Metabolism of Clentiazem in Rats

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Following oral dosing of [14C]clentiazem to rats the metabolites in urine and bile were separated and their chemical structures were investigated by HPLC and GC-MS analyses. Fifteen basic, 6 acidic, 2 neutral and 4 conjugated metabolites were found in urine and/or bile.

Eight basic metabolites (MB1–8) were identified as the synthetic compounds; deacetyl clentiazem (MB1), N-monodemethyl clentiazem (MB2), deacetyl-N-monodemethyl clentiazem (MB3), deacetyl-O-demethyl clentiazem (MB4), N-monodemethyl-O-demethyl clentiazem (MB5), deacetyl-N-monodemethyl-O-demethyl clentiazem (MB6), O-demethyl clentiazem (MB7) and N-didemethyl clentiazem (MB8). The chemical structures of seven basic metabolites (MB9–15) were assigned as follows, deacetyl-N-didemethyl clentiazem (MB9), O-demethyl-N-didemethyl clentiazem (MB10), deacetyl-N-demethyl-N-didemethyl clentiazem (MB11), N-monodemethyl-2-hydroxy-methoxyphenyl clentiazem (MB12), deacetyl-2-hydroxy-methoxyphenyl clentiazem (MB13), deacetyl-N-monodemethyl-2-hydroxy-methoxyphenyl clentiazem (MB14) and deacetyl-N-didemethyl-2-hydroxy-methoxyphenyl clentiazem (MB15).

Four acidic metabolites were identified as the synthetic compounds: (+)-(2S,3S)-3-(acetoxy)-8-chloro-3,4-dihydro-2-(4-methoxyphenyl)-4-oxo-1,5-benzothiazepin-5(2H)-acetic acid (MA1), deacetyl-MA1 (MA2), O-demethyl-MA1 (MA3) and deacetyl-O-demethyl-MA1 (MA4) and the two remaining acidic metabolites, MA5 and MA6, were also detected. Two neutral metabolites were identified as the synthetic compounds: (+)-(2S,3S)-3-(acetoxy)-8-chloro-3,4-dihydro-2-(4-methoxyphenyl)-4-oxo-1,5-benzothiazepin-5(2H)-acetonitrile (MN1) and deacetyl MN1 (MN2). Other two metabolites conjugated with glucuronic acid were found in bile and the structures were presumed to be 8-chloro-2,3-dihydro-3-hydroxy-5-(2-hydroxyethyl)-2-(4-hydroxyphenyl)-1,5-benzothiazepin-4(5H)-one (MN3) and 2-methoxybenzyl MN3 (MN4). The glucuronide or sulfate of MA4 was also detected.

These metabolites were formed by a number of pathways including deacetylation, deamination, N-demethylation, O-demethylation, aromatic hydroxylation and conjugation.

Keywords clentiazem; drug metabolism; rat; urine; bile

Introduction

A new Ca2+-channel blocking agent, clentiazem, (2S,3S)-3-acetoxy-8-chloro-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5H)-one maleate, TA-3090, is a derivative of diltiazem in which a chlorine atom is introduced at the 8-position of the benzothiazepine skeleton. Diltiazem is widely used in the treatment of a variety of cardiovascular disorders such as angina pectoris, arrhythmia and hypertension. In animal experiments, clentiazem has shown long-lasting hypotensive and antihypertensive effects, cardiovascular effects and calcium antagonistic spasmytic activities.

The long duration of its pharmacological actions is in accordance with its pharmacokinetic profile; namely, after oral administration to rats, the plasma half-life of clentiazem was about 2.4 times longer than that of diltiazem. It was thought that the long duration of action of clentiazem might be related to its high affinity for organs and tissues and/or its metabolism, different from that of diltiazem.

Diltiazem is metabolized extensively to a number of basic, acidic and conjugated metabolites by deacetylation, N-demethylation, O-demethylation, aromatic hydroxylation, oxidative deamination or conjugation.

In the present paper, we describe the separation and characterization of the acidic, basic, neutral and conjugated metabolites present in urine and bile after oral dosing of [14C]clentiazem to rats.

Materials and Methods

Chemicals [14C]Clentiazem (Fig. 1) was synthesized as described in the previous report from [3H]ethyldimethylaminoethyl chloride hydrochloride (380.36 MBq/mmol, New England Nuclear, MA, U.S.A.) as the starting radioactive material. The specific radioactivity was 1.35 MBq/mg. The radiochemical purity determined by thin-layer chromatography was greater than 98%.

[14C]Clentiazem was diluted with unlabeled clentiazem to obtain a suitable specific radioactivity when the dosing solutions were prepared. N-(3-trimethylsilyl)trifluoroacetamide (BSTFA) and N-methylisobutyl (fluoroacetamide) (MBTFA) were obtained from Nacalai Tesque (Kyoto, Japan). Dimethylethylsilylimidazole (DMESI) was purchased from Tokyo Kasei (Tokyo, Japan). β-Glucuronidase from Helix pomatia (type H-1) was purchased from Sigma Chemical Co. (MO, U.S.A.). Other chemicals used, including solvents, were the best grade available.

Animals Male Sprague-Dawley rats were purchased from Charles River Japan (Kanagawa, Japan). The rats were fasted overnight prior to dosing with [14C]clentiazem, but had free access to water.

Dosage [14C]Clentiazem was dissolved in water at a concentration of 6 mg/ml. The drug solution was administered by gastric intubation at a dose of 30 mg/kg.

