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Metabolism of an Antiallergic Agent, Azelastine (4-(*p*-Chlorobenzyl)-2-〔N-Methylperhydroazepinyl-(4)〕-1-(2H)-Phthalazinone Hydrochloride) in Rats and Guinea Pigs*)

Kiyoshi TATSUMI[1)], Hideyuki YAMADA[2)], Hidetoshi YOSHIMURA[2)], Yukio NISHIZAWA[3)], Misao SAKAI,[3)] Hitoshi MIZUO[3)] and Chiyuki YAMATO[3)]

1) *Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, 1-2-3, Kasumi, Minami-ku, Hiroshima 734, Japan*
2) *Faculty of Pharmaceutical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812, Japan*
3) *Tsukuba Research Laboratories, Eisai Co., Ltd., 1-3, Tokodai 5-chome Toyosato-machi, Tsukuba-gun, Ibaragi 300-26, Japan*

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ABSTRACT

The *in vivo* metabolism of an antiallergic agent, azelastine, (4-(*p*-chlorobenzyl)-2-[N-methylperhydroazepinyl-(4)]-1-(2H)-phthalazinone hydrochloride) was examined following oral administration to rats and guinea pigs. As a result, it was found that the drug was metabolized to 4-(*p*-chlorobenzyl)-2-[N-methyl-7-oxo-perhydroazepinyl-(4)]-1-(2H)-phthalazinone, 6-hydroxy-4-(*p*-chlorobenzyl)-2-[N-methylperhydroazepinyl-(4)]-1-(2H)-phthalazinone (6-hydroxyazelastine), 7-hydroxy-4-(*p*-chlorobenzyl)-2-[N-methylperhydroazepinyl-(4)]-1-(2H)-phthalazinone and 4-(*p*-chlorobenzyl)-2-[perhydroazepinyl-(4)]-1-(2H)-phthalazinone (desmethylazelastine) in these animal bodies. A part of the hydroxy derivatives of the drug was excreted into urine as their glucuronides. In addition, the incubation of azelastine with rat liver microsomes resulted in the formation of 6-hydroxyazelastine and desmethylazelastine in the presence of an NADPH-generating system.

INTRODUCTION

Azelastine (4-(*p*-chlorobenzyl)-2-[N-methylperhydroazepinyl-(4)]-1-(2H)-phthalazinone hydrochloride) was developed as an antiallergic agent in the Research Laboratories of Asta-Werke AG, Bielefeld, W. Germany. Strong antiallergic actions of the drug were demonstrated by Tasaka and Akagi[7)], Zechel et al.[10)], Fischer and Schmutzler[2)], Katayama et al.[5)] and Yamada et al.[9)]

In a previous paper[8)], we showed the metabolic fate of the drug in rats and guinea pigs following oral or intravenous administration of the ^{14}C-labelled compound; the blood level, the tissue distribution, the urinary, biliary and fecal excretion, and the placental transfer of the radioactivity. The present study was carried out in order to elucidate the metabolism of the drug in rats and guinea pigs.

MATERIALS AND METHODS

1) Chemicals

Azelastine and its ^{14}C-labelled compound (phthalazinone-1, 4-^{14}C, 112 μCi/mg) were kindly supplied by Asta-Weke AG. The radiochemical purity of the labelled compound was ascertained to be more than 99% by thin-layer

*) 辰巳 淳, 山田英之, 吉村英敏, 西沢幸夫, 酒井操, 水尾 均, 大和千畝：ラットおよびモルモットにおける抗アレルギー剤 azelastine の代謝

chromatography as described previously[8]. 4-(*p*-Chlorobenzyl)-2-[N-methyl-2-oxo-perhydroazepinyl-(4)]-1-(2H)-phthalazinone (2-oxoazelastine), 4-(*p*-chlorobenzyl)-2-[N-methyl-7-oxo-perhydroazepinyl-(4)]-1-(2H)-phthalazinone (7-oxoazelastine), 4-(*p*-chlorobenzyl)-2-[perhydroazepinyl-(4)]-1-(2H)-phthalazinone (desmethylazelastine), 6-hydroxy-4-(*p*-chlorobenzyl)-2-[N-methylperhydroazepinyl-(4)]-1-(2H)-phthalazinone (6-hydroxyazelastine), and a mixture of 6-hydroxyazelastine and 7-hydroxy-4-(*p*-chlorobenzyl)-2-[N-methylperhydroazepinyl-(4)]-1-(2H)-phthalazinone (7-hydroxyazelastine) (9 : 1, hydroxyazelastine (9:1)) or (1:1, hydroxyazelastine (1:1)) were also donated by the company.

2) Administration of drug

Male Donryu-strain rats weighing 150–230 g, Sprague-Dawley rats weighing about 230 g and guinea pigs weighing 300–350 g were fed a commercial pellet diet (Oriental Kobo Co., Ltd) and fasted overnight prior to use. Azelastine and its ^{14}C-labelled compound were given orally by stomach tube as an aqueous solution.

