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Studies on the Metabolism of Atenolol. Characterization and Determination of a New Urinary Metabolite in the Rat[1]

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The characterization and quantitation of a new urinary metabolite, M2, from rats orally given atenolol, a β-adrenergic blocking drug, are described. 4-(2-Hydroxy-3-isopropylaminopropoxy)phenylglyoxylic acid amide was synthesized as an authentic sample by an unequivocal route. The structure of M2 was definitively established by direct comparison with the synthetic specimen. Determination of M2 in urine was achieved by means of selected ion monitoring (SIM) using [$^2H_6$]-atenolol as an internal standard. The amount of M2 excreted in urine was estimated to be 1.04% of the dose.

Keywords——atenolol; β-adrenergic blocking drug; rat urine; metabolite; 4-(2-hydroxy-3-isopropylaminopropoxy)phenylglyoxylic acid amide; selected ion monitoring; mass fragmentography; quantitation of atenolol

The metabolism of atenolol [4-(2-hydroxy-3-isopropylaminopropoxy)phenylacetamide], which is widely used as a β-adrenergic blocking drug, has been investigated by several groups.[2-4] In a preceding paper of this series we reported the identification of a novel urinary metabolite, Ml, which is formed by hydroxylation at the methylene group of the acetamide side chain in the rat.[5] In the previous study the occurrence of an additional metabolite besides Ml was indicated by the reconstructed ion profile, but its complete structure has remained unclear. The present paper describes the characterization of the second new metabolite, M2, by direct comparison with an authentic sample prepared by an unequivocal route and the determination of its urinary excretion in the rat.

Materials and Methods

Reagents——4-Hydroxymandelic acid was purchased from Sigma Chemical Co. (St. Louis, MO), [$^2H_4$]-methanol (99.5%) and [$^2H_6$]-acetone (99%) from E. Merck AG (Darmstadt), trifluoroacetic anhydride (TFAA) and *N,O*-bis(trimethylsilyl)acetamide (BSA) from Tokyo Kasei (Tokyo) and *n*-butylboronic acid from Aldrich Chemical Co. (Milwaukee, WI). 4-Hydroxyphenylacetamide was kindly supplied by ICI Pharm. Co. All other reagents used were of analytical-reagent grade. Piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) was prepared in these laboratories by the method reported previously.[6]

Gas Chromatography-Mass Spectrometry (GC-MS)——A Shimadzu Model LKB-9000B gas chromatograph-mass spectrometer connected on-line with a Shimadzu GC-mass pack 500-FDGT computer was used. A coiled glass column (1 m × 3 mm i.d.) was packed with 2% OV-1 on Gass Chrom Q (80—100 mesh). The flow-rate of carrier gas (helium) was 30 ml/min. The column temperatures were kept at 175°C for the trifluoroacetate, 220°C for the trimethylsilyl derivative and 210°C for the *n*-butylboronate. The injection port and ion source were kept at 250°C. The accelerating voltage, ionization voltage and trap current were 3.5 kV, 70 eV and 60 μA, respectively.

Syntheses of Authentic Samples——Melting points were taken on a micro hot-stage apparatus and are uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded on a Hitachi Model R-24A spectrometer at 60 MHz using tetramethylsilane as an internal standard. Abbreviations used: s=singlet, d=doublet, t=triplet, q=quartet and m=multiplet.

Ethyl 4-Hydroxyphenylglyoxylate (I): Chromium trioxide (500 mg) in 98% AcOH (3 ml) was added dropwise to a stirred solution of ethyl 4-hydroxymandelate (488 mg) in AcOH (5 ml), and the whole was allowed to stand at room temperature for 30 min. MeOH (2 ml) was added to decompose the excess reagent, and the resulting solution was diluted with AcOEt, then washed with ice-cold 5% Na_2CO_3 (5 ml) and water. The organic layer was dried over anhydrous Na_2SO_4 and evaporated down. The residue obtained was

