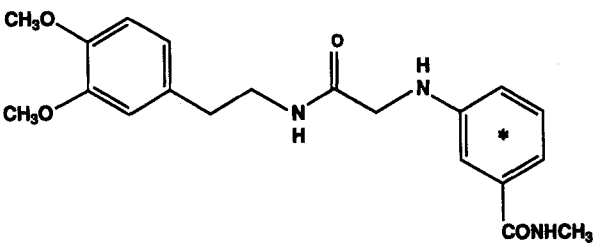


Figure 1. Chemical structure of ecabapide (DQ-2511). * ^{14}C-labelled position.

Materials and methods

Chemicals

Ecabapide and ^{14}C-ecabapide (labelled in the benzene ring of the benzamide moiety, Lot No. CP-1000; figure 1) were synthesized by Daiichi Pharmaceutical Co., Ltd and Daiichi Pure Chemical Co., Ltd respectively (Hosokami *et al.* 1992; Japanese Patent Application No. 85-280428). The specific activity of ^{14}C-ecabapide was 2·92 MBq/mg, and its radiochemical purity was > 98%. ^{14}C-Ecabapide was appropriately diluted with unlabelled ecapabide before use.

β-Glucuronidase (Type IX-A from *E. coli*) and D-saccharic acid 1,4-lactone were obtained from Sigma Chemical Co. (St Louis, MO, USA).

Synthesis of authentic metabolites

5-Acetylamino-2-hydroxy-N-methylbenzamide (**1**). To methanol (50 ml) was added $SOCl_2$ (15·6 g, 0·13 mol) at − 50°C, then 5-amino-2-hydroxybenzoic acid (5·0 g, 0·033 mol) was added with iced-water cooling. The resulting suspension was stirred at room temperature for 2 days and concentrated, the residue dissolved in water, and the solution made alkaline with 5% (w/v) aqueous $NaHCO_3$. The resulting solution was extracted with $CHCl_3$ (800 ml), and the organic layer was concentrated to give methyl 5-amino-2-hydroxybenzoate (**2**). To a suspension of **2** in $CHCl_3$ (100 ml), acetic anhydride (18·6 g) was added with stirring in an ice bath. Concentrated H_2SO_4 (1·5 ml) was added to the resulting mixture and the solution concentrated to yield an oil, which was dissolved in $CHCl_3$. The solution was washed with 5% (w/v) aqueous $NaHCO_3$, brine, and then dried. The solvent was removed, and the resulting solid was recrystallized from a mixture of acetonitrile, diethyl ether and hexane to give methyl 5-acetylamino-2-acetyloxybenzoate (**3**). A suspension of **3** in 40% aqueous methylamine (65 ml) was stirred at room temperature, and concentrated to dryness. The residue was recrystallized from a mixture of water and ethanol to give 2·8 g of **1** as crystals, m.p. 197–199°C (lit. m.p. 192°C). ^{1}H-nmr (DMSO-d_6) δ: 2·01 (3H, s), 2·81 (3H, d, J = 4·8 Hz), 6·85 (1H, d, J = 8·7 Hz), 7·45 (1H, dd, J = 2·4/8·7 Hz), 7·94 (1H, d, J = 2·4 Hz), 8·65 (1H, br d, J = 4·8 Hz), 9·78 (1H, s), 11·96 (1H, s). Calculated for $C_{10}H_{12}N_2O_3$: C, 57·69; H, 5·81; N, 13·45. Found: C, 57·60; H, 5·67; N, 13·39.

5-Acetylamino-2-hydroxybenzamide (**4**). A suspension of **3** (3·6 g, 0·014 mol) in 28% (w/v) aqueous ammonia (50 ml) was stirred at room temperature and the resulting clear solution concentrated under reduced pressure to dryness. The solid was recrystallized from ethanol to give 2·0 g of **4** as crystals, m.p. 210·5–211·5°C. ^{1}H-nmr (DMSO-d_6) δ: 2·00 (3H, s), 6·84 (1H, d, J = 8·7 Hz), 7·46 (1H, dd, J = 2·4/8·7 Hz), 7·76 (1H, br s), 7·89 (1H, d, J = 2·4 Hz), 8·23 (1H, br s), 9·75 (1H, s), 12·29 (1H, br s). Calculated for $C_9H_{10}N_2O_3$: C, 55·67; H, 5·19; N, 14·43. Found: C, 55·65; H, 5·33; N, 14·38.

3-Acetylamino-4-hydroxy-N-methylbenzamide (**5**). To a mixture of methanol (100 ml) and concentrated H_2SO_4 (19·0 g) was added 3-amino-4-hydroxybenzoic acid (10·0 g, 0·065 mol), and the resulting mixture refluxed for 24 h. The mixture was then concentrated, and poured into iced-water after cooling. The solution was brought to pH 7 with $NaHCO_3$ powder, and the resulting precipitate was collected by filtration. The solid was recrystallized from methanol, and dried *in vacuo* at 60–80°C to give 7·6 g methyl 3-amino-4-hydroxybenzoate (**6**). Compound **5** was synthesized from **6** by the same method as was used for the synthesis of **1**, as pale yellow crystals, m.p. 234–235°C. ^{1}H-nmr (DMSO-d_6) δ: 2·10 (3H, s), 2·73 (3H, d, J = 4·8 Hz), 6·87 (1H, d, J = 8·7 Hz), 7·45 (1H, dd, J = 1·6, 8·7 Hz), 8·14 (1H, br d, J = 4·8 Hz), 8·18 (1H, br s), 9·35 (1H, s) 10·31 (1H, br s). Calculated for $C_{10}H_{12}N_2O_3$: C, 57·69; H, 5·81; N, 13·45. Found: C, 57·69; H, 5·70; N, 13·40.