3) Thin-layer chromatography (TLC)

Silica gel plates (Wako gel B-5 FM, Wako Pure Chemical Industries, Ltd., 0.25 mm thick) was used in this study. The thin-layer plates were developed in (A) benzene-acetone-methanol (7:2:1), (B) $CHCl_3$-methanol (7:3), (C) $CHCl_3$-diethylamine (9:1) or (D) $CHCl_3$-methanol-diethylamaine (40:3:3). The chromatograms were visualized under ultraviolet light (wave length 254 nm), or by spraying with a Dragendorff's or Folin-Ciocalteau's reagent.

4) Analytical procedure

Nuclear magnetic resonance (NMR) spectra were recorded with a Japan Electron Optics (JEOL) JNM-PS-100 spectrometer using tetramethylsilane as an internal standard. Electron impact mass spectra were determined with a Japan Electron Optics (JEOL) JMS-01SG mass spectrometer or JMS DX-300 GC/MS spectrometer. Ultraviolet (UV) spectra were taken with a Hitachi 340 spectrophotometer. Infrared (IR) spectra were recorded with a Japan Spectroscopic DS-701 G IR spectrophotometer. Radioactivity was determined with a Packard Tri-Carb Liquid Scintillation Spectrometer 3375.

5) Isolation of *in vivo* metabolites

After azelastine was administered to fifteen Donryu-strain rats at a dose of 100 mg/kg/day for three days, the feces was collected over a period of four days. The combined feces was dried over P_2O_5 *in vacuo*, powdered with a blender and then extracted with $CHCl_3$ continueously for 20 hr by using a Soxhlet extraction apparatus. Next, the $CHCl_3$ solution (Fraction A) was extracted three times each with an equal volume of 1 N HCl and 1 N NaOH, successively, by shaking. The remaining $CHCl_3$ phase was evaporated to dryness *in vacuo* to leave 3.4 g of residue (Fraction B). The 1 N HCl phase described above was adjusted to pH 10 with 2 N NaOH and then extracted three times with an equal volume of $CHCl_3$. The combined $CHCl_3$ extract was evaporated to dryness *in vacuo* to leave 0.55 g of residue (Fraction C). Fraction B was dissolved in a small volume of $CHCl_3$ and chromatographed on a silica gel column (Kiselgel 60, Merck, 3.1×42 cm) using 1.5 liters each of $CHCl_3$-acetone (9:1), $CHCl_3$-acetone (4:1) and acetone, successively. When each fraction (100 ml) was subjected to TLC with solvent system A, a Dragendorff's reagent-positive metabolite with Rf value of 0.54 (metabolite 1) was detected from both $CHCl_3$ and $CHCl_3$-acetone (9:1) eluates. The crude metabolite 1 from these eluates was purified by preparative TLC (solvent system A) and by recrystallization from a mixture of benzene and *n*-hexane to give 5 mg of white crystals. Fraction B was also dissolved in a small volume of $CHCl_3$ and put on an alumina column (Aluminum oxid activ neutral, Merck, 1.7×22 cm). The column was eluted with 500 ml each of $CHCl_3$, $CHCl_3$-methanol (99:1), $CHCl_3$-methanol (98:2), $CHCl_3$-methanol (95:5), $CHCl_3$-methanol (90:10) and methanol, successively. Each fraction (50 ml) was examined by TLC using the solvent system B. As a result, a Dragendorff's reagent-positive spot with Rf value of 0.39 was detected from $CHCl_3$-methanol (99:1) eluate, and those with Rf values of 0.32 (metabolite 2) and 0.25 (metabolite 3) from both $CHCl_3$-methanol (95:5) and $CHCl_3$-methanol (90:10) eluates, respectively. The $CHCl_3$-methanol (99:1) eluate was evaporated to dryness *in vacuo* and the residue was purified by preparative TLC (solvent system C) to give 3 mg of white crystals. The compound was identified as unchanged azelastine by its comparison with the authentic sample on thin-layer chromatographic behavior, and UV and IR spectra.

Furthermore, the $CHCl_3$–methanol (95:5) and the $CHCl_3$–methanol (90:10) eluates were combined and eveporated to dryness *in vacuo* to leave 2 mg of a mixture of metabolites 2 and 3 as white crystals. However, an attempt to purify metabolites 2 and 3 separately by using preparative TLC or column chromatography was unsuccessful. When fraction A was subjected to TLC with solvent system D, metabolite 4 (Rf 0.56) was found together with other metabolites described above. However, no attempt was made to further purify the metabolite, because of its very small quantity.