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subjected to column chromatography on silica gel. Elution with $CHCl_3$ and recrystallization of the eluate from benzene gave I (117 mg) as colorless needles. mp 68—70°C. *Anal.* Calcd for $C_{10}H_{10}O_4$: C, 61.85; H, 5.19. Found: C, 61.64; H, 5.50. MS *m/z*: 194 ($M^{+\cdot}$), 121 $(M-73)^+$, 93 $(M-101)^+$. NMR (5% $C^2H_3O^2H$ solution) δ: 1.35 (3H, t, $-CH_2C\underline{H}_3$), 4.31 (2H, q, $-C\underline{H}_2CH_3$), 4.70 (solvent OH), 6.75 (2H, d, C_6H_4), 7.69 (2H, d, C_6H_4).

4-Hydroxyphenylglyoxylic Acid Amide (II): A solution of I (30 mg) dissolved in conc. NH_4OH was allowed to stand overnight in the refrigerator. The resulting solution was evaporated down and the residue obtained was subjected to column chromatography on silica gel. Elution with $CHCl_3$ and recrystallization of the eluate from benzene–acetone gave II (17 mg) as colorless needles. mp 165—166°C. *Anal.* Calcd for $C_8H_7NO_3$: C, 58.18; H, 4.27; N, 8.48. Found: C, 57.94; H, 4.17; N, 8.25. MS *m/z*: 165 ($M^{+\cdot}$), 121 $(M-44)^+$, 93 $(M-72)^+$. NMR (5% $C^2H_3O^2H$ solution) δ: 4.69 (solvent OH), 6.70 (2H, d, C_6H_4), 7.82 (2H, d, C_6H_4).

4-(2,3-Oxidopropoxy)phenylglyoxylic Acid Amide (III): Triethylamine (0.1 ml) was added to a solution of II (87 mg) in epichlorohydrin (5 ml), and the whole was stirred at 90°C for 1 h. After removal of the solvent by evaporation the residue was subjected to preparative thin-layer chromatography (TLC) using MeOH–$CHCl_3$ (1:9) as a developing solvent. Elution of the adsorbent corresponding to the spot (*Rf* 0.69) with acetone and recrystallization of the product obtained from the eluate from acetone–hexane gave III (25 mg) as colorless needles. mp 155—158°C. *Anal.* Calcd for $C_{11}H_{11}NO_4$: C, 59.72; H, 5.01; N, 6.33. Found: C, 60.23; H, 5.40; N, 6.51. MS *m/z*: 221 ($M^{+\cdot}$), 177 $(M-44)^+$, 121 $(M-100)^+$. NMR (5% $C^2H_3O^2H$ solution) δ: 2.99 (2H, m, $-\overset{O}{CH-C\underline{H}_2}$), 3.97 (3H, m, $-C\underline{H}_2\overset{O}{C\underline{H}-CH_2}$), 4.64 (solvent OH), 6.90 (2H, d, C_6H_4), 7.95 (2H, d, C_6H_4).

4-(2-Hydroxy-3-isopropylaminopropoxy)phenylglyoxylic Acid Amide (IV): A solution of III (20 mg) dissolved in isopropylamine (3 ml) was stirred at 60—70°C for 20 min. The solution was concentrated *in vacuo*, and the residue was subjected to preparative TLC using toluene–EtOH–AcOEt–conc. NH_4OH (6:4:2:1) as a developing solvent. Elution of the adsorbent corresponding to the spot (*Rf* 0.67) with MeOH and recrystallization of the product obtained from the eluate from acetone gave IV (16 mg) as colorless needles. mp 166—168°C. *Anal.* Calcd for $C_{14}H_{20}N_2O_4$: C, 59.98; H, 7.19; N, 9.99. Found: C, 59.70; H, 7.42; N, 9.56. MS *m/z*: 265 $(M^{+\cdot}-15)$, 121 $(M-159)^+$, 72 $(M-208)^+$. NMR (5% $C^2H_3O^2H$ solution): 1.13 (6H, d, $J=6$ Hz, $-CH(C\underline{H}_3)_2$), 2.90 (3H, m, $-C\underline{H}_2NHC\underline{H}\langle$), 4.03 (2H, m, $-OC\underline{H}_2CH\langle$), 7.03 (2H, d, C_6H_4), 8.09 (2H, d, C_6H_4).