Sample Collection Urine: [14C]Clentiazem was administered orally to 5 rats weighing 200–215 g (7 weeks old) or to 10 rats weighing 200–215 g. The samples were collected for 3 days after dosing.
280—300 g (9 weeks old). The urine was collected in containers cooled with dry ice for a period of 0—24 h (24-h urine) or 0—6 h (6-h urine).

Bile [14C]Clentiazem was given orally to rats that had undergone bile duct cannulation weighing 220—230 g (8 weeks old). The bile excreted through the cannula was collected into test tubes cooled with dry ice for a period of 0—24 h.

Fractionation and Separation of Metabolites in Urine and Bile. The metabolites in urine and bile were fractionated by solvent extraction. First, the urine and bile samples were acidified to pH 2 with 1 N HCl. These samples were then extracted 3 times with 2 volumes of tert-butyl methyl ether (t-BME) to obtain the acidic and neutral metabolite fractions. The residual aqueous layer was adjusted to pH 8 with 1 N NaOH and extracted 3 times with 2 volumes of t-BME to obtain the basic metabolite fraction. The residual aqueous layers, containing water-soluble metabolites, were subjected to enzymatic hydrolysis. The t-BME layers containing acidic and neutral metabolites were shaken once with 0.1 M Na2HPO4. The remaining t-BME contains the neutral metabolites. The aqueous layer was adjusted to pH 2 with 1 N HCl and extracted 3 times with 2 volumes of t-BME to obtain acidic metabolites.

Each t-BME fraction was evaporated to dryness using a Concentrator (CC-180, TOMY Seiko, Tokyo, Japan) in vacuo. The residues dissolved in 50% dioxane were passed through a membrane filter (0.45 μm, Japan Merck, Darmstadt, Germany). The acidic, basic and neutral metabolite fractions were separated by HPLC with collecting fractions at 0.5 min intervals. The radiochromatograms were obtained after measurement of the radioactivity of the aliquots for every fraction. The fractions of each radioactive peak were combined. The combined fractions from the basic metabolite fraction were applied to a Sep-Pak C18 cartridge (Waters, MA, U.S.A.). After washing the cartridge with water, the metabolites were eluted with methanol. The eluates were evaporated to dryness using a Concentrator in vacuo. The combined fractions from the acidic and neutral metabolite fractions were evaporated to dryness in vacuo using a Concentrator.

Each of the dried samples was dissolved in a mixture of water and dioxane (50:50, v/v). After passage through a membrane filter, the filtrates were subjected to HPLC analysis. HPLC was carried out using a Hitachi chromatograph equipped with a 655-61 processor, a 655-15 liquid chromatograph and a 638 UV absorbance monitor (at 244 nm). The column used was a Nova Pak C18 cartridge (8 mm i.d. x 10, 5 μm, Waters, MA, U.S.A.) and it was used with a Radial Compression Separation System RCM-100 (Waters).

Two mobile phases for the separation of metabolites from 24-h urine and bile were prepared: (A) acetonitrile–tetrahydrofuran (7:3, v/v), (B) 0.1 M sodium phosphate buffer at pH 7.9 (adjusted with triethylamine). First, the metabolite fractions were diluted with phosphate acid for acidic metabolites. These solutions were used for the combined isocratic and gradient elution in which the ratio of solvent (A): (B) changed from 25:75 to 25:75 then 50:50 and finally 50:50, v/v at 0, 1, 25 and 30 min after sample injection. The flow rate was 2.0 ml/min. The basic metabolites from the 6-h urine were also separated by HPLC under similar conditions.

GC-MS Derivatization: The dried samples from the basic metabolite fraction were treated with 20 μl of 10% BSTFA (or DMSF/acetoni trie for 30 min at room temperature, and then with 5 μl of MBTFA. The resultant solution was allowed to stand for 30 min at room temperature.

The samples from the acidic and neutral metabolite fraction were treated with 20 μl of 10% BSTFA acetoni trie for 30 min at room temperature.

Measurement of GC-MS: Mass spectra were obtained with a Hitachi M-80A gas chromatograph-mass spectrometer equipped with a Hitachi M-003 data processing system in the positive ion electron ionization (PIE I) or negative ion chemical ionization (NIC I) mode. The conditions in the PIE I mode were as follows: column, G-100 (Chemicals Inspection and Testing Institute, Tokyo, Japan); column oven temperature, 250—280 °C; injection port temperature, 270 °C; interface temperature, 280 °C; ion-source temperature, 180 °C; carrier gas, He 20 ml/min; ionization energy, 20 eV; total emission current, 100 μA; ion accelerating voltage, 3 kV.

The conditions in the NIC I mode were as follows: column, 1.5% OV-17 on 100/120 Gas Chrom Q (Gasukuro Kogyo, Tokyo, Japan); column oven temperature, 250—280 °C; injection port temperature, 270 °C; interface temperature, 270 °C; ion-source temperature, 170 °C; carrier gas, He 30 ml/min, ionization energy 100 eV; total emission current, 100 μA; ion accelerating voltage, 3 kV; reagent gas, isobutane (ca. 1 Torr).

Measurement of Radioactivity An aliquot of each sample was dis- solved in a toluene-Triton X-100 based scintillation cocktail (2,5-diphenyloxazole 12 g, dimethyl 1,4-bis-(2,5-phenyloxazol)benzene 0.2 g, Triton X-100 1 L, toluene 2 L). The radioactivity was measured with a liquid scintillation spectrometer (Tri-Carb 460CD, Packard Instrument Co., Inc., IL, U.S.A.). Quenching was corrected by the external standard method.