6) Isolation *in vitro* metabolites

Sprague-Dawley rats were used in this experiments. The liver was homogenized in 3 volumes of 0.25 M sucrose, the homogenate was centrifuged for 20 min at 10,000 × g, and the supernatant was centrifuged for 60 min at 105,000 × g. Microsomes were resuspended in 0.1 M phosphate buffer (pH 7.4). An incubation mixture consisted of 0.5 mmol of ^{14}C-azelastine, microsomes equivalent to 73 g of liver, 0.5 mmol of NADP, 2 mmol of glucose-6-phosphate, 800 units of glucose-6-phosphate dehydrogenase and 0.5 mmol of $MgCl_2$ in a final volume of 100 ml of 0.1 M phosphate buffer (pH 7.4). The incubation was performed at 37°C for 5 hr in an open vessel. After incubation, the mixture was heated for 1 min in a boiling water bath, adjusted to pH 10 with 2 N NaOH and then extracted three times with 2 volumes of ethyl acetate containing isoamylalcohol at a concentration of 1.5%. The combined ethyl acetate extract was evaporated to dryness *in vacuo*. When the residue was subjected to TLC with solvent system D, two spots corresponding to metabolites 2 and 4 were detected at Rf 0.14 and 0.56, respectively. Metabolite 4 was further purified by silica gel column chromatography as follows: The residue was dissolved in a small volume of $CHCl_3$ and charged on a silica gel column (Silica Gel 60, Wako Pure Chemical Industries, Ltd., 2 × 16 cm). The column was eluted with 200 ml each of $CHCl_3$–methanol (19:1), $CHCl_3$–methanol (17:3), $CHCl_3$–methanol (4:1) and $CHCl_3$–methanol (7:3), successively. When each fraction was subjected to TLC with solvent system D, metabolite 4 was detected from both $CHCl_3$–methanol (4:1) and $CHCl_3$–methanol (7:3) eluates. For further purification, the crude metabolite from these eluates was chromatographed on a silica gel column (1 × 21 cm) using $CHCl_3$–methanol-diethylamine (87:6.5:6.5) and then rechromatographed on a column of the same size using $CHCl_3$–methanol-diethylamine (91:4.5:4.5).

7) Quantitative determination of metabolites in blood and tissues

Sprague-Dawley rats were given ^{14}C-azelastine at a dose of 10 mg/kg and sacrificed by cervical fracture. The blood samples were collected from the carotid artery and the tissues of interest were excised from bodies. The blood (2 ml) and tissue (2 g) samples were homogenized in 4 volumes of water and the homogenates were extracted with 2 volumes of ethyl acetate containing isoamylalcohol at a concentration of 1.5%, respectively. The ethyl acetate extract was subjected to TLC (solvent system D) and the silica gel on the plate was counted for radioactivity as described above.

8) Quantitative determination of metabolite in unine and feces

After administration of ^{14}C-azelastine at a dose of 1 mg/kg to Donryu-strain rats, the 24 hr-urine and feces were collected, respectively. The urine was adjusted to pH 10 with 2 N NaOH and then extracted three times with 2 volumes of ethyl acetate. The combined ethyl acetate extract was evaporated to dryness *in vacuo* and the residue was subjected to TLC (solvent system D).

The aqueous phase after extraction was adjusted to pH 6 with 2 N HCl, mixed with 2 volumes of 0.1 M phosphate buffer (pH 6.0), and the mixture was incubated with β-glucuronidase (Type 1, bacterial, 62,000 units/g, Sigma Chemical Co.) at 37°C for 24 hr. After incubation, the mixture was adjusted to pH 10 with 2 N NaOH and extracted with ethyl ecetate as described above. The ethyl acetate extract, after removal of the solvent, was also subjected to TLC (solvent system D). After development, the silica gel on the thin-layer plate was scraped as 0.5 cm wide bands and counted for radioactivity.

The combined feces was dried, powdered and extracted with $CHCl_3$ for 20 hr according to the method described in the section of "Isolation of *in vivo* metabolites". The $CHCl_3$ extract was evaporated to dryness *in vacuo* and the residue was subjected to TLC (solvent system

D). After development, the radioactivity on the thin-layer plate was measured as described above.

RESULTS AND DISCUSSION

Our previous study[8] showed that when ^{14}C-azelastine was administered orally to rats and guinea pigs, the major excretion pathway of the drug was by way into feces in both species. In the present study, therefore, we attempted the isolation and identification of azelastine metabolites from rat feces. As described in MATERIALS AND METHODS, metabolite 1, and a mixture of metabolites 2 and 3 were isolated from the feces of rats given azelastine, respectively. The mass spectral analysis of metabolite 1 (Fig. 1 and 2) showed that the metabolite seems to be the oxoperhydroazepinyl derivative of azelastine. The spectrum of the metabolite resembled closely that of the synthetic candidate, 2-oxoazelastine or 7-oxoazelastine. The UV spectrum of the metabolite was also identical with those of these two synthetic compounds, in which an absorption maximum was observed at 290 nm (in ethanol). On the other hand, upon TLC examination using the solvent system A, it was found that 2-oxoazelastine had the Rf value of 0.64, whereas 7-oxoazelastine had that of 0.54, and that the thin-layer chromatographic behavior of metabolite 1 coincided completely with that of the latter oxoazelastine. In addition, the IR spectrum of the metabolite was identical with that of the 7-oxo compound as shown in Fig. 3, but not with that of the 2-oxo compound (data not shown). From these results, metabolite 1 was identified as 7-oxoazelastine which has a lactam structure of seven member ring in its molecule. It is known that other drugs such as nicotine[4,6], and tremoline[1,3] also undergo the oxidation of pyrrolidine rings to form the corresponding lactams in animal bodies. Such lactams seem to be formed via the corresponding carbinolamines from these cyclic amines[3,4].

