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Structural Determination of Dog and Human Urinary Metabolites of Nipradilol (K-351), a New Antihypertensive Agent

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The metabolic fate of Nipradilol (K-351: NIP), a new potent antihypertensive and antianginal agent, was investigated in the dog and human.

From dog urine, the parent drug and 11 metabolites were isolated by column chromatography on Amberlite XAD-2 resin and silica gel. Their chemical structures were deduced from spectral comparisons (mass, proton and carbon-13 nuclear magnetic resonance, infrared) with synthetic samples, and quantitative determination was accomplished by gas chromatography-mass spectrometry (GC-MS) (selected ion monitoring method). NIP was metabolized by four principal pathways in the dog: (a) denitration, giving denitro NIP; (b) aliphatic and aromatic hydroxylation of the 3,4-dihydro-2*H*-1-benzopyran ring, giving 4- or 5-hydroxy NIP or denitro NIP; (c) degradation of the isopropylaminopropanol side chain, including N-deisopropylation followed by N-methylation or deamination; (d) glucuronidation. 4-Hydroxy metabolites had a *trans*-conformation with 3- and 4-substituents in a pseudoaxial position.

In the human, 7 metabolites out of the 11 were identified; aromatic hydroxylation and N-methylation were not found, suggesting the existence of pronounced species differences. Moreover, degradation of the side chain was a minor route among the above pathways.

Keywords——Nipradilol (K-351); 3,4-dihydro-8-(2-hydroxy-3-isopropylamino)propoxy-3-nitroxy-2*H*-1-benzopyran; antihypertensive, antianginal agent; metabolism; structural determination; ^{13}C-NMR analysis; quantitative determination; GC-MS (SIM); dog; human

Nipradilol (K-351: 3,4-dihydro-8-(2-hydroxy-3-isopropylamino)propoxy-3-nitroxy-2*H*-1-benzopyran, NIP) is a new type of potent beta-adrenoceptor blocking agent having dilator action not only on arterial but also on venous vessels, resulting in regulatory effects on pre- or after-loads.[1] The dilator action of NIP is based on direct vasodilation (similar to that caused by glyceryl trinitrate) and alpha-adrenoceptor blockage.[2] Unlike traditional beta-adrenoceptor blocking agents, NIP possesses long-lasting antihypertensive action after a single oral administration to spontaneously hypertensive rats (SHR).[2]

In the cases of some beta-adrenoceptor blocking agents such as propranolol[3,4] and alprenolol,[5] ring-hydroxylated metabolites possess the significant pharmacological effects, suggesting a contribution of the metabolites to the activity of the parent drug following oral administration to man. Indeed, the denitro compound of NIP possessed beta-adrenergic blocking activity, though it lacked the vasoactive property.[2] Therefore, it seems to be interest to elucidate the metabolic fate and the pharmacological activity of the metabolites. The present investigations were undertaken to isolate, identify and determine the urinary metabolites of NIP in the dog and human.

Materials and Methods

Materials——The authentic samples used for the identification and determination of urinary metabolites were

prepared in our laboratories, as will be described in a succeeding paper. Trifluoroacetic and heptafluoro-*n*-butyric anhydride were purchased from Tokyo Kasei Co. All other chemicals and solvents used in this study were of reagent grade.

Drug Administration and Urine Collection——Dog: NIP was administered orally at a daily dose of 40 mg/kg to two dogs (beagle, *ca.* 10 kg) for 4 d, and the urine was combined and used for the isolation of metabolites. In the quantitative determination of urinary metabolites, six dogs were given a dose of 10 mg/kg. Dogs were fasted for 18 h prior to the experiment and kept individually in stainless steel metabolic cages with free access to water. Urine was collected daily and stored at −20 °C until used.

Human: Human urine was obtained from participants in the phase-I clinical study, which was performed by the single oral administration of NIP in the dose range of 1 to 24 mg. Six healthy male volunteers each took an NIP tablet together with 150 ml of water after a common meal. Urine was collected during 24 h after administration.