3-Acetylaminobenzamide (**7**). To a solution of 3-aminobenzamide (15·0 g, 0·11 mol) in dry pyridine (70 ml) was added acetic anhydride (70 ml) with stirring at room temperature, and the reaction mixture added to iced-water with stirring. The reaction mixture was concentrated to dryness, and the residue was recrystallized from methanol to yield 16·0 g of **7** as needles, m.p. 218·5–219·5°C. ^{1}H-nmr (DMSO-d_6) δ: 2·06 (3H, s), 7·32 (1H, br s), 7·35 (1H, t, J = 8·0 Hz), 7·52 (1H, d, J = 8·0 Hz), 7·75 (1H, br

d, $J = 8.0$ Hz), 7·91 (1H, br s,) 8·02 (1H, s), 10·04 (1H, s). Calculated for $C_9H_{10}N_2O_2$: C, 60·67; H, 5·66; N, 15·72. Found: C, 60·75 H, 5·65; N, 15·85.

*3-Acetylamino-*N*-hydroxymethylbenzamide* (**8**). A mixture of **7** (2·0 g, 0·011 mol) and 37% formaldehyde solution (5 ml) in tetrahydrofuran (30 ml) containing K_2CO_3 (2·0 g) was refluxed for 5 h. The organic layer was separated, concentrated *in vacuo* and the residue dissolved in water, and chromatographed on HP-20 (400 ml) eluting with 2 litres of water, and then with 1 litre of 3% tetrahydrofuran. The eluate which was obtained by eluting with 3% tetrahydrofuran was concentrated under reduced pressure at 40°C, and the residue dried *in vacuo* at room temperature to afford 1·8 g (77%) of **8** as crystals, m.p. 136–140°C. ^{1}H-nmr (DMSO-d_6) δ: 2·05 (3H, s), 4·69 (2H, t, $J = 7.2$ Hz), 5·64 (1H, t, $J = 7.2$ Hz), 7·37 (1H, t, $J = 8.0$ Hz), 7·51 (1H, d, $J = 8.0$ Hz), 7·76 (1H, d, $J = 8.0$ Hz), 8·04 (1H, s), 9·05 (1H, br t, $J = 6.4$ Hz), 10·07 (1H, s). Calculated for $C_{10}H_{12}N_2O_3$: C, 57·69; H, 5·81; N, 13·45. Found: C, 57·39; H, 5·93; N, 13·51.

*3-Acetylamino-*N*-methylbenzamide* (**9**). Compound **9** was synthesized from 3-amino-*N*-methylbenzamide as was used for the synthesis of **7**, as colourless needles, m.p. 174–175°C. ^{1}H-nmr (DMSO-d_6) δ: 2·05 (3H, s), 2·77 (3H, d, $J = 4.8$ Hz), 7·35 (1H, t, $J = 8.0$ Hz), 7·46 (1H, d, $J = 8.0$ Hz), 7·73 (1H, br d, $J = 8.0$ Hz), 8·00 (1H, s), 8·36 (1H, br d, $J = 4.8$ Hz), 10·05 (1H, s). Calculated for $C_{10}H_{12}N_2O_2$: C, 62·49; H, 6·29; N, 14·57. Found: C, 62·27; H, 6·25; N, 14·54.

Animal studies

Eight male Donryu rats (Japan SLC), weighing 200–220 g, were kept individually in metabolic cages and ^{14}C-ecabapide administered orally at a dose of 100 mg/kg (3·33 MBq/kg). Four animals were anaesthetized with diethyl ether at 2 h after dosing, and blood samples were taken from the abdominal aorta. Plasma was separated from the blood by centrifugation at 1800*g* for 15 min. For the remaining animals, urine and faeces were collected at intervals of 0–24 and 24–48 h after dosing, and stored at − 20°C until analysis.

Twelve unfasted male rats, weighing 250–300 g, were kept in stainless-steel metabolic cages. Ecabapide in 0·5% (w/v) sodium carboxymethylcellulose suspension was administered orally at a daily dose of 150 mg per animal to rats for 5 days. On the sixth day all rats received a single 40 mg per animal (740 kBq per animal) oral dose of ^{14}C-ecabapide. Urine was collected until 48 h after the last dose in dry ice-cooled bottles and used for isolation of the metabolites.

Instrumentation

The hplc system consisted of L-6000 and L-6200 pumps with a L-4000 UV detector (Hitachi, Tokyo, Japan), a Rheodyne injector (model 7120) with a 500 μl loop, and an on-line radioactivity detector Ramona 5-LS (Raytest, Straubenhardt, Germany).

Field-desorption mass (FD/MS) spectra and fast atom bombardment mass (FAB/MS) spectra were obtained using a JMX-HX 110 mass spectrometer (JEOL, Tokyo, Japan).