As regards the mixture of metabolites 2 and 3, the mass spectral analysis showed that these metabolites seem to be two kinds of hydroxyazelastine possessing a hydroxy group on a benzene ring of phthalazinone moiety (Fig. 4 and 5). The presence of a phenolic hydroxyl group in the molecule was supported by the facts that both metabolites 2 and 3 were positive to Folin-Ciocalteau's reagent and that the UV spectrum of the mixture showed the bathochromic shift of absorption maxima in an alkaline solution as described below. Furthermore, the mass spectrum of the metabolite mixture was almost completely identical with that of the synthetic candidate, hydroxyazelatine (9:1) or hydroxyazelastine (1:1). The TLC examination using the solvent system B also demonstrated that all of these samples gave the two spots at Rf 0.32 and 0.25, respectively. In the case of hydroxyazelastine (1:1), the relative intensity of these spots was nearly equal, whereas in the case of hydroxyazelastine (9:1), the intensity of the upper spot was much stronger than that of the lower one. This fact indicated that the upper spot is due to 6-hydroxyazelastine and the lower one due to

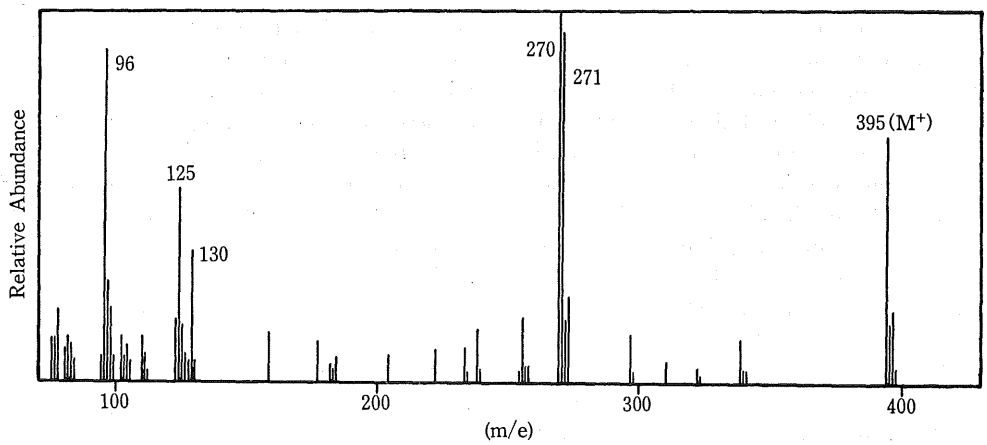

Fig. 1. Electron impact mass spectrum of metabolite 1.

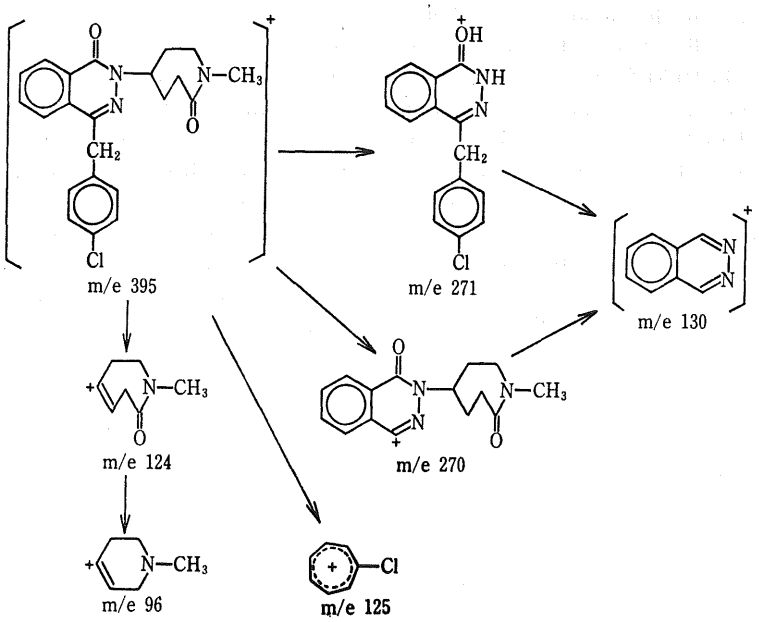

Fig. 2. Fragmentation paths of metabolite 1 in electron impact mass spectrometry.