4-(2,3-Oxidopropoxy)phenylacetamide (V): Triethylamine (0.15 ml) was added to a solution of 4-hydroxyphenylacetamide (5.0 g) in epichlorohydrin (50 ml), and the whole was stirred at 90°C for 4 h. The solution was concentrated *in vacuo*, and the residue was rinsed with hexane and recrystallized from MeOH to give V (4.84 g) as colorless plates. mp 163.5—167°C. MS *m/z*: 207 ($M^{+\cdot}$), 189 $(M-18)^+$. This compound was subjected to further reaction without elemental analysis.

4-(3-Amino-2-hydroxypropoxy)phenylacetamide (VI): Ammonia gas was bubbled into a solution of V (500 mg) in MeOH (80 ml) for 6 h, and the solution was allowed to stand at room temperature overnight. The resulting solution was concentrated *in vacuo*, and the residue was rinsed with acetone and recrystallized from MeOH to give VI (475 mg) as colorless needles. mp 169—173°C. Trifluoroacetate, MS *m/z*: 398 ($M^{+\cdot}$), 266 $(M-132)^+$. This compound was subjected to further reaction without elemental analysis.

[$^2H_6$]-Atenolol (VII): [$^2H_6$]-Acetone (1 ml) was added to a solution of VI (50 mg) in $C^2H_3O^2H$ (5 ml), and the whole was refluxed for 3 h. After addition of $NaBH_4$ (50 mg) under ice-cooling, the solution was allowed to stand at 0°C for 30 min. The resulting solution was concentrated *in vacuo*, and the residue was dissolved in 5% NH_4OH. The solution was extracted with AcOEt. The organic layer was dried over anhydrous Na_2SO_4 and concentrated. Recrystallization of the crude product from acetone–hexane gave VII (4.2 mg) as colorless needles. mp 139—145°C. The melting point showed no depression on admixture with nonlabeled authentic sample. Trifluoroacetate, MS *m/z*: 446, 314, 267.

Derivatization for GC-MS——The trifluoroacetate was prepared by treatment with trifluoroacetic anhydride–ether (1:1) in the usual manner. The trimethylsilyl derivative was obtained by treatment with *N*,*O*-bis(trimethylsilyl)acetamide for 30 min at room temperature. The *n*-butylboronate was formed on column by applying a dry sample together with *n*-butylboronic acid in dimethylformamide.[7]

Characterization of Metabolite M2 in Rat Urine——The urine was extracted with *tert*-BuOH–hexane (1:1) (400 ml×3) under the condition of pH >11. The organic layer was back-extracted with 0.2 N HCl. The aqueous layer was washed with AcOEt, adjusted to pH >11 with conc. NH_4OH and reextracted with *tert*-BuOH–hexane (1:1) (500 ml×3). The organic layer was concentrated, and the residue was purified twice by means of preparative TLC using toluene–EtOH–AcOEt–conc. NH_4OH (6:4:2:1) as a developing solvent. The adsorbent corresponding to the spot (*Rf* 0.65) was eluted with acetone. Portions of the eluate were derivatized into the trifluoroacetate, trimethylsilyl derivative and *n*-butylboronate. The remaining portion was treated with $NaBH_4$ in MeOH in the usual manner. The reduction product was trimethylsilylated and subjected to GC–MS.

Quantitation of Metabolite M2 in Rat Urine——Five male Sprague-Dawley rats weighing 250—255 g were orally given a single dose of atenolol (50 mg/kg) in capsules. A 24-h urine specimen was collected and diluted with 20 ml of water. After addition of the internal standard ([$^2H_6$]-atenolol), the urine sample was

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percolated through a column of Amberlite XAD-2 resin (30 cm × 10 mm i.d.). The column was washed with water (100 ml), and the metabolite was eluted with 30% MeOH (50 ml). The effluent was concentrated *in vacuo*, and the residue was redissolved in EtOH (5 ml). An aliquot of the solution was applied to PHP-LH-20 (0.5 ml). The eluate obtained with EtOH (15 ml) was derivatized into the trifluoroacetate and subjected to SIM.