Enzymatic Hydrolysis The aqueous metabolite fractions of rat bile were applied to an Amberlite XAD-2 (Organos Co., Ltd., Tokyo, Japan) column. The adsorbed radioactive metabolites were eluted with CH3OH. The CH3OH eluate was evaporated to dryness under reduced pressure. The residue was dissolved in 0.2 M acetate buffer (pH 5). The solution was divided into five samples for enzymatic hydrolysis as follows: to sample No. 1 was added β-glucuronidase, HPO4 (76 mm) and saccharic acid 1,4-lactone (4 mm); to sample No. 2 was added β-glucuronidase and HPO4 (76 mm); to sample No. 3 was added β-glucuronidase and saccharic acid 1,4-lactone; to sample No. 4 was added only β-glucuronidase; sample No. 5 was treated as a control (without enzyme). The β-glucuronidase, from Helix pomatia, contained sulfatase as impurity and the β-glucuronidase and sulfatase activities were 5500 and 360 units per sample, respectively. Saccharic acid 1,4-lactone and HPO4 were used to inhibit the β-glucuronidase and sulfatase activities.

The mixtures were incubated for 1 h at 37 °C. The reaction was stopped by addition of 0.1 N NaOH. The reaction mixture was then treated with t-BME under acidic conditions followed by extraction under basic condition. The t-BME extracts were dried using a Concentrator under reduced pressure. The residues were dissolved in a mixture of water and dioxane (50:50, v/v). Subsequently, these samples were analyzed by HPLC using a radio detecting detector (LAMONA TLS, Laytest, Straubenhardt, FRG).

Results

Urinary and Biliary Excretion of Metabolites The urinary and biliary excretion of radioactivity up to 24 h after oral dosing of [14C]Clentiazem (30 mg/kg) was 8.6% and 70.9% of the dose, respectively. The radioactive metabolites in the urine and bile were separated into lipophilic acidic, basic and neutral metabolite fractions, and an aqueous metabolite fraction by solvent extraction. The radioactivity in the basic metabolite fraction of 24-h urine and bile was 53.5% of the total urinary radioactivity and 4.4% of the total biliary radioactivity, respectively. The radioactivity in the acidic metabolite fraction extracted from the 24-h urine was only 6.5% of the total urinary radioactivity, while that from the bile amounted to 25.9% of the total biliary radioactivity. The radioactivity in the neutral metabolite fraction of bile was 3.0% of the total biliary radioactivity.

The urinary excretion was 3.3% up to 6 h after oral dosing. The lipophilic basic metabolite fraction of the 6-h urine accounted for 71.2% of the total urinary radioactivity.

HPLC of Basic Metabolites Fourteen and thirteen radioactive peaks were found in the HPLC radiochromatograms of the basic metabolite fractions in the 24-h urine and bile, respectively (Fig. 2a, b).

In the HPLC-radiochromatograms of the urinary basic metabolite fraction (Fig. 2a), peaks No. 14, 12, 10, 8, 9, 6, 4, 10 and 10 appeared at the same retention times as those of clentiazem and the synthetic MB1, MB2, MB3, MB4, MB5, MB6, MB7 and MB8, respectively. The structures of the metabolites contained in the HPLC peaks No. 9, 6 and 3 were assigned by GC-MS to be MB9, MB11, MB14 and MB15, respectively. MB4 in peak No. 9 could not be confirmed by GC-MS analysis. No metabolites could be detected by GC-MS analyses in peaks No. 1, 2, 5, 7, 11 and 13.

In the HPLC-radiochromatogram of the biliary basic
metabolite fraction (Fig. 2b), the retention times of peaks No. 13, 11, 9, 7, 8, 6, 4, 9 and 10 were in accordance with those of clentiazem and the synthetic MB1, MB2, MB3, MB4, MB5, MB6, MB7 and MB8, respectively. It was confirmed by GC-MS that MB9, MB11, MB12 and MB14 were contained in peaks No. 6, 5, 6 and 6, respectively, but the presence of MB7 in peaks No. 9 could not be confirmed. In peaks No. 1—3 and 12 no mass spectra were obtained suggesting the presence of metabolites.

**Chemical Structures of Basic Metabolites**

The structures of the metabolites in each radioactive peak were analyzed by GC-MS after derivatization with BSTFA and/or MBTFA.

The major fragmentation patterns and the fragment ions and mass spectra of clentiazem and its basic metabolites in PIEI mode are shown in Figs. 3 and 4.