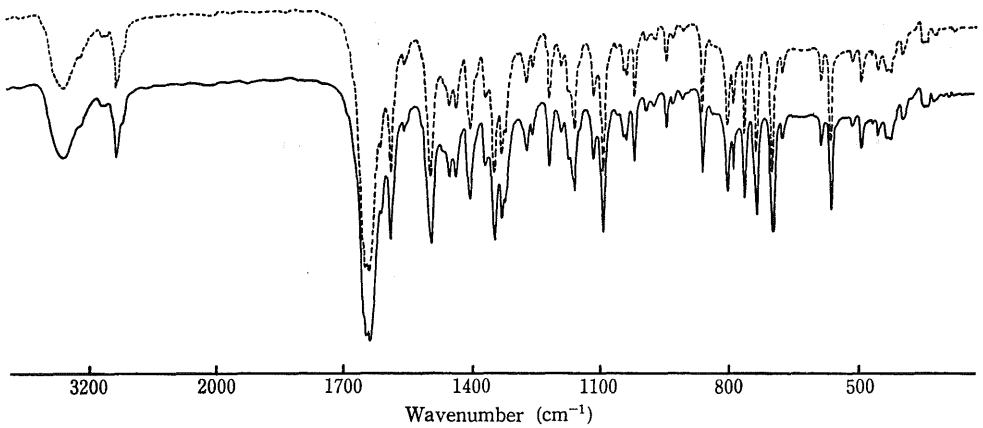

Fig. 3. Infrared spectra of synthetic 7-oxoazelastine and metabolite 1 (KBr).
——— synthetic compound, ····· metabolite

7-hydroxyazelastine, respectively. The chromatograms of the metabolite mixture was rather similar to these of hydroxyazelastine (9:1). As shown in Fig. 6, the UV spectrum of the metabolite mixture in ethanol had the absorption maxima at 222, 254 and 295 nm, which were shifted to 270 and 300 nm in alkaline ethanol. These UV spectra in both neutral and alkaline media coincided completely with those of hydroxyazelastine (9:1). Based on these data, metabolites 2 and 3 were tentatively identified as 6-hydroxyazelastine and 7-hydroxyazelastine, respectively. However, complete resolution and purification of these two hydroxy metabolites were unsuccessful. In addition, the thin-layer chromatographic behavior of metabolite 4 was entirely identical with that of authentic desmethylazelastine, suggesting that azelastine undergoes metabolic N-demethylation in animal bodies.

When azelastine was incubated with rat liver microsomes in the presence of an NADPH-

generating system, the formation of metabolite 4 as well as metabolite 2 was also observed as described in MATERIALS AND METHODS. The mass spectral analysis of metabolite 4 (Fig. 7 and 8) showed that the metabolite is a compound corresponding to N-demethylated azelastine. In fact, the mass spectrum was completely identical with that of authentic desmethylazelastine, supporting the above idea that the metabolite is formed *in vivo*.

The presence of these metabolites in feces seems to be due to their biliary excretion and the exsorption, because the radioactivity was almost completely recovered from bile, and gastrointestinal tracts and its contents following i. v. administration of ^{14}C-azelastine (1 mg/kg) to rats as described previously[8]. Upon TLC examination, metabolites 1, 2, 3 and 4 were also detected from feces of guinea pigs, and from urine of rats and guinea pigs.

Fig. 9 shows the concentration of azelastine and its metabolites in blood and tissues. In

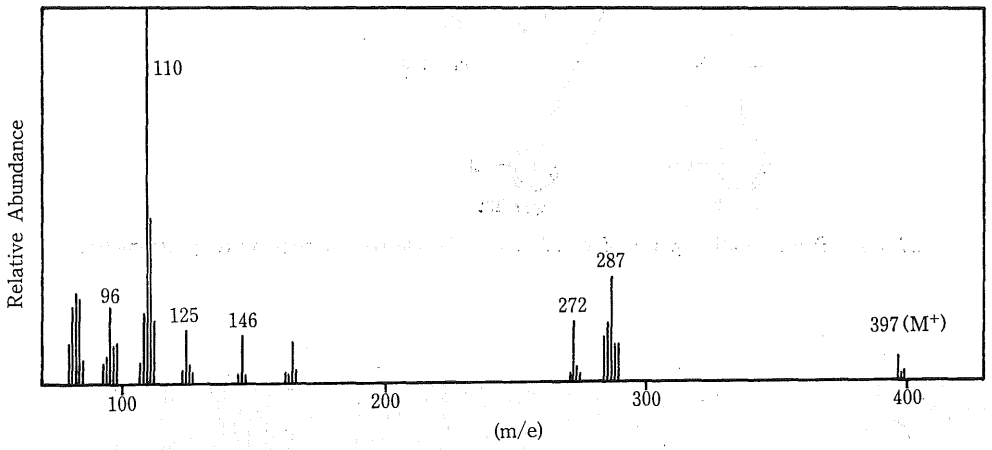

Fig. 4. Electron impact mass spectrum of a mixture of metabolites 2 and 3.