Thin-Layer Chromatography (TLC)——Precoated Silica gel $60F_{254}$ plates (E. Merck) with 0.25 mm layers were used for analytical separation and plates with 2 mm layers were used for isolation. Analytical and preparative chromatograms were developed to 12 cm from the origin with the following solvent systems (v/v): A, toluene–ethyl acetate–ethanol–28% aqueous ammonia (6 : 2 : 5 : 1); B, chloroform–methanol–acetic acid (15 : 5 : 2). Spots on the chromatograms were detected under ultraviolet (UV) light at 254 nm.

High Performance Liquid Chromatography (HPLC)——Analytical separations were accomplished by HPLC on a Finpak SIL-C_{18} column (25 cm × 0.46 cm i.d., Japan Spectroscopic Co.) with the following mobile phase (v/v): 0.1 M acetic acid (adjusted to pH 3.0 with 10% tetramethylammonium hydroxide methanol solution)–acetonitrile (a, 65 : 35; b, 85 : 15). In some cases, final purification of metabolites was performed by HPLC on a Partisil-10 column (10 μm silica gel, 20 cm × 0.46 cm i.d., Whatman Inc.) with the following mobile phase (v/v): chloroform–acetonitrile–isopropylamine (I, 76 : 20 : 4; II, 66 : 30 : 4; III, 90 : 5 : 5).

Characterization of Urinary Metabolites——Aliquots of urine (1 ml), following pH adjustment to 10 or 2, were poured through a column of Extrelut® 1 (E. Merck), and the metabolites were eluted with ethyl acetate or chloroform, respectively. Each eluate was concentrated *in vacuo*, and the residue was directly subjected to TLC and HPLC, or gas chromatography-mass spectrometry (GC-MS) after derivatization. The basic and neutral metabolites were derivatized into the trifluoroacetates (TFA) with trifluoroacetic anhydride and trimethylamine as a catalyst.[6] On the other hand, acidic metabolites were esterified with ethereal diazomethane and then derivatized to the TFA.

Procedure for the Isolation of NIP and Its Metabolites——For the identification of the metabolites excreted in urine, a large-scale experiment was carried out as follows. Pooled urine (2 l) was adjusted to pH 7 with HCl, and passed through a column of Amberlite XAD-2 (30 cm × 4 cm i.d.). The column was washed with water (0.5 l), and the metabolites were eluted with MeOH (1 l). The eluate was concentrated *in vacuo*, and the residue was separated by silica gel column chromatography (30 cm × 3 cm i.d.) into six fractions, which were eluted successively with the following solvent system (v/v): chloroform–MeOH–isopropylamine, 96 : 0 : 4 (0.4 l), 92 : 4 : 4 (0.4 l) and 81 : 15 : 4 (0.6 l). Fraction I (0.6—0.7 l) was further purified by preparative HPLC (mobile phase I). The residue was recrystallized from ethyl acetate to give 5 mg of M-1 as a white, crystalline compound. Fractions II (0.7—0.75 l) and III (0.75—0.8 l) were purified by preparative HPLC (mobile phases I and II, respectively), affording M-2 (5 mg) and M-3 (50 mg), respectively, as oily substances, which were chromatographically pure. Fraction IV (0.8—1.0 l) contained M-4 and M-5, but these metabolites could not be isolated because their amounts were too small. Fraction V (1.0—1.15 l) was further separated into two fractions by extraction with three 30 ml portions of chloroform containing 10% isopropanol under acidic conditions. The organic phase was further purified by preparative TLC (solvent-A), and the product was recrystallized from chloroform–*n*-hexane to give 40 mg of M-6 as a white, crystalline compound. Following the extraction of fraction V, the aqueous phase was applied to an XAD-2 column. The methanol eluate was separated into two fractions by preparative HPLC (mobile phase III), yielding M-7 (10 mg) as a white, crystalline compound and M-8 (10 mg) as a pale yellow substance. Fraction VI (1.15—1.3 l) contained M-9 and M-10, but these metabolites could not be isolated because their amounts were too small.