Results and Discussion

It has previously been demonstrated that the principal urinary metabolites in rats given atenolol are the unchanged drug and a hydroxylated metabolite, M1.[5] However, characterization of another biotransformation product, M2, remained to be achieved. The chromatographic behavior of M2 was similar to that of atenolol, and these two compounds gave similer times on gas chromatography. Separation of the desired fraction was accomplished by preparative TLC (Fig. 1).

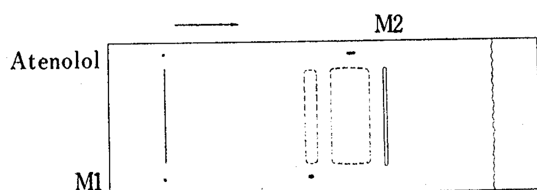

Fig. 1. Preparative Thin-layer Chromatogram of Urinary Metabolites of Atenolol in the Rat

The metabolite M2 was derivatized into the trifluoroacetate and subjected to GC–MS. The mass spectrum is illustrated in Fig. 2a. The occurrence of the characteristic ions (*m/z* 308 and 266) indicated that the isopropylaminopropanol group remained intact.[8] The molecular ion at *m/z* 454 suggested the formation of the nitrile structure (A) upon trifluoroacetylation, with concomitant loss of H_2O from the amide side chain, as in the case of heptafluorobutylation.[9] The characteristic ions at *m/z* 409 $(M-15)^+$ for the trimethylsilyl derivative (B) and at *m/z* 346 $(M^{+\cdot})$ for the cyclic *n*-butylboronate (C)[7] provided evidence for an increment of 14 in the molecular weight during this biotransformation (Figs. 2b and 2c). The formation of a fragment ion at *m/z* 308 with the trimethylsilyl derivative was ascribable to migration of the trimethylsilylisopropylamino group from the side chain to the positively charged aromatic ring.[10] These findings led us to assume that an oxygen function had been introduced at the methylene group of the acetamide side chain or the aromatic ring. On the other hand, reduction of M2 with sodium borohydride afforded the hydroxyl compound. The trimethylsilyl derivative of the reduction product (D) showed a mass spectrum identical with that of M1. The results confirmed that atenolol undergoes oxidative transformation to M2 having an oxo function at the methylene group of the acetamide side chain.

In order to establish the structure of M2 by direct comparison, preparation of an authentic sample was undertaken. First, ethyl 4-hydroxymandelate was transformed into ethyl 4-hydroxyphenylglyoxylate (I) by oxidation with chromium trioxide in acetic acid. Upon brief exposure to aqueous ammonia, I was readily converted to the corresponding amide (II). Condensation of II with epichlorohydrin in the presence of triethylamine gave 4-(2,3-oxidopropoxy)phenylglyoxylic acid amide (III) together with a small amount of the corresponding chlorohydrin. On treatment with isopropylamine, III was transformed into the desired compound (IV) in a satisfactory yield. The structures of these new compounds were supported by the usual criteria, *i.e.*, elemental analyses, and mass and NMR spectra. The assignment of the structure 4-(2-hydroxy-3-isopropylaminopropoxy)phenylglyoxylic acid amide to metabolite M2 was definitively established by direct comparison with the authentic sample.

We next sought to quantitate M2 in rats orally given atenolol by means of SIM. For this purpose, deuterium-labeled atenolol was synthesized as an internal standard. A key intermediate, 4-(3-amino-2-hydroxypropyl)phenylacetamide (VI), was prepared from 4-hydroxyphenylacetamide through 4-(2,3-oxidopropoxy)phenylacetamide (V) in a manner similar to that described above. On being refluxed with $[^2H_6]$-acetone in $[^2H_4]$-methanol, VI provided