^{1}H-nmr spectra were measured with a GSX-500 FT-nmr spectrometer (JEOL, Tokyo, Japan), using CD_3OD or DMSO-d_6 solutions with tetramethylsilane as an internal standard.

Radioactivity analysis

Radioactivity in urine samples (100 μl) was measured directly using 10 ml Aquasol-2 (Dupont/NEN, USA) as scintillation cocktail. Radioactivity in faeces was measured after combustion of lyophilized samples. Faecal samples were lyophilized and ground to powder. About 10 mg of each powdered faeces sample was burned by combustion system (ASC-113 (Aloka, Tokyo, Japan)), and the trapping mixture of $^{14}CO_2$ was counted with 12 ml scintillation cocktail containing 0·58% PPO/0·02% Bis MSB in methanol/toluene (5:7, v/v).

Radioactivity in all samples was counted in a liquid scintillation spectrometer LSC-900 (Aloka). A quench correction was accomplished by external standardization.

Isolation of metabolites

Pooled urine (100 ml) was extracted with chloroform (fraction A). The remaining aqueous layer was lyophilized to dryness, redissolved in 50 ml water, and then applied to a Cosmosil 75C_{18} OPN column (100 × 18 mm i.d., Nacalai Tesque, Kyoto, Japan). The adsorbed radioactivity was eluted with 60 ml 10% (v/v) acetonitrile/water and 50 ml acetonitrile, successively, and fractionated in three groups (fractions B, C and D). For the purification of ecabapide metabolites, each fraction was evaporated and subjected to preparative hplc. The chromatographic systems were as follows: the column was a YMC-pack ODS-AM (300 × 10 mm i.d., YMC, Kyoto, Japan) or a TSKgel ODS-80$_{TM}$ (300 × 7·8 mm i.d., TOSOH, Tokyo, Japan); the eluents were (A) 35% (v/v) acetonitrile in 0·25 M phosphate buffer (pH 6·5), (B) 10% (v/v) acetonitrile in 0·1 M ammonium acetate (pH 7·0), (C) 10% (v/v) acetonitrile in 0·1 M ammonium acetate (pH 7·0), or 7% (v/v) acetonitrile in 0·3 M phosphate buffer (pH 6·5) and (D) 5% (v/v) acetonitrile in 17 mM phosphate buffer (pH 4·5); the flow rate was 2–3 ml/min. Ecabapide metabolites in fractions A–D were separated with the eluent system A, B, C and D respectively.

Analysis of metabolite profiles

Metabolite profiles in rat plasma, urine and faecal samples were obtained using the radio hplc system described below. Aliquots of the urine (0–24 h) and plasma (2 h) samples were directly analysed by hplc. Aliquots of powdered faeces were extracted with methanol, the extract concentrated and the residue redissolved in a small amount of methanol and injected on to the hplc column.

Chromatographic conditions were as follows; the column was a TSKgel ODS-80$_{TM}$ (150 × 4·6 mm i.d., TOSOH); the eluents were (E) 2% (v/v) acetonitrile in 40 mM phosphate buffer (pH 6·7), and (F) 70% (v/v) acetonitrile in 40 mM phosphate buffer (pH 6·7). Analyses were performed starting from F = 0–5% linearly in 10 min, F = 5% for 30 min, and then up to F = 50% linearly in 30 min. The flow rate was maintained at 1·0 ml/min.

Metabolites were quantified by a radioactive peak integration using a RAMONA Radio-Chromato-Graphic-System (Nuclear Interface, Munster, Germany). Recovery tests were performed by comparing the amount of radioactivity eluted the hplc column with the known amounts of radioactive samples injected.

Results

Excretion of radioactivity

After oral administration of ^{14}C-ecabapide at a single dose of 100 mg/kg to rat, approximately half of the total radioactive dose was excreted in urine and the rest was recovered from faeces; urinary and faecal excretion accounted for 36·7 ± 5·4 and 55·7 ± 11·8% of the radioactive dose respectively within 48 h after dosing.

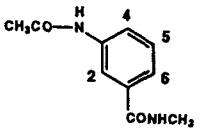
Identification of metabolites

The structures of ecabapide metabolites that possess the benzamide moiety were determined by spectroscopic techniques (FD/MS, FAB/MS, ^{1}H-nmr), and by comparison with synthetic reference compounds. ^{1}H-nmr spectral data for the isolated metabolites are summarized in table 1.