In PIEI mass spectra, the molecular ion peaks of most of the basic metabolites are as small as that of clentiazem. The base peak ion at m/z 58 in the mass spectra of clentiazem, MB1, MB4 or MB7 is produced by α-cleavage of the N-dimethylaminoethyl group (fragment ion (b) in Fig. 3). The N-monodemethyl metabolites after derivatization with MBTFA, on the other hand, yielded a fragment ion at m/z 154 by fission of the C-N bond, leaving the charge on the trifluoroacetylated side-chain (fragment ion (c)). The peaks at m/z 58 and m/z 154 show the substituent groups at the nitrogen atom on the aminoethyl side chain, i.e., N-dimethyl- or N-monomethyl-. The
Fig. 4. Mass Spectra of Clentiazem and Basic Metabolites
Each basic metabolite was analyzed by GC-MS in PIEI mode after TMS and/or TFA derivatization.
primary amine group in MB8, MB9, MB11 and MB15 (N-didemethyl metabolites) after treatment with MBTFA might form the ion of the N-monoTFA-aminoethyl group (m/z 140). However, the ion peak at m/z 140 is almost undetectable. In PIEIMS the characteristic fragment ion (a) indicates the substituents (R₃ and R₄) at the 2- and 3-positions of the benzothiazepine skeleton. In the 3-O-acetyl compounds these ions are formed by further loss of a ketone from the acetyl moiety of the corresponding ions. In the mass spectra of the 2-(4-methoxyphenyl)-3-O-acetyl compounds, 2-(4-methoxyphenyl)-3-OTMS compounds (deacetyl metabolites, 2-(4-OTMS-phenyl)-3-O-acetyl compounds (O-demethyl metabolite) or 2-(4-OTMS-phenyl)-3-OTMS compounds (deacetyl-O-demethyl metabolites) this ion peak is seen at m/z 150, 222, 208 or 280. The 3-O-acetyl metabolites form the fragment ion (M⁺ – 60) by liberating acetic acid. The same fragmentation was observed in diltiazem and some of its metabolites.⁸¹

A prominent peak, the (M⁺ – 90) ion, afforded by liberation of TMSOH is seen in the spectra of the TMS derivatives of MB3 and MB6.

The chemical structures of the basic metabolites, MB1—15 (except MB10 and MB13), were assigned on the basis of the appearance of those characteristic ions described above. Furthermore, MB1—8 were identified as the synthetic compounds, i.e., deacetyl clentiazem (MB1), N-monomethyl clentiazem (MB2), deacetyl-N-monomethyl clentiazem (MB3), deacetyl-O-demethyl clentiazem (MB4), N-monomethyl-O-demethyl clentiazem (MB5), deacetyl-N-monomethyl-O-demethyl clentiazem (MB6), O-demethyl clentiazem (MB7) and N-didemethyl clentiazem (MB8).

The structures of the basic metabolites (MB9, MB11, MB12, MB14, and MB15) were characterized by GC-MS in PIEI mode as follows.

1) MB9 In the mass spectrum of the TMS-TFA derivative of MB9 (Fig. 4), the molecular ion (M⁺) peak was observed at m/z 546 with the base peak ion at m/z 222 and characteristic ions at m/z 531 and 427. On the other hand, the peaks at m/z 58 and at m/z 154 were not seen in the spectrum. The base peak ion at m/z 222 shows deacetylated metabolites such as MB1 and MB3. Furthermore, the m/z value of the M⁺ (m/z 546) was 68 amu larger than that of the TMS derivative of MB1, and 14 amu less than that of the TMS-TFA derivative of MB3. The difference of 68 or 14 amu suggests a structural change from –N(CH₃)₂ (MB1) or –N(CH₂)COCF₃ (MB3) to –NHCOCF₃ (MB9). These findings, therefore, suggest that MB9 is deacetylated and N-didemethylated clentiazem.

2) MB11 In the mass spectrum of the TMS-TFA derivative of MB11 (Fig. 4), the apparent M⁺ ion was present at m/z 604. The base peak ion at m/z 280 indicated that MB11 is a deacetylated and O-demethylated metabolite like MB4 or MB6. No fragment ions appeared at m/z 58 and 154. The m/z value of the M⁺ of MB11 was 68 amu larger than that of MB4 or 14 amu less than that of MB6. These differences suggest the same structural change as that between MB9 and MB1 or MB3, showing that MB11 is a N-didemethylated metabolite like MB9. This suggests, therefore, that MB11 is deacetylated, O-demethylated and N-didemethylated clentiazem.

3) MB12 In the mass spectrum of the TMS-TFA derivative of MB12 (Fig. 4), the M⁺ was observed at m/z 618 which was 88 amu larger than that of MB2. The base peak ion appeared at m/z 238 and the characteristic ions were detected at m/z 558 (M⁺ – 60), 530 (M⁺ – 88), 362 and 154 (fragment ion c). The (M⁺ – 60) ion showed that this metabolite is a 3-O-acetyl compound. The m/z value of the base peak ion (m/z 238) was 88 amu larger than that of the corresponding ion at m/z 150 of clentiazem, MB2 or MB8. The same amu difference was observed between the ions at m/z 362 and the corresponding ion (m/z 274) of MB2 or MB8. The difference of 88 amu suggests that the 2-phenyl group of clentiazem has an additional OH group which is derivatized to OTMS. Therefore, MB12 is a metabolite having an additional OH group at the 2-phenyl group of MB2.

4) MB14 In the mass spectrum of the TMS-TFA derivative of MB14 (Fig. 4), the M⁺ at m/z 648 and the base peak ion at m/z 310 were 88 amu larger than those of MB3 at m/z 560 and 222, respectively. This amu difference suggests that MB14 has an additional OH group at the 2-phenyl group of MB3. Furthermore, the fragment ion at m/z 154 indicates an N-monomethylated metabolite such as MB2 and MB3. Therefore, MB14 is presumed to be a metabolite having an additional OH group at the 2-phenyl group of MB3.