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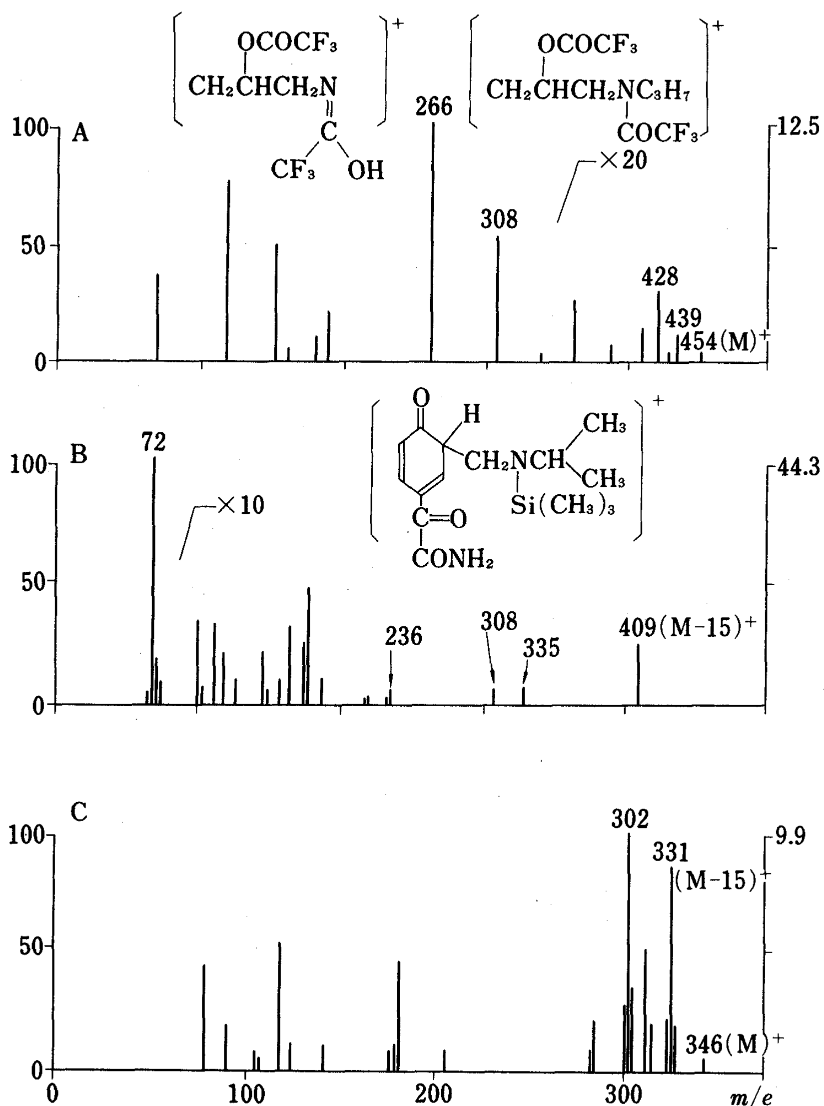

Fig. 2. Mass Spectra of Derivatives of Metabolite M2 from Rat Urine

A: trifluoroacetate, B: trimethylsilyl derivative, C: *n*-butylboronate.