Quantitative Determination of Urinary Metabolites——NIP and its metabolites in urine were determined by means of GC-MS with selected ion monitoring (SIM). The acidic and neutral metabolites were extracted from urine (0.5 ml) into 5 ml of ethyl acetate containing 3,4-dihydro-3-hydroxy-8-(2-chloro-3-hydroxy)propoxy-2*H*-1-benzopyran (2.5 μg/ml) as an internal standard (I.S.) at pH 2. After removal of the ethyl acetate under a stream of nitrogen, the metabolites were derivatized into the methylesters with ethereal diazomethane then to the trimethylsilyl ethers with trimethylsilyl reagent (hexamethyldisilazane–trimethylchlorosilane–pyridine = 2 : 1 : 4) for SIM. The selected ions used were *m/z* 456 for M-6, *m/z* 412 for M-11, *m/z* 310 for M-12 and *m/z* 402 for the I.S.

To the aqueous layer that remained after the extraction with ethyl acetate, an aqueous solution of 3,4-dihydro-3-hydroxymethyl-8-(2-hydroxy-3-isopropylamino)propoxy-2*H*-1-benzopyran (2.5 μg/ml, 0.2 ml) as an I.S. was added, and the basic metabolites were extracted with ethyl acetate containing 10% isopropanol at pH 10. The metabolites were derivatized into the corresponding acyl compounds with heptafluoro–*n*-butyric anhydride and triethylamine for SIM. The selected ions used were *m/z* 362 (M-9), 480 (M-10) and 508 (M-3, 7, 8 and I.S.).

The determination of the metabolites possessing a nitroxy moiety was performed by the above method after reduction of the nitroxy moiety to a hydroxy group with zinc dust in HCl.[7,8] Glucuronides were determined as the

difference between the concentrations of the metabolites before and after enzymatic hydrolysis as described below. Urine samples were adjusted to pH 5 with 1 N HCl and divided into three 0.5 ml portions. The first was used as the unconjugated fraction and the second portion was treated with β-glucuronidase (10000 Fishman units, Type B-3, Sigma, U.S.A.) and arylsulfatase (240 units, Type H-1, Sigma, U.S.A.). The third portion was supplemented with 10 mg of saccharo-1,4-lactone together with the above enzyme mixture. Each portion was incubated for 3 h at 37 °C.

When various metabolites (0.1 μg) were added to the control urine and analyzed by the method described above, mean recoveries of the compounds were more than 90%, except for M-7 (80%). The calibration curves obtained by plotting peak height ratio *vs.* concentration of various metabolites showed good linearity, and the detection limits after extraction from 0.5 ml of urine were less than 25 ng in all cases.

Spectrometry——The low- and high-resolution MS obtained in the electron impact (EI) mode, were recorded with a JEOL JMS-DX300 mass spectrometer (Japan Electron Optics Lab. Co.) equipped with a JMA-3500 data analysis system, at an ionization potential of 70 eV. Chemical ionization (CI)-MS were obtained with a direct inlet system using ammonia as the reagent gas, at an ionization potential of 250 eV. GC-MS was performed on the above spectrometer coupled to a JEOL G05 gas chromatograph. A glass column (1 m × 2 mm i.d.) was packed with 3% OV-1 or OV-17 on Gas Chrom Q (80—100 mesh) and maintained at 170 or 260 °C, respectively, with a carrier gas (He) flow rate of 40 ml/min. The accelerating voltage, ionization voltage and trap current were 3.0 kV, 70 eV, 300 μA, respectively.