Mb-1. The FAB/MS spectrum of Mb-1 gave the protonated molecular ion $[M + H]^+$ and the $[2M + H]^+$ ion at m/z 209 and 417 respectively. Furthermore, when Mb-1 was derivatized with acetic anhydride in pyridine, it was converted to a new compound that showed $[M + H]^+$ at m/z 251 in the FAB/MS spectrum, 42 amu greater than that of Mb-1, indicating the addition of an acetyl group to the molecule. The ^{1}H-nmr spectrum of Mb-1 in DMSO-d_6 contained six protons corresponding to two methyl groups at 2·00 ppm (3H, s) and 2·80 ppm (3H, d, J = 4·8 Hz), three aromatic protons at 6·85 ppm (d, J = 8·7 Hz), 7·44 ppm (dd, J = 8·7/2·4 Hz) and 7·94 ppm (d, J = 2·4 Hz) respectively, and three NH and/or OH protons at 8·64, 9·79 and 11·96 ppm respectively. The presence of only three aromatic protons indicated that substitution occurred in the benzene ring. These data suggested that Mb-1 was a monohydroxylated derivative of 3-acetylamino-*N*-methylbenzamide. The position of the hydroxy group substitutent was thought to be either at the carbon-4 (C-4) or C-6 of 3-acetylamino-*N*-methylbenzamide by analysis of the splitting pattern of the three aromatic protons in the ^{1}H-nmr spectrum. These resonances are observed as an *ortho–meta* split doublet of doublets, an *ortho* split doublet and a *meta* split doublet. Metabolite Mb-1 was conclusively identified as 5-acetylamino-2-hydroxy-*N*-methylbenzamide by chemical synthesis of both of the authentic compounds, i.e. 3-acetylamino-*N*-methylbenzamide derivatives hydroxylated at the C-4 or C-6, and by comparison of their ^{1}H-nmr spectra with that of Mb-1 (figure 2).

Mb-2. FAB/MS spectrum of Mb-2 gave $[M + H]^+$ ion at m/z 193, which was 16 amu lower than that of Mb-1. ^{1}H-nmr spectrum of Mb-2 in DMSO-d_6 showed

Table 1. ^{1}H-nmr data for ecabapide metabolites.



						Aromatic								Glucuronyl		
Metabolite	Solvent	$-COCH_3$	$-NCH_3$	$-NC\underline{H}_2OH$	$-NCH_2O\underline{H}$	H-2	H-4	H-5	H-6	$-NH_2$	–NH–		Ar-OH	H1	H2-4	H5
Mb-1	DMSO-d_6	2·00 (s)	2·80 (d, J = 4·8 Hz)	—	—	7·94 (d, J = 2·4 Hz)	7·44 (dd, J = 2·4/8·7 Hz)	6·85 (d, J = 8·7 Hz)	—	—	8·64 (d, J = 4·0 Hz)	9·79 (s)	11·96 (s)	—	—	—
Mb-2	DMSO-d_6	2·05 (s)	2·76 (d, J = 4·8 Hz)	—	—	8·00 (s)	7·45 (d, J = 7·2 Hz)	7·35 (t, J = 8·0 Hz)	7·74 (d, J = 7·9 Hz)	—	8·37 (d, J = 4·8 Hz)	10·07 (s)	—	—	—	—
Mb-3	DMSO-d_6	2·00 (s)	—	4·73 (d, J = 5·6 Hz)	5·73 (br s)	7·99 (d, J = 2·4 Hz)	7·52 (dd, J = 2·4/8·7 Hz)	6·87 (d, J = 8·7 Hz)	—	—	9·23 (br t)	9·83 (s)	11·60 (s)	—	—	—
Mb-4	DMSO-d_6	2·00 (s)	—	—	—	7·90 (d, J = 2·4 Hz)	7·47 (dd, J = 2·4/8·7 Hz)	6·84 (d, J = 8·8 Hz)	—	7·76/8·25 (br s)	—	9·79 (s)	12·25 (s)	—	—	—
Mb-5	DMSO-d_6	2·05 (s)	—	4·69 (br t, J = 6·0 Hz)	5·66 (bt t)	8·04 (s)	7·51 (d, J ≈ 8·0 Hz)	7·37 (t, J = 8·0 Hz)	7·76 (d, J = 8·0 Hz)	—	9·07 (br t)	10·08 (s)	—	—	—	—
Mb-6	DMSO-d_6	2·05 (s)	—	—	—	8·01 (s)	7·51 (d, J ≈ 8·0 Hz)	7·35 (t, J = 8·0 Hz)	7·74 (d, J = 8·0 Hz)	7·31/7·90 (br s)	—	10·05 (s)	—	—	—	—
Mb-7	CD_3OD	2·10 (s)	2·92 (s)	—	—	7·94 (d, J = 2·4 Hz)	7·68 (dd, J = 2·4/8·7 Hz)	7·42 (d, J = 8·7 Hz)	—	—	—	—	—	4·93 (d, J = 6·4 Hz)	3·53 (m)	3·74 (m)