5) MB15 The mass spectrum of the TMS-TFA derivative of MB15 (Fig. 4), showed the M⁺ at m/z 634, the base peak ion at m/z 310 and the characteristic fragment ions at m/z 619, 605 and 544. The base peak ion at m/z 310 suggests a deacetylated metabolite having an additional OH group at the 2-phenyl group as described in MB14. No fragment ions appeared at m/z 154 and 58. From these results the mass number of the partial structure of MB15, excluding the substituent at the 5-position, is calculated to be 494 which is 140 amu less than that of M⁺. This value is the same as CH₃CH₂NH–TFA. Therefore, MB15 has an additional OH group at the 2-phenyl group of MB9.

The basic metabolites MB10 and MB13 were not found in the 24-h-urine when analyzed by GC-MS in PIEI mode. These metabolites were characterized by analyzing the sample from the 6-h-urine by GC-MS in NICI mode.

6) MB10 The mass spectrum of the DMESI–TFA derivative of MB10 in NICI mode (Fig. 5) showed prominent peaks at m/z 588, 528 and 324. The first two ions are presumed to be M⁺ and the M⁺ – 60 ion. The ion at m/z 528 suggests that this metabolite contains an acetyl group at the 3-position of the benzothiazepine. The M⁺ and M⁺ – 60 ion were 72 amu larger than the M⁺ and M⁺ – 60 ions (m/z 516, 456) of the TFA derivative of MB8. The difference of 72 amu suggests the difference between 4-O-DMESI (MB10) and 4-OCH₃ (MB8) at the 2-phenyl

![Fig. 5. Mass Spectrum of MB10](image-url)

Basic metabolite, MB10, was analyzed by GC-MS in NICI mode after DMESI and TFA derivatization.
group. Hence, MB10 might be O-demethylated and N-dimethylated clenitazem.

7) MB13 When the TMS or DMESI derivative of MB13 was analyzed by GC-MS in NICI mode, a prominent peak was seen at m/z 566 or 594 (spectra not shown). There were no other peaks to suggest the structures of fragment ions from the compound. Thus, the peak at m/z 566 or 594 was presumed to be M⁻ peak, the mass number being 30 amu larger than that of the TMS or DMESI derivative of MB4. Since there was no fragment ion indicating M⁻ − 60, this metabolite might be deacetylated.

The difference of 30 amu was thought to suggest the same structural difference as that between the derivatives of MB5 and MB12, MB6 and MB14 or MB11 and MB15. Hence, MB13 could be a metabolite having an additional OH group at the 2-phenyl group of MB1.

Acidic Metabolites Seven and six radioactive peaks were found during HPLC of the acidic metabolite fractions from urine and bile, respectively.

In the HPLC-radiochromatogram of the acidic metabolite fraction of urine shown in Fig. 6a, peaks No. 7 and 5 corresponded to those of authentic MA2 and MA4, respectively. No radioactive peak corresponding to MA1

![Diagram](image_url)

Fig. 6. HPLC-Radiochromatograms of Acidic Metabolite Fraction of Urine a) and Bile b)

Rat urine and bile, excreted over 24h after oral dosing of [¹⁴C]clenitazem, were extracted with r-BME at pH 2. The r-BME extract was shaken once with 0.1 m Na₂HPO₄. The aqueous layer was adjusted to pH 2 with 1 m HCl and extracted 3 times with 2 volumes of r-BME to remove the acidic metabolites. The r-BME extract containing acidic metabolites was analyzed by HPLC. The radioactivity was measured in each fraction of the eluates by radiochromatography.

![Diagram](image_url)

Fig. 7. Structures and m/z Values of Characteristic Ions in the PIEI MS of Acidic Metabolites

<table>
<thead>
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<th>compounds R₁</th>
<th>R₂</th>
<th>M⁺</th>
<th>(a)</th>
<th>(b)</th>
<th>(c)</th>
<th>(d)</th>
<th>(e)</th>
<th>(f)</th>
<th>other prominent ions</th>
</tr>
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<tbody>
<tr>
<td>MA1</td>
<td>X</td>
<td>Ac</td>
<td>507</td>
<td>150</td>
<td>121</td>
<td>274</td>
<td>300</td>
<td>419</td>
<td>447 77 151</td>
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<tr>
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<td>X</td>
<td>TMS</td>
<td>537</td>
<td>222</td>
<td>121</td>
<td>274</td>
<td>300</td>
<td>419</td>
<td>447 249 508 522</td>
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<tr>
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<td>Y</td>
<td>Ac</td>
<td>565</td>
<td>208</td>
<td>179</td>
<td>332</td>
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<td>209</td>
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<td>(300)</td>
<td>507</td>
<td>535 121 436 596 610</td>
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</tbody>
</table>

: base peak ion
( ) : postulated ion
and MA3 was found and no metabolites could be detected in the other peaks.

In the HPLC-radiochromatogram of the acidic metabolite fraction of bile shown Fig. 6b, MA1 (peak 6), MA2 (peak 5), MA3 (peak 4) and MA4 (peak 3) were identified as authentic MA1—4 by GC-MS and HPLC. The peaks of MA3 and MA4 contained MA5 and MA6, respectively. No metabolites could be detected in peaks 1 and 2 by GC-MS.

The metabolites in these radioactive peaks were analyzed by GC-MS in PBEI mode after derivatization with BTF. Six acidic metabolites, MA1—6, were characterized as follows.

1) **MA1—4** The characteristic fragment ions and mass spectra of the TMS derivatives of these acidic metabolites are shown in Figs. 7 and 8.