The proton (^{1}H-) and carbon-13 (^{13}C-)nuclear magnetic resonance (NMR) spectra were recorded at 200 MHz in CD_3OD solution with tetramethylsilane (TMS) as an internal reference on a JEOL model JNM-FX200 spectrometer. Off-resonance decoupling or selective proton decoupling were used in the assignment of ^{13}C-NMR signals.

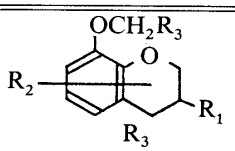
Infrared spectra (IR) were determined in KBr tablets with a JASCO model FT/IR-3 spectrophotometer (Japan Spectroscopic Co.).

Results

Dog

Isolation and Identification of Urinary Metabolites——The ethyl acetate and chloroform extracts obtained from urine on an Extrelut® 1 column under basic and acidic conditions, respectively, were directly subjected to TLC and HPLC, or GC-MS after TFA or methylester-TFA derivatization. TLC, HPLC, reconstructed ion chromatogram (RIC) and MS data are shown in Table I, Figs. 1 and 2, and Table II, respectively. The basic and acidic extracts contained 10 (M-1—M-10) and 2 (M-11 and M-12) metabolites, respectively, which were not observed in control urine. M-1, -2, -4 and -5 which were detected on TLC and HPLC did not afford peaks on GC, but gave peaks corresponding to M-3, -7, -9 and -10, respectively, when

Table I. *Rf* Value on TLC, Retention Index on GC and Retention Time on HPLC of the Urinary Metabolites

Metabolite				TLC *Rf* value		GC t_R index		HPLC t_R (min)	
No.	R_1	R_2	R_3	A	B	OV-1	OV-17	a	b
M-1	ONO_2	H	$CH(OH)CH_2NHCH(CH_3)_2$	0.62	0.59	—	—	22.20	—
M-2	ONO_2	OH	$CH(OH)CH_2NHCH(CH_3)_2$	0.50	0.51	—	—	11.18	—
M-3	OH	H	$CH(OH)CH_2NHCH(CH_3)_2$	0.43	0.47	2154	2339	5.26	13.40
M-4	ONO_2	H	$CH(OH)CH_2NH_2$	0.43	0.47	—	—	9.60	—
M-5	ONO_2	H	$CH(OH)CH_2NHCH_3$	0.43	0.47	—	—	13.64	—
M-6	OH	H	$CH(OH)CH_2OH$	0.27	0.59	1806	1946	—	7.08
M-7	OH	OH	$CH(OH)CH_2NHCH(CH_3)_2$	0.25	0.35	2178	2276	—	5.64
M-8	OH	OH	$CH(OH)CH_2NHCH(CH_3)_2$	0.23	0.29	2075	2115	—	4.66
M-9	OH	H	$CH(OH)CH_2NH_2$	0.18	0.29	2014	2235	—	6.88
M-10	OH	H	$CH(OH)CH_2NHCH_3$	0.18	0.29	2066	2285	—	8.10
M-11	OH	H	COOH	0.08	0.44	1825	2177	—	7.12
M-12	OH	H	CH(OH)COOH	0.06	0.34	1905	2162	—	7.44

TLC, GC and HPLC conditions: see the text.