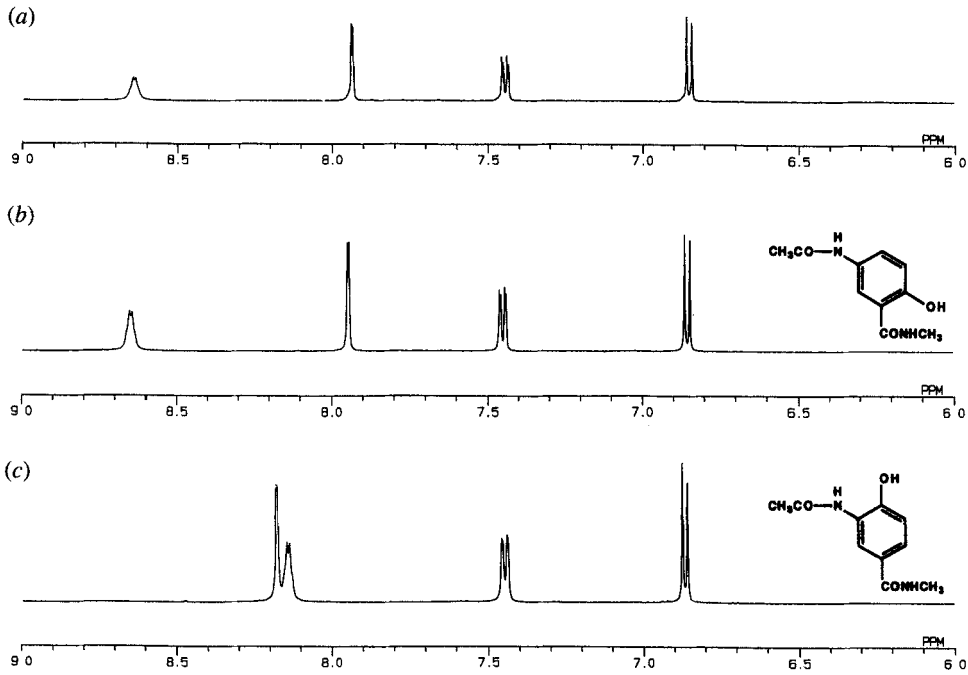


Figure 2. ^{1}H-nmr spectra of (*a*) Mb-1 isolated from rat urine, (*b*) authentic 5-acetylamino-2-hydroxy-*N*-methylbenzamide, and (*c*) authentic 3-acetylamino-4-hydroxy-*N*-methylbenzamide.

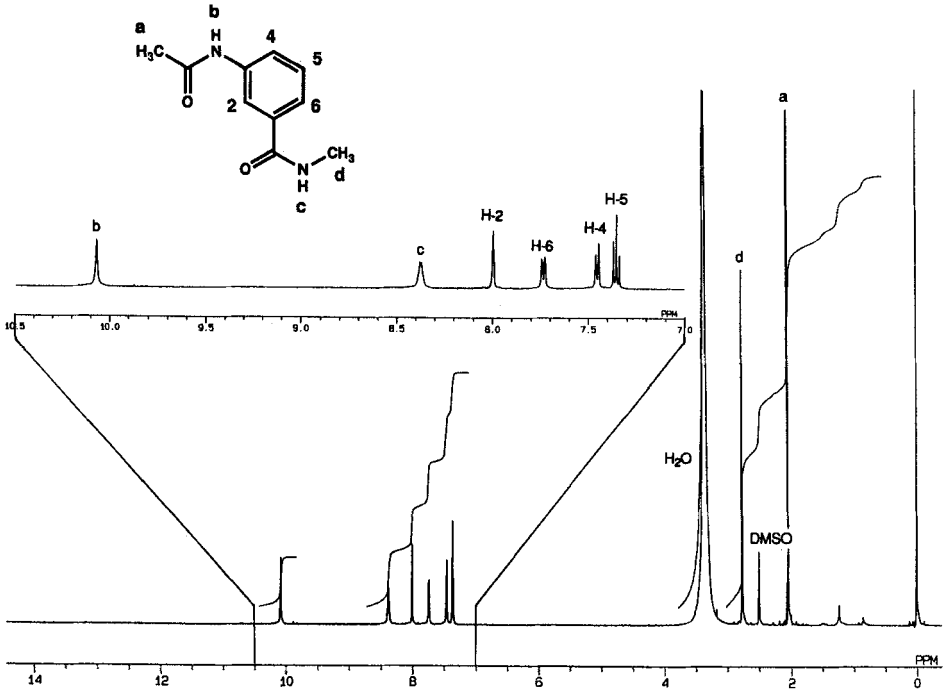


Figure 3. ^{1}H-nmr spectrum of Mb-2 isolated from rat urine.

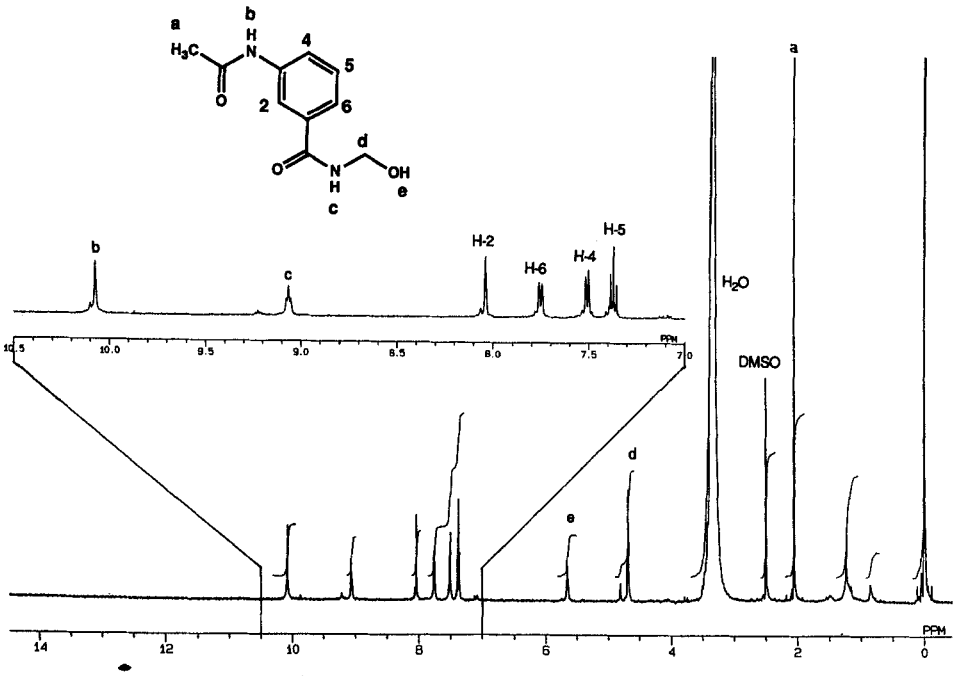


Figure 4. ^{1}H-nmr spectrum of Mb-5 isolated from rat urine.

signals for two methyl groups (2·05 ppm (s) and 2·76 ppm (d, $J = 4{\cdot}8$ Hz)), four aromatic protons (7·35 ppm (t, $J = 8{\cdot}0$ Hz), 7·45 ppm (d, $J = 7{\cdot}2$ Hz), 7·74 ppm (d, $J = 7{\cdot}9$ Hz) and 8·00 ppm (s)), and two NH and/or OH protons (8·37 ppm (d, $J = 4{\cdot}8$ Hz) and 10·07 ppm (s)) (figure 3). The aromatic protons are observed as one *ortho–ortho* split doublet of doublets, which appears as a triplet, two *ortho* split doublets with a small *meta* coupling, and one broad singlet. From these spectral characteristics, Mb-2 was assumed to be 3-acetylamino-*N*-methylbenzamide. Positive identification of Mb-2 was made by comparison of its spectral data and co-chromatography with an authentic sample of 3-acetylamino-*N*-methylbenzamide prepared by chemical synthesis.

Mb-5. The ^{1}H-nmr spectrum of Mb-5 in DMSO-d_6 showed three methyl protons at 2·05 ppm as a singlet, four aromatic protons at 7·37 ppm (t, $J = 8{\cdot}0$ Hz), 7·51 ppm (d, $J = 8{\cdot}0$ Hz), 7·76 ppm (d, $J = 8{\cdot}0$ Hz) and 8·04 ppm (s) respectively, methylene protons at 4·69 ppm (t, $J = 6{\cdot}0$ Hz), and three NH and/or OH protons at 5·66 ppm (br t, $J = 6{\cdot}0$ Hz), 9·07 ppm and 10·08 ppm respectively (figure 4). By comparison of the ^{1}H-nmr spectrum with that of Mb-2, it was suggested that the *N*-methyl group of Mb-2 was converted to an *N*-carbinol derivative in metabolite Mb-5. In addition, the FD/MS spectrum of Mb-5 showed a molecular ion at m/z 208, indicating that its molecular weight was 16 amu greater than that of Mb-2. Based on comparison of the spectral data of an authentic sample prepared by chemical synthesis, the structure of Mb-5 was identified as 3-acetylamino-*N*-hydroxymethylbenzamide.

Mb-6. ^{1}H-nmr spectrum of Mb-6 in DMSO-d_6 showed a single methyl group and four aromatic protons as seen in that of Mb-5. The remaining signals were broad singlets of three NH or OH protons. Thus, Mb-6 appeared to be the *N*-demethylated

derivative of Mb-2. The FAB/MS spectrum of Mb-6 also supported this assumption; it showed $[M + H]^+$ at m/z 179, indicating that the molecular weight of Mb-6 was 14 amu lower than that of Mb-2. The spectral data for Mb-6 is consistent with 3-acetylaminobenzamide, which was confirmed by comparison with an authentic sample.

Mb-3 and Mb-4. ^{1}H-nmr spectra of Mb-3 and Mb-4 in DMSO-d_6 were very similar to those of Mb-5 and Mb-6 respectively, and differed from Mb-5 and Mb-6 only in the part of the ^{1}H-mmr spectra corresponding to the aromatic protons. Mb-3 and Mb-4 each have only three aromatic protons. FD/MS spectrum of Mb-3 and FAB/MS spectrum of Mb-4 showed $[M]^+$ at m/z 224 and $[M + H]^+$ at m/z 195, which were 16 amu greater than those of Mb-5 and Mb-6 respectively. These data suggested that Mb-3 and Mb-4 were monohydroxylated derivatives of Mb-5 and Mb-6 respectively. Hydroxylation was assumed to have occurred at the C-2 both in MB-3 and Mb-4, corresponding to the position of hydroxylation observed in Mb-1. Thus, Mb-4 was proposed to be 2-hydroxylated derivative of Mb-6, and this was confirmed by comparison with a chemically synthesized authentic sample of 5-acetylamino-2-hydroxybenzamide. The structure of Mb-3 can be inferred directly as 5-acetylamino-2-hydroxy-*N*-hydroxymethylbenzamide, because of its similarity to Mb-5.

Mb-7. FAB/MS spectrum of Mb-7 gave $[M + H]^+$ and $[M + Na]^+$ ion peaks at m/z 385 and 407 respectively. Thus, the molecular weight of Mb-7 was 176 amu higher than that of Mb-1, and corresponded to a glucuronidated derivative of Mb-1. Mb-7 was hydrolysed by β-glucuronidase from *E. coli* and the reaction was inhibited by the addition of D-saccharic acid 1,4-lactone, a β-glucuronidase inhibitor. The retention time of the aglycone of Mb-7 on hplc was identical with that of Mb-1. ^{1}H-nmr spectrum of Mb-7 in CD_3OD, when compared with that of Mb-1, was similar and characteristic of a glucuronide. The splitting pattern of signals at 3·53 ppm (3H, m), 3·74 ppm (1H, m) and 4·93 ppm (1H, d, $J = 6{\cdot}4$ Hz) respectively was typical of that attributable to glucuronyl protons. No obvious change was observed in the signals for the two methyl groups of Mb-7 compared with those of Mb-1. From these findings Mb-7 was identified as a glucuronide of 5-acetylamino-2-hydroxy-*N*-methylbenzamide.

Metabolite profiles in rat plasma, urine and faeces

Seven metabolites and unchanged ecabapide were separated and analysed by radio-hplc under the chromatographic conditions described in *Materials and methods* in order to determine the metabolic pattern (figure 5).

The most abundant compound excreted into the urine was the unchanged drug, which accounted for 37·1% of the urinary radioactivity. The percentage composition of the metabolites in urine was Mb-1 (7·5%), Mb-2 (2·6%), Mb-3 (8·1%), Mb-4 (1·1%), Mb-5 (6·0%), Mb-6 (4·7%), and Mb-7 (8·5%). These metabolites and the unchanged drug represented approximately 80% of the total urinary radioactivity.

The major compound in plasma at 2 h after dosing was also found to be the unchanged drug and comprised 28·9% of the radioactivity present. Several unknown metabolites were also detected, but Mb-7 and Mb-1 were found in relatively high proportions and accounted for 9·9 and 6·4% respectively of the total radioactivity in the plasma samples.

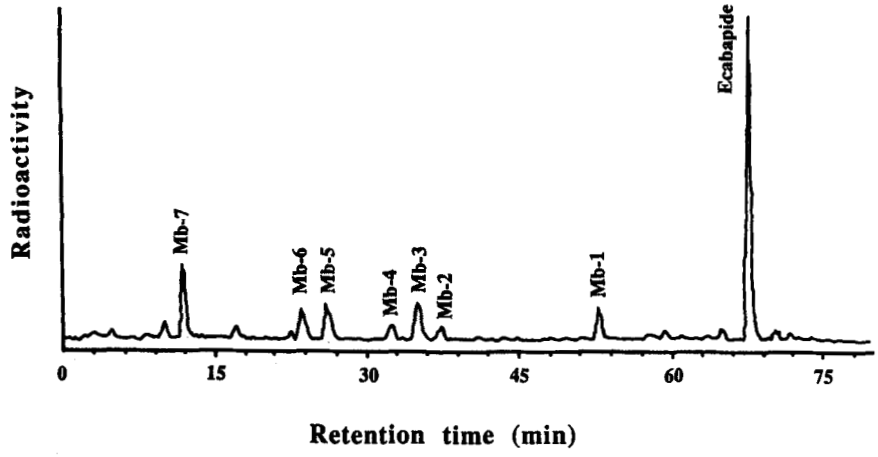


Figure 5. Hplc radiochromatogram showing the separation of ecabapide and its metabolites in rat urine.

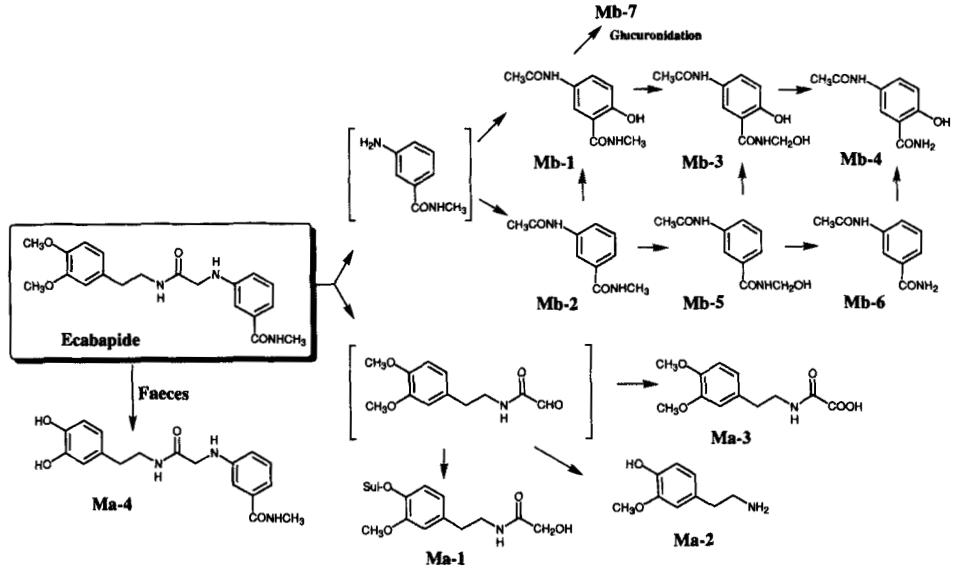


Figure 6. Postulated metabolic pathways of ecabapide in rat.

The metabolite profile of ecabapide in faeces was different from that in urine; the catechol derivative of ecabapide (Ma-4; figure 6) identified in the previous study (Tsumura *et al.* 1988) was confirmed to be the major component, and accounted for > 50% of the radioactivity in faeces.

Discussion

The previous study on ecabapide metabolism suggested that the main metabolic pathway appeared to be the C-N cleavage of aminoacetyl moiety, as four metabolites of the phenylethylamine derivatives (e.g. *N*-[2(3,4-dimethoxyphenyl)ethyl]oxamic acid, Ma-3; figure 6) were identified in rat urine (Tsumura *et al.* 1988).

The structures of ecabapide metabolites identified in this and previous studies

are summarized in figure 6. Seven new metabolites (Mb-1–7) have been isolated and identified in rate urine. These structures support the assumption that ecabapide was metabolized via *N*-dealkylation leading to the loss of 3-amino-*N*-methylbenzamide. The 3-amino-*N*-methylbenzamide moiety of ecabapide produced by C-N cleavage was found to undergo *N*-acetylation immediately to form 3-acetylamino-*N*-methylbenzamide, and was further metabolized exensively via *N*-demethylation and/or aromatic hydroxylation followed by glucuronidation. Since Mb-1 (5-acetylamino-2-hydroxy-*N*-methylbenzamide) and Mb-7 (the glucuronide of Mb-1) were relatively predominant metabolites, the aromatic hydroxylation pathway followed by glucuronidation was suggested to be the major metabolic pathway of the *N*-methylbenzamide moiety of ecabapide. The present study in rat showed that > 90% of the radioactive dose was recovered from urine and faeces within 48 h after administration, and that the urinary and faecal excretion profiles of the orally administered ^{14}C-ecabapide labelled in the benzene ring of the benzamide were almost the same as those of ^{14}C-ecabapide labelled at the C-1 position of the ethylamine moiety conducted previously (Tsumura *et al.* 1988). Therefore, it was confirmed that ecabapide and its metabolite do not accumulate in the body, and the disposition of the ecabapide metabolites which have the benzamide moiety was considered to be similar to those which have the phenylethylamine moiety.

In the process of *N*-demethylation of Mb-4 and Mb-6, *N*-hydroxymethyl derivatives (Mb-3 and MB-5 respectively) were isolated as their intermediates. In general, a carbinolamine is known to be an inherent unstable intermediate in *N*-demethylation. The presence of an α-carbonyl group adjacent to the nitrogen, however, increases the stability of the α-hydroxyalkyl metabolite (Gorrod and Temple 1976). Several studies have reported the isolation of the carbinolamine metabolites from this type of compounds in free and/or conjugated forms (Bull and Lindquist 1964, McMahon 1966, Allen *et al.* 1971, Lucier and Menzer 1971). In this study, carbinolamine intermediates such as Mb-3 and Mb-5 were purified and identified, which also revealed their stability.

References

ALLEN, J. G., BLACKBURN, M. J., and CALDWELL, S. M., 1971, The metabolism by man of an anticonvulsant oxadiazole. An unusual metabolic route for tertiary amides. *Xenobiotica*, **1**, 3–12,

ASANO, M., KUREBAYASHI, Y., RYOKAWA, Y., HASIZUME, T., and AKASHI, A., 1990, Antiulcer effects of 3-[[[2-(3,4-dimethoxyphenyl)ethyl]carbamoyl]methyl]amino-*N*-methylbenzamide in experimental gastric and duodenal ulcers. *Arzneimittel Forschung Drug Research*, **40**, 276–281.

BULL, D. L., and LINDQUIST, D. A., 1964, Metabolism of 3-hydroxy-*N*,*N*-dimethylcrotonamide dimethyl phosphate by cotton plants, insects, and rats. *Journal of Agricultural Federation Chemistry*, **12**, 310–317.

GORROD, J. W., and TEMPLE, D. J., 1976, The formation of an *N*-hydroxymethyl intermediate in the *N*-demethylation of *N*-methylcarbazole *in vivo* and *in vitro*. *Xenobiotica*, **6**, 265–274.

HOSOKAMI, T., KURETANI, M., HIGASHI, K., ASANO, M., OHYA, K., TAKASUGI, N., MAFUNE, E., and MIKI, T., 1992, Syntheses of 2-(3,4-dimethoxyphenyl)ethylamine derivatives and their antiulcer activities. *Chemical and Pharmaceutical Bulletin*, **40**, 2712–2719.

LUCIER, G. W., and MENZER, R. E., 1971, Nature of neutral phosphorus ester metabolites of phosphamidon formed in rats and liver microsomes. *Journal of Agricultural Federation Chemistry*, **9**, 1249–1255.

MCMAHON, R. E., 1966, Microsomal dealkylation of drugs. *Journal of Pharmaceutical Science*, **55**, 457–466.

TSUMURA, M., UDAGAWA, T., TANAKA, M., YAMADA, M., ONO, K., TAKEGOSHI, T., YOKOYAMA, I., and SHIGEMOTO, M., 1988, *In vivo* behaviour of novel antiulcer drug DQ-2511 in rat, dog, and human. In: *Proceedings of 108th Annual Meeting of the Pharmaceutical Society of Japan*, Abs 4E03 4-2, edited by the Pharmaceutical Society of Japan (Hiroshima: Nishiki print).