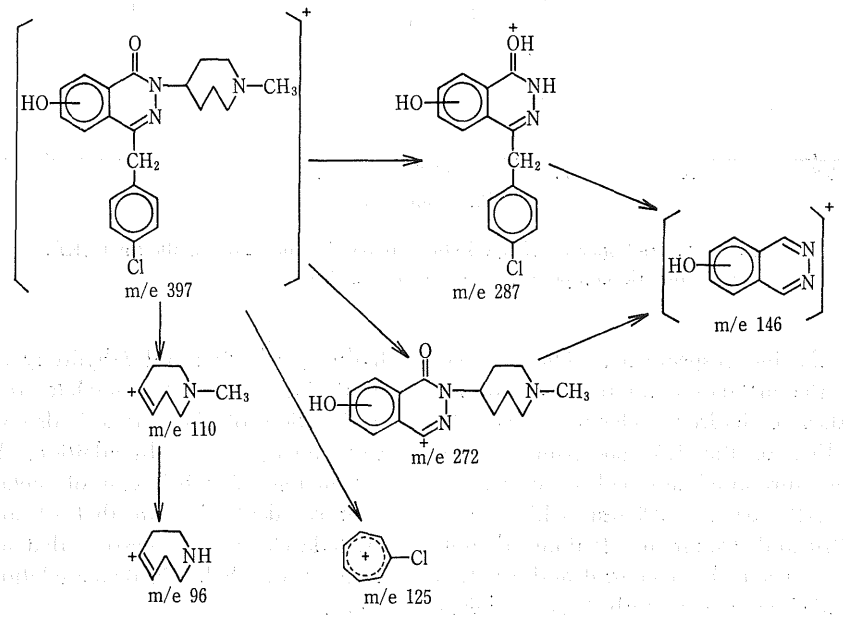

Fig. 5. Fragmentation paths of a mixture of metabolites 2 and 3 in electron impact mass spectrometry.

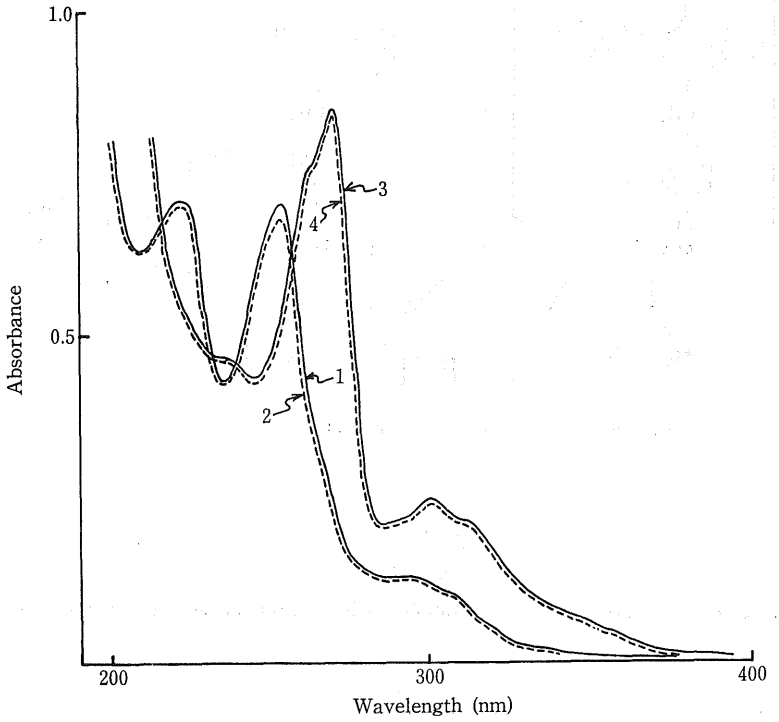

Fig. 6. Ultraviolet spectra of a mixture of synthetic 6-hydroxyazelastine and 7-hydroxyazelastine (9:1), and that of metabolites 2 and 3.
————, the mixture of synthetic compounds: spectrum 1, in ethanol; spectrum 3, in alkaline ethanol.
----------, the mixture of metabolites: spectrum 2, in ethanol; spectrum 4, in alkaline ethanol.