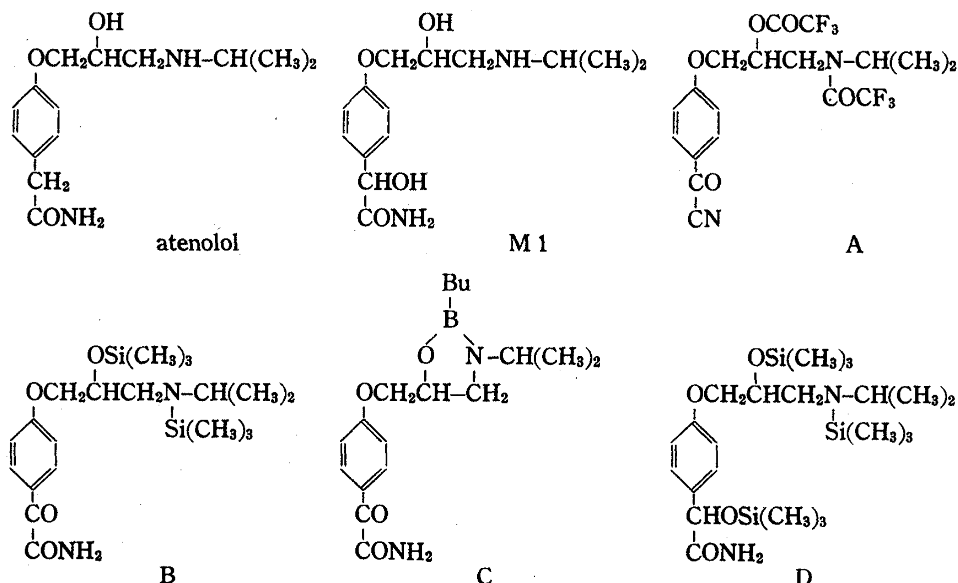

Chart 1

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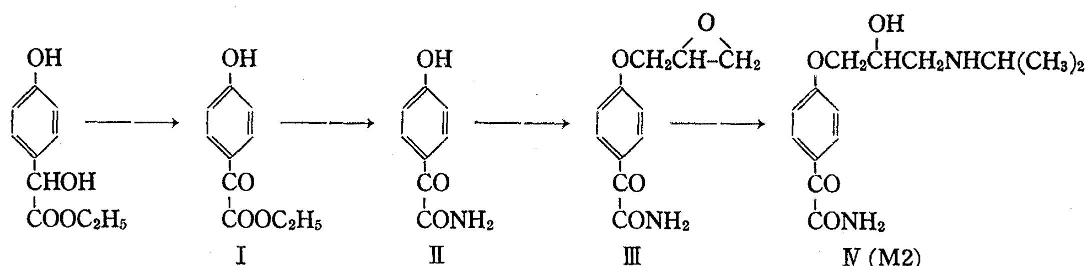

Chart 2

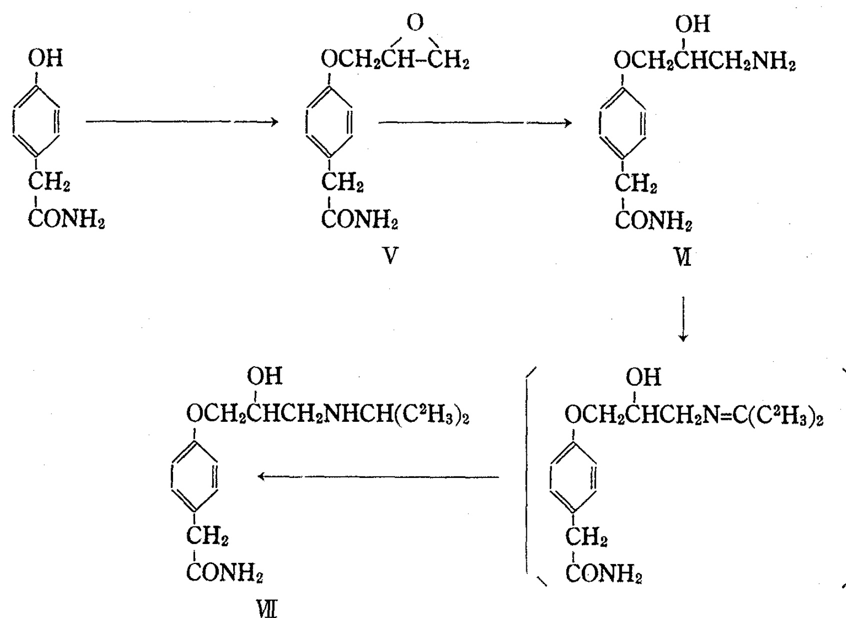

Chart 3

the Schiff base. Subsequent reduction with sodium borohydride in [$^2H_4$]-methanol gave the desired [$^2H_6$]-atenolol (VII). The incorporation of the heavy isotope into the isopropyl group was confirmed by the existence of a molecular ion at m/z 446, and two characteristic fragment ions at m/z 314 and 267 in the mass spectrum.