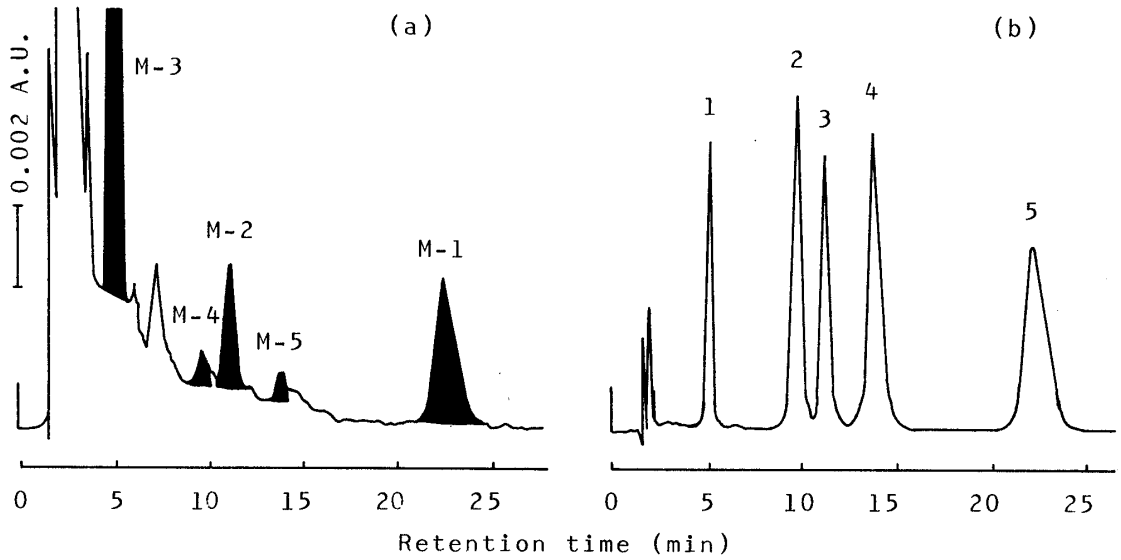


Fig. 1. HPLC Separation of Metabolites Possessing a Nitroxy Moiety from Dog Urine (a) and Synthetic Mixtures (b)

Peaks: 1, DNIP; 2, deisopropyl NIP; 3, 5-hydroxy NIP; 4, *N*-methyldeisopropyl NIP; 5, NIP. Conditions: column, Finpak SIL-C_{18}, 25 cm × 0.46 cm i.d.; mobile phase, system a; flow rate, 1.5 ml/min; detector, UV 254 nm.

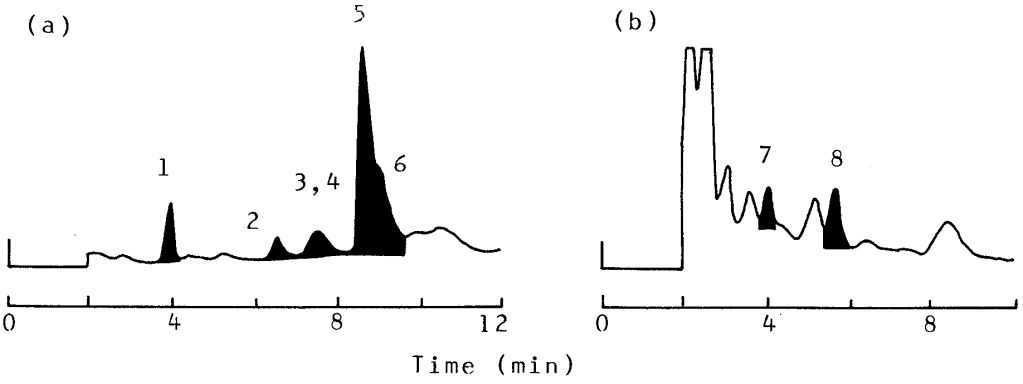


Fig. 2. Reconstructed Ion Chromatograms of Dog Urinary Basic (a) and Acidic (b) Extracts as TFA or Methylester-TFA Derivatives

GC conditions: column, 3% OV-1, 1 m × 2 mm i.d.; column temperature, 190 °C.