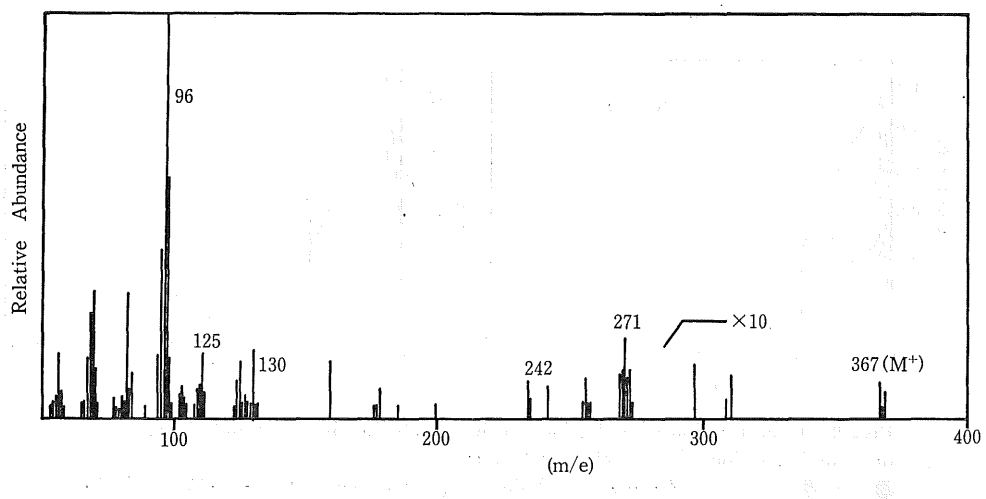

Fig. 7. Electron impact mass spectrum of metabolite 4.

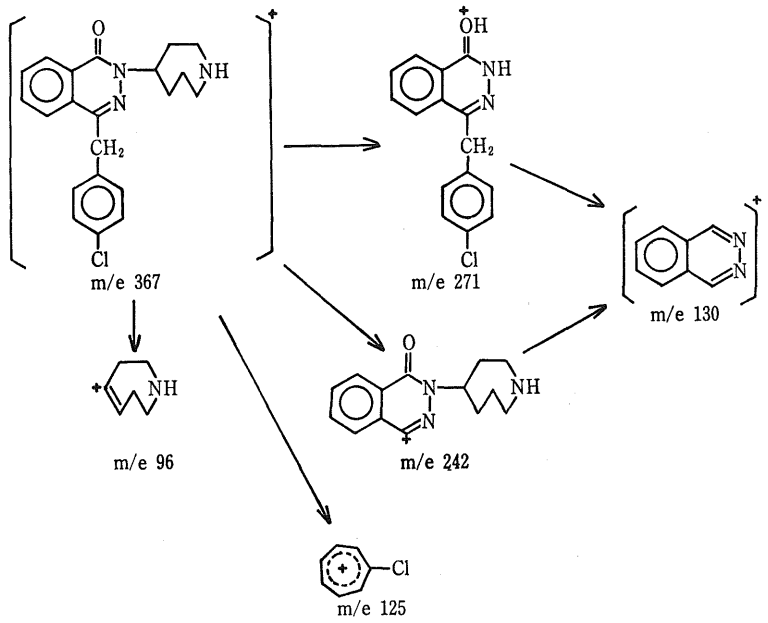

Fig. 8. Fragmentation paths of metabolite 4 in electron impact mass spectrometry.

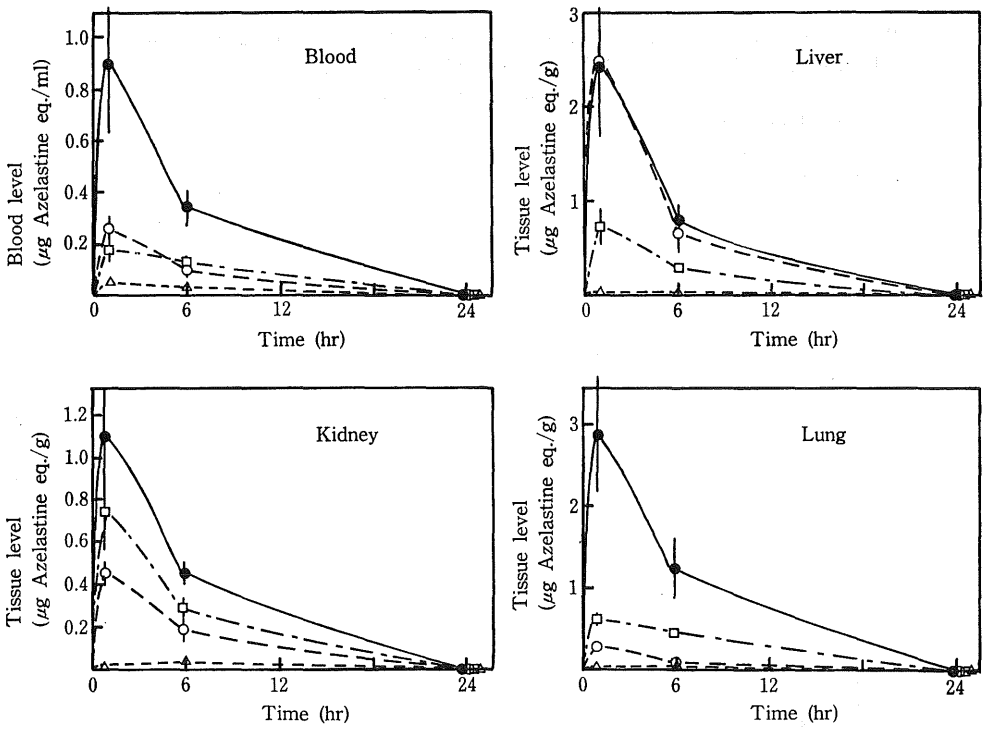

Fig. 9. Concentration of azelastine and its metabolites in blood and tissues after oral administration of ^{14}C-azelastine to rats.
●——● azelastine, △·····△ metabolite 1 (7-oxoazelastine), ○- - -○ metabolite 2 (6-hydroxyazelastine), □— - —□ metabolite 4 (desmethylazelastine). Each value represents the mean of three experiments with S. E.

all samples, azelastine, metabolite 2 and metabolite 4 showed the highest concentration at 1 hr and disappeared almost completely at 24 hr after medication. In the liver, the concentration of azelastine was nearly equal to that of metabolite 2, whereas in the blood, kidney and lung, the highest concentration was observed with unchanged azelastine. In the kidney and lung, metabolite 4 exhibited higher concentration compared with metabolite 2. In all samples, the level of metabolite 1 was the lowest among the metabolites.

Table 1 shows the results of the determination of azelastine and its metabolites in the 24 hr-urine and feces. Hydroxyazelastines (metabo-

Table 1. Determination of Azelastine and its Metabolites in 24 hr-Urine and Feces after Oral Administration of ^{14}C-Azelastine to Rats and Guinea Pigs

	Rat		Guinea pig	
	Urine	Feces	Urine	Feces
	% of dose			
Azelastine	0.1	3.7	2.7	7.0
Metabolite 1	0.1	9.1	1.6	6.2
Metabolite 2+3	1.1	56.3	4.5	15.5
Glucuronides of metabolite 2+3	0.1	—	1.5	—
Metabolite 4	trace	trace	trace	trace

Each value represents the mean of three experiments.

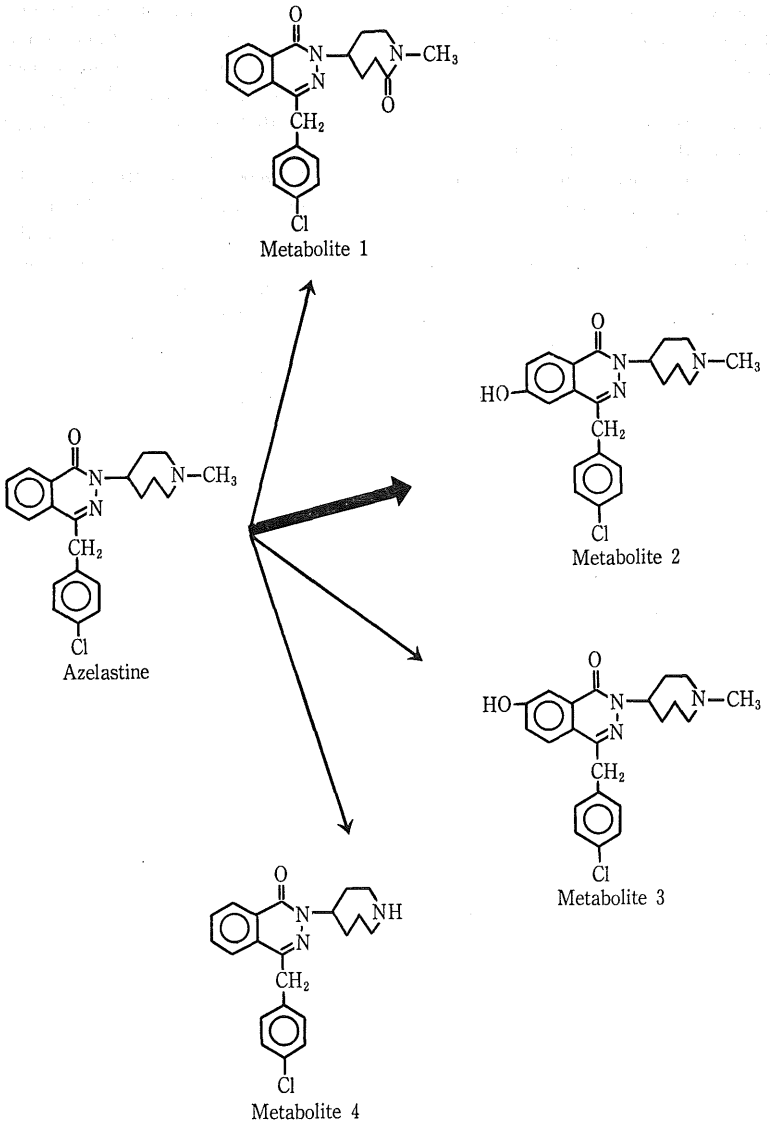

Fig. 10. Metabolites of azelastine in rats and guinea pigs.

lites 2 and 3) were excreted into the feces of rats and guinea pigs as main metabolites of azelastine. A small amount of their glucuronides were also detected from the urine of both species.

The metabolites of azelastine, which were found in the present study, were depicted in Fig. 10.

ACKNOWLEDGEMENTS

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