Unchanged diltiazem and clenitiazem, and their basic metabolites form a characteristic fragment ion at m/z 58 or 154 which is derived from the side chain, 5-CH$_2$-CH$_2$N-(CH$_3$)$_2$ or 5-CH$_2$-CH$_2$N(CH$_3$)$_2$-TFA. These fragment ions, however, were not found in the acidic metabolites of clenitiazem or in those of diltiazem. These observations suggest that the side chains of the acidic metabolites are different from those of clenitiazem and its basic metabolites. On the other hand, the characteristic fragment ions (fragment ion (a)) appearing in the mass spectra of the basic metabolites were also seen in MA1, MA2, MA3 and MA4 at m/z 150, 222, 208 and 280, respectively. The mass number of these fragment ions depends on the substituents at the 4-position in the 2-phenyl moiety and the 3-position in the benzothiazepine skeleton. The fragmentation patterns and structures of these ions (fragment ion (a)) produced from MA1, MA2, MA3 and MA4 suggest that the substituents at the 2- and 3-positions of the benzothiazepine skeleton in these acidic metabolites are the same as those in the basic metabolites. The acidic metabolites, MA1—4, of clenitiazem and the corresponding metabolites of delitzamex exhibited similar mass spectra.

The molecular ions of all the acidic metabolites have odd mass numbers, indicating that the numbers of nitrogen atoms in the structures are odd, according to the nitrogen rule. These data such as the mass numbers of the molecular ions and the structures of the proposed fragment ions (fragment ions (a)—(f) in Fig. 7) support the structures of MA1—4. From these mass spectral data, it was postulated that these acidic metabolites of clenitiazem have an acidic side chain, CH$_2$COOH, at the 5-position and their various substituents at the 2- and 3-positions of the benzothiazepine skeleton as described for the acidic metabolites of diltiazem.

The acidic metabolites (MA1—4) were identified by comparison with the HPLC and GC-MS data for the corresponding synthesized compounds, (+)-(2S,3S)-3-acetyl-8-chloro-3,4-dihydro-2-(4-methoxyphenyl)-4-oxo-1,5-benzothiazepin-5(2H)-acetic acid (MA1), deacetyl MA1 (MA2), O-demethyl MA1 (MA3) and deacetyl-O-demethyl-MA1 (MA4), respectively.

Synthetic reference compounds for MA5 and MA6 were not available. Therefore, the structures of these metabolites deduced as follows.

2) **MA5** In the mass spectrum of the TMS derivative of MA5 (Fig. 9), the ion of largest mass number, m/z 595, was assumed to be the M$^+$. The base peak ion of m/z 238 was thought to be a fragment ion (a) (Fig. 7). Other characteristic fragment ions are seen at m/z 535 (M$^+ - 60$, fragment ion (f)), 507 (M$^+ - 88$) and 362 (fragment ion (c)). The fragment ion (f) suggests that the substituent at the 3-position of the benzothiazepine skeleton is an acetyloxy group. The mass numbers of these M$^+$ and

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**Fig. 8.** Mass Spectra of Acidic Metabolites (MA1—4)

Each acidic metabolite was analyzed by GC-MS in PBEI mode after TMS derivatization.

**Fig. 9.** Mass Spectra of MA5 and MA6
characteristic ions were 88 and 30 amu larger than those of the corresponding ions of MA1 (m/z 507, 150, 419, 274) and MA3 (m/z 565, 208, 477, 332), respectively. These differences in mass numbers correspond to the changes of substituents, i.e., H→OTMS (+88) or H→OCH₃ (+30). Thus, MA5 is suggested to have an additional OH group in the 2-phenyl ring of MA1 or an additional OCH₃ group in the 2-phenyl ring of MA3.

3) MA6 In the mass spectrum of the TMS derivative of MA6 (Fig. 9), an ion at m/z 625 was assumed to be M⁺. A base peak ion m/z 310 and characteristic ions m/z 596 (M⁺−29), 535 (M⁺−90, fragment ion (f)) and 364 were seen. The ion m/z 535 suggests that R₃ in Fig. 7 is OH (OTMS) as shown in MA2 and MA4. The m/z values of M⁺ and these characteristic ions of this metabolite were 88 amu or 30 amu larger than the corresponding ions of MA2 or MA4. These findings suggest that MA6 is a metabolite having an additional OH group in the 2-phenyl ring of MA2 or an additional OCH₃ group in the 2-phenyl ring of MA4.

HPLC of Neutral Metabolites Twelve radioactive peaks were found in the HPLC-radiochromatograms of the neutral metabolite fraction in the bile, as shown in Fig. 10.

No metabolite could be found in the neutral metabolite fraction of the urine. On the other hand, two neutral metabolites (MN1 and MN2 in peaks 12 and 10, respectively) were found in the bile.

Chemical Structure of Neutral Metabolites These two metabolites were characterized by GC-MS. The structures of the major ions (Fig. 11) are analogous to those of the ions formed from acidic or basic metabolites. The mass spectra of the TMS derivatives of MN1 and MN2 are shown in Fig. 12.