TABLE II. MS Data for TFA or Methylester–TFA Derivatives of Dog Urinary Metabolites

Peak No.	Metab. No.	Diagnostic ion (relative intensity), *m/z*				
		M^+	Side chain^{+}	Side chain − $C_3H_6$$^{+}$	MH − side chain^{+}	Other
1	M-6	528 (67)	267 (100)	—	262 (5)	
2	M-9	527 (16)	266 (26)	—	262 (100)	
3	M-10	541 (5)	280 (100)	—	262 (18)	
4	M-8	681 (5)	308 (100)	266 (98)	374 (2)	
5	M-3	569 (13)	308 (89)	266 (100)	262 (13)	
6	M-7	681 (6)	308 (100)	266 (93)	374 (3)	
7	M-11	334 (100)	—	—	—	220 (16), 147 (64)
8	M-12	460 (16)	199 (100)	—	262 (4)	171 (68)

Basic (peak nos. 1—6 on RIC in Fig. 2) and acidic (peak nos. 7 and 8) metabolites were derivatized into TFA and methylester–TFA, respectively.

they were derivatized into the reduction compounds with zinc dust under acidic conditions. These results suggest that these metabolites possess a nitroxy moiety in the molecule, like the parent drug. The MS of metabolites as TFA derivatives (summarized in Table II) exhibited characteristic fragment ions of the drug, such as two principal fragment ions of *m/z* 308 (isopropylaminopropanol side chain) and 262 (MH − side chain), resulting from cleavage at the ether linkage. The metabolites isolated and purified as described in the experimental section were analyzed by CI- and EI-MS, ^{1}H- and ^{13}C-NMR, and IR spectrometry to determine their structures. The MS and ^{1}H-NMR spectra are shown in Tables III and IV, respectively.

M-1 (NIP): The IR spectrum showed characteristic absorption bands due to the nitroxy moiety at 1620, 1280 and 879 cm^{-1}. The high-resolution MS gave a quasimolecular ion (MH^+) at *m/z* 327.1563, which corresponds to $C_{15}H_{23}N_2O_6$ (error, +0.6 mmu). All the

TABLE III. EI- and CI-MS Data for the Isolated Metabolites of NIP

Metab. No.	Diagnostic ion (relative intensity), *m/z*					
	EI					CI
	M^+	M − $C_2H_4O^{\neg+}$	M − $ONO_2^{\neg+}$	MH − side chain$^{\neg+}$	Other	
M-1	327 (MH^+, 2)	282 (100)	264 (14)	211 (18)	166 (82)	327 (MH^+)
M-2	—	298 (60)	280 (7)	227 (43)	182 (53)	343 (MH^+)
M-3	281 (1)	237 (44)	—	166 (100)	—	282 (MH^+)
M-6	240 (32)	—	—	166 (100)	—	253 ($M+NH_4^{\neg+}$)
M-7	297 (3)	253 (12)	—	182 (100)	—	298 (MH^+)
M-8	297 (2)	253 (92)	—	182 (100)	—	298 (MH^+)

TABLE IV. ^{1}H-NMR Spectral Data for the Ring System in the Isolated Metabolites of NIP