The separation and clean-up of M2 in rat urine was then carried out in the manner reported previously.[5] A urine specimen was percolated through a column of Amberlite XAD-2 resin. The fraction eluted with 30% methanol was applied to PHP–LH-20, a lipophilic ion-exchange gel, for the elimination of endogenous acidic substances. The eluate obtained with ethanol was derivatized into the trifluoroacetate for SIM. The selected ions used were m/z 454 for M2 and m/z 446 for the internal standard. A typical selected ion recording is illustrated in Fig. 3.

A calibration curve was constructed by plotting the ratio of peak area of the authentic M2 to that of the internal standard against the weight ratio of the two; satisfactory linearity was observed in the range of 10—100 ng of M2 (Fig. 4). A known amount of the authentic M2 was added to the rat urine, and the recovery rate was estimated by the standard procedure. The spiked M2 was recovered at a mean rate of 83.52±7.69% (n=5). The urinary excretion of M2 in 24 h after oral administration of atenolol was determined in the rat. The bioconversion rate of atenolol into M2 was found in the range of 0.86—1.60% with a mean of 1.04% (Table I). The amount of M2 excreted was approximately one-fifth of that of M1.

It should be noted that atenolol undergoes successive oxidative biotransformations at the position adjacent to both the aromatic ring and the carbamoyl group, providing two

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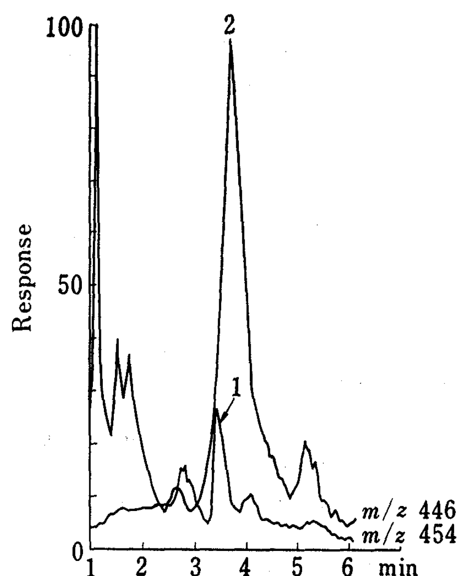

Fig. 3. A Selected Ion Recording of Metabolite M2 (Trifluoroacetate) from Rat Urine

1, metabolite M2; 2, internal standard (I.S.).

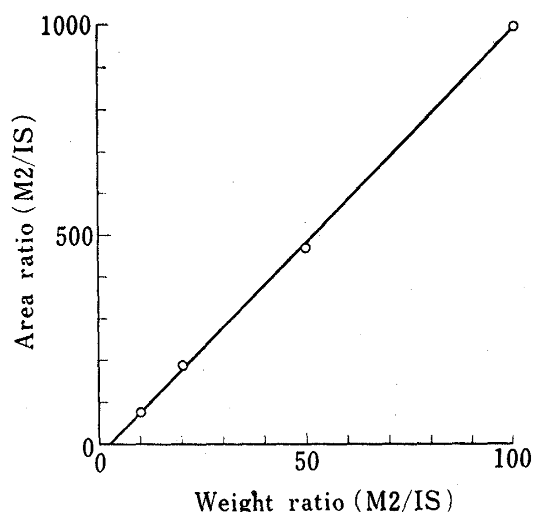

Fig. 4. Calibration Curve for Metabolite M2 (Trifluoroacetate)

TABLE I. Urinary Excretion of Metabolites in 24 h after Oral Administration of Atenolol in the Rat[a]

Metabolite	Excretion rate (%)					Mean
	A	B	C	D	E	
M2	0.87	0.98	1.60	0.89	0.86	1.04
M1						5.86[b]
Unchanged						26.12[b]

a) Given at a single dose of 50 mg/kg.
b) Taken from the previous report.[5]

principal metabolites, M1 and M2. These two products were formed in the rat, whereas only M1 was detectable in the dog. Detailed knowledge of the metabolic fate of atenolol in animals may lead to a better understanding of the pharmacological activities of this drug.

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