1) MN1 The ion at m/z 416 was assumed to be the M⁺. The base peak ion m/z 150 was assumed to be derived by cleavage at the point of (a) (Fig. 11). Therefore, the substituents, R₁ and R₂, of this metabolite were suggested to be 4-methoxyphenyl and acetyl groups, respectively. This characteristic ion was demonstrated in the mass spectra of clentiazem and the basic and acidic metabolite MB₂, MB₈ and MA₁, and those of diltiliazem and its metabolites.²)

The M⁺ and the fragment ions at m/z 416, 373, 356 and 328 had 2 amu higher mass clusters at m/z 418, 375, 358 and 330, showing the presence of a chlorine atom. Thus, MN1 and clentiazem have the same benzothiazepine skeleton. The mass number of the partial structure of clentiazem, excluding the dimethylaminoethyl group, was 376, while the M⁺ of MN1 was 416. The difference (40 amu) in mass numbers would arise from the difference in the mass numbers of the substituents at the S-positions of MN1 and clentiazem. The mass number of the M⁺ was even, so the

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![Figure 10](image_url)  
**Fig. 10.** HPLC-Radiochromatograms of Neutral Metabolite Fraction in Bile

Rat bile excreted over 24h after oral dosing of [¹⁴C]clentiazem, was extracted with t-BME at pH 2. The t-BME extracts were shaken once with 0.1 M Na₂HPO₄. The remaining t-BME layer containing neutral metabolites was analyzed by HPLC. The radioactivity was measured in each fraction by radiochromatography.

![Figure 11](image_url)  
**Fig. 11.** Structures and m/z Values of Characteristic Ions in the PIEI MS of Neutral Metabolites

<table>
<thead>
<tr>
<th>Compounds</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>m⁺</th>
<th>(a)</th>
<th>(b)</th>
<th>(c)</th>
<th>(d)</th>
<th>(e)</th>
<th>Other Prominent Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN1</td>
<td>X</td>
<td>Ac</td>
<td>CN</td>
<td>416</td>
<td>150</td>
<td>121</td>
<td>274</td>
<td>328</td>
<td>356</td>
<td>43 288 373</td>
</tr>
<tr>
<td>MN2</td>
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<td>TMS</td>
<td>CN</td>
<td>446</td>
<td>222</td>
<td>121</td>
<td></td>
<td></td>
<td>356</td>
<td>73 148 207 316 417 431</td>
</tr>
<tr>
<td>MN3</td>
<td>Y</td>
<td>TMS</td>
<td>CH₂OTMS</td>
<td>581</td>
<td>280</td>
<td>179</td>
<td></td>
<td>463</td>
<td>491</td>
<td>73 307 463 552 566</td>
</tr>
<tr>
<td>MN4</td>
<td>Z</td>
<td>TMS</td>
<td>CH₂OTMS</td>
<td>611</td>
<td>310</td>
<td>209</td>
<td></td>
<td></td>
<td>73 280 365 489 579 596</td>
<td></td>
</tr>
</tbody>
</table>

: base peak ion
number of nitrogen atoms in this compound is even. Therefore, it is suggested that this substituent at the 5-position in MN1 is CH$_3$CN (40 amu). The fragment ion at m/z 288 might come from the m/z 328 ion by liberation of this substituent. It was assumed that the fragment ions at m/z 373 and 356 resulted from a loss of COCH$_3$ (43 amu) and CH$_3$COOH (60 amu), respectively, from M$. The fragment ion at m/z 356 might break, forming the fragment ion m/z 328 by loss of CO (28 amu). The mass spectrum of the metabolite MN1 is identical to that of the synthetic compound.

2) MN2  The mass spectrum of the TMS derivative of MN2 is shown in Fig. 12. The ion at m/z 581 accompanied by the M$^+15$ ion, which was the ion of the largest mass number, was assumed to be M$^{+}$. The base peak ion at m/z 280 suggests that the substituents at the 4-position of R$_1$ and R$_2$ of MN3 are OTMS and TMS, respectively, as shown in Fig. 11.

The mass numbers of M$^{+}$ ion and characteristic ions at m/z 566, 552, 491, 463 and 286 were 14amu less than those of MA4. The mass number of the M$^{+}$ ion is odd, therefore the number of nitrogen atoms is odd. Accordingly, the substituent at the 5-position does not contain an odd-number of nitrogen atoms. The mass numbers of ions at m/z 375 and 335 were 116 amu less than those of the ions at m/z 491 and 451, respectively. Furthermore, the ion at m/z 117 might be formed by heterolytic cleavage of the C-N bond between the side chain carbon and the nitrogen at the 5-position. From these findings this substituent was presumed to be CH$_3$CH$_2$OH. Therefore, it is assumed that MN3 is 8-chloro-2,3-dihydro-3-hydroxy-5-(2-hydroxy-ethyl)-2(4-hydroxyphenyl)-1,5-benzothiazepin-4(5H)-one.

2) MN4  In the mass spectrum of the TMS derivative of MN4 (Fig. 12), the ion at m/z 611 was assumed to be the M$. The base peak at m/z 310 was the same as that of MA6, suggesting that the 2-phenyl ring of MN4 has one OH group and one OCH$_3$ group. The mass number of M$^{+}$ and the prominent peak ions at m/z 596, 582 and 521 were 14amu less than those of the ions at m/z 625, 610, 596 and 535 of MA6. The difference of 14amu suggests that the substituent at the 5-position is CH$_3$CH$_2$OH as described for MN3. This shows that MN4 is a metabolite
Fig. 13. Structures of Metabolites and Possible Metabolic Pathways in Rat
having an additional OCH₃ group or an additional OH group on the 2-phenyl ring.

Discussion

Clentiazem has a chlorine atom at the 8-position of the benzothiazepine skeleton of diltiazem. The introducing of a halogen atom such as chlorine to the molecule generally increases the lipophilicity. The changes in lipophilicity might give rise to some differences in metabolism. However, the biotransformation pathways of clentiazem would be expected to be fundamentally analogous to those of diltiazem because of the structural similarity of the two compounds. Therefore, it could be assumed that the metabolites of clentiazem would be similar to those of diltiazem.