Metab. No.	Chemical shift (ppm)
M-1	4.24 (1H, dd, $J_{gem}=-12.2$ Hz, $J_{a,b}=1.2$ Hz, H_a), 4.47 (1H, ddd, $J_{gem}=-12.2$ Hz, $J_{a',b}=2.9$ Hz, $J_{a',c}=2.6$ Hz, $H_{a'}$), 5.53 (1H, m, H_b), 2.98 (1H, ddd, $J_{gem}=-17.9$ Hz, $J_{a,c}=2.6$ Hz, $J_{b,c}=2.6$ Hz, H_c),[a] 6.6—6.9 (3H, m, $H_{d,e,f}$)
M-2	4.19 (1H, d, $J_{gem}=-12.1$ Hz, H_a), 4.45 (1H, d, $J_{gem}=-12.1$ Hz, $H_{a'}$), 5.55 (1H, m, H_b), 2.92 (1H, d, $J_{gem}=-18.6$ Hz, H_c), 3.09 (1H, dd, $J_{gem}=-18.6$ Hz, $J_{b,c'}=5.4$ Hz, $H_{c'}$), 6.31 (1H, d, $J_{e,f}=8.8$ Hz, H_e), 6.71 (1H, d, $J_{f,e}=8.8$ Hz, H_f)
M-3	4.1—4.2 (3H, m, $H_{a,a',b}$), 2.71 (1H, dd, $J_{gem}=-16.5$ Hz, $J_{b,c}=6.1$ Hz, H_c), 3.04 (1H, dd, $J_{gem}=-16.5$ Hz, $J_{b,c'}=4.4$ Hz, $H_{c'}$), 6.6—6.8 (3H, m, $H_{d,e,f}$)
M-6	4.0—4.2 (3H, m, $H_{a,a',b}$), 2.71 (1H, dd, $J_{gem}=-16.6$ Hz, $J_{b,c}=5.8$ Hz, H_c), 3.06 (1H, dd, $J_{gem}=-16.6$ Hz, $J_{b,c'}=4.5$ Hz, $H_{c'}$), 6.6—6.8 (3H, m, $H_{d,e,f}$)
M-7	4.0—4.2 (3H, m, $H_{a,a',b}$), 2.58 (1H, dd, $J_{gem}=-14.2$ Hz, $J_{b,c}=5.6$ Hz, H_c), 2.93 (1H, dd, $J_{gem}=-14.2$ Hz, $J_{b,c'}=5.1$ Hz, $H_{c'}$), 6.26 (1H, d, $J_{e,f}=8.6$ Hz, H_e), 6.65 (1H, d, $J_{f,e}=8.6$ Hz, H_f)
M-8	4.15 (1H, dd, $J_{gem}=-11.4$ Hz, $J_{a,b}=3.2$ Hz, $J_{a,c}=1.0$ Hz, H_a), 4.23 (1H, dd, $J_{gem}=-11.4$ Hz, $J_{a',b}=2.6$ Hz, $H_{a'}$), 3.88 (1H, m, H_b), 4.44 (1H, d, $J_{b,c}=4.2$ Hz, H_c), 6.78—7.0 (3H, m, $H_{d,e,f}$)

a) The $H_{c'}$ signal is masked by the solvent resonance.

spectroscopic data, together with the *Rf* value on TLC and retention time on HPLC, were identical with those of NIP.

M-2 (5-Hydroxy NIP): The IR spectrum showed bands at 1631, 1276 and 856 cm^{-1} with the characteristic pattern of the nitroxy moiety. The CI-MS spectrum of M-2 exhibited MH^+ at *m/z* 343, which is 16 amu greater than that of NIP (M-1), suggesting that M-2 could be an oxygenated derivative of NIP. No molecular ion was observed in the EI-MS spectrum (Table III), but principal fragment ions were seen at *m/z* 298 ($M^+ - C_2H_4O$), 280 ($M^+ - ONO_2$) and 227 (MH − side chain), indicating the introduction of a hydroxyl moiety into the ring system. The absence of a molecular ion and the appearance of the base peak at *m/z* 298 can be explained by the facile loss of $CH_2=CHOH$ from the isopropylaminopropanol side chain, as has been observed in aryloxy beta-blocking drugs possessing this side chain.[9,10] In the ^1H-NMR spectrum of M-2 (Fig. 3), the signals due to the aromatic ring protons at the 6- and 7-position appeared at δ 6.31 (1H, d) and δ 6.71 (1H, d) with an *ortho*-coupling constant (J= 8.8 Hz). The ^{13}C-NMR data (Fig. 3) show that the signal of the carbon at the 5-position, as compared with that of NIP, was shifted downfield and appeared at about δ 150. From these results, M-2 was assumed to be 5-hydroxy NIP. A synthetic sample was proved to be identical with M-2 by comparison of their IR, NMR and MS.

M-3 (Denitro NIP, DNIP): The MS of M-3 showed a molecular ion at *m/z* 281.1640 ($C_{15}H_{23}NO_4$; error, +1.3 mmu) together with fragment ions at *m/z* 237 ($M^+ - C_2H_4O$) and 166 (MH − side chain), which are equivalent to the loss of 45 amu (NO_2) from NIP (Table III).