The structures of MB1—15 were identified or assumed by examination of HPLC profiles and GC-MS analyses. Fifteen basic metabolites were found in urine and bile.

N-Demethylation has been reported to be a pathway for the biotransformation of many compounds. It is thought that such metabolism is carried out by cytochrome P-450. Recently, it was reported that N-demethylation of diltiazem could be carried out by cytochrome P-450 IIIA in in vitro experiments using rabbit and human liver microsomes and hepatocytes. The same mechanism might be involved in the N-demethylation of clentiazem.

In the present study, many deacetylated metabolites (MB1, MB3, MB4, MB6, MB9, MB11, MB13, MB14 and MB15) were detected in rat urine and bile. In in vitro experiments using monolayer primary cultures of rat hepatocytes, deacetylation was one of main metabolic pathways. Deacetylation usually occurs by an esterase-catalyzed hydrolysis. It has been reported that diltiazem was deacetylated by a specific esterase of the liver microsome fraction. Deacetylated metabolites of clentiazem might be produced in the same way.

Similar metabolic pathways have been reported with diltiazem, but the O-demethyl metabolite, which is thought to be produced directly from diltiazem, and corresponds to the MB7 of clentiazem, has not been detected yet. The differences in the amount of this type of O-demethylated metabolite arising from clentiazem and diltiazem might be one of the differences in metabolism between these drugs.

The major oxidative metabolic pathways of clentiazem to give basic metabolites in rats are considered to be deacetylation, N-demethylation, O-demethylation and aromatic hydroxylation of the 2-phenyl ring. In addition to these pathways, it has been shown in this study that deamination is also an important metabolic pathway of clentiazem. The deaminations of other basic metabolites may occur also, but they were omitted from the illustration in Fig. 13. Similar metabolic pathways have been reported with diltiazem.

In the present study, the structures of 6 acidic metabolites were confirmed to be those of the 8-chlorinated acidic metabolites of diltiazem. No change in metabolic pathways following the introduction of a chlorine atom was found.

Only 2 acidic metabolites (MA2 and MA4) were found in the urine, while there were 6 acidic metabolites (MA1, MA2, MA3, MA4, MA5 and MA6) in the bile. Each of the acidic metabolites (MA1—6) has a CH₂COOH group at the 5-position of the benzothiazepine skeleton, which may be produced by oxidative deamination of the CH₂N(CH₃)₂ moiety. Two deamination pathways for alkylamines have been proposed. One is via carbamidom-amine; i.e., hydroxylation at the methylene carbon atom adjacent to the nitrogen atom of the N-dimethylaminomethyl group to form a carbamidomamine, followed by deamination with dehydration and translocation of an electron to give an aldehyde. The other is via formation of the N-oxide; the nitrogen atom at the N-dimethylaminomethyl group is oxidized directly to the N-oxide or hydroxylamine, which is transformed to an aldehyde by way of an immoinon ion. The aldehyde is oxidized to a carboxylic acid by aldehyde dehydrogenase and/or oxygenase in the microsomal fraction.

No intermediates in the metabolism of clentiazem, such as the carbamidom-amine, N-oxide or hydroxylamine, were detected in this study. In an in vitro study the deamination of the side chain of diltiazem has been shown to be catalysed by cytochrome P-450 to produce the aldehydes. The same mechanism would be expected for clentiazem because of similarity of their chemical structures.

We found two novel neutral metabolites (MN1 and MN2) having a nitrile group in the side chain at the 5-position of the benzothiazepine. The metabolite having a nitrile group was found in the urine of rat that received TMB4 intraperitoneally. It may be that the nitrile group of the metabolite was biotransformed through an intermediate having a better leaving group from the organic oxime. In the present study, such an intermediate was not found. However, it might be possible for the basic metabolite having a primary amine group such as MB8, MB9, MB10 and MB11 to be transformed to metabolites possessing an oximino group such as the unstable intermediate in the metabolism of amphetamine. It could be that the metabolic process to form the nitrile metabolite through an oximino intermediate is similar to the N-dealkylation (deamination) mechanism. The oximino intermediate would be further metabolized to nitrile metabolites by the same mechanism as seen in TMB-4 metabolism.

Other neutral metabolites (MN3 and MN4) were found as glucuronide conjugates in the aqueous fraction of the rat bile. These metabolite have a -CH₂CH₂OH substituent at the 5-position of the benzothiazepine. The metabolite having an alcohol moiety might be produced by the reduction of an aldehyde precursor. The aldehyde precursor was not found in the series of metabolic studies of clentiazem. Diltiazem was metabolized to a compound having an aldehyde moiety in an in vitro experiment using rat microsomes. It could be that the aldehyde precursor was oxidized or reduced to acidic or alcohol metabolites. Then most of the alcohol metabolites could be conjugated with glucuronic acid. MA4 was one of the main acidic metabolites in rat urine and bile. However, little MA4 was found to be glucuronide or sulfate. About 75% of the aqueous metabolite fraction was not cleaved by reaction with glucuronidase or sulfatase, and this is thought to be conjugated metabolites other than glucuronide and sulfate.

Figure 13 illustrates the structures of the acidic, basic, neutral and conjugated metabolites identified or postulated in the study and the possible metabolic pathways.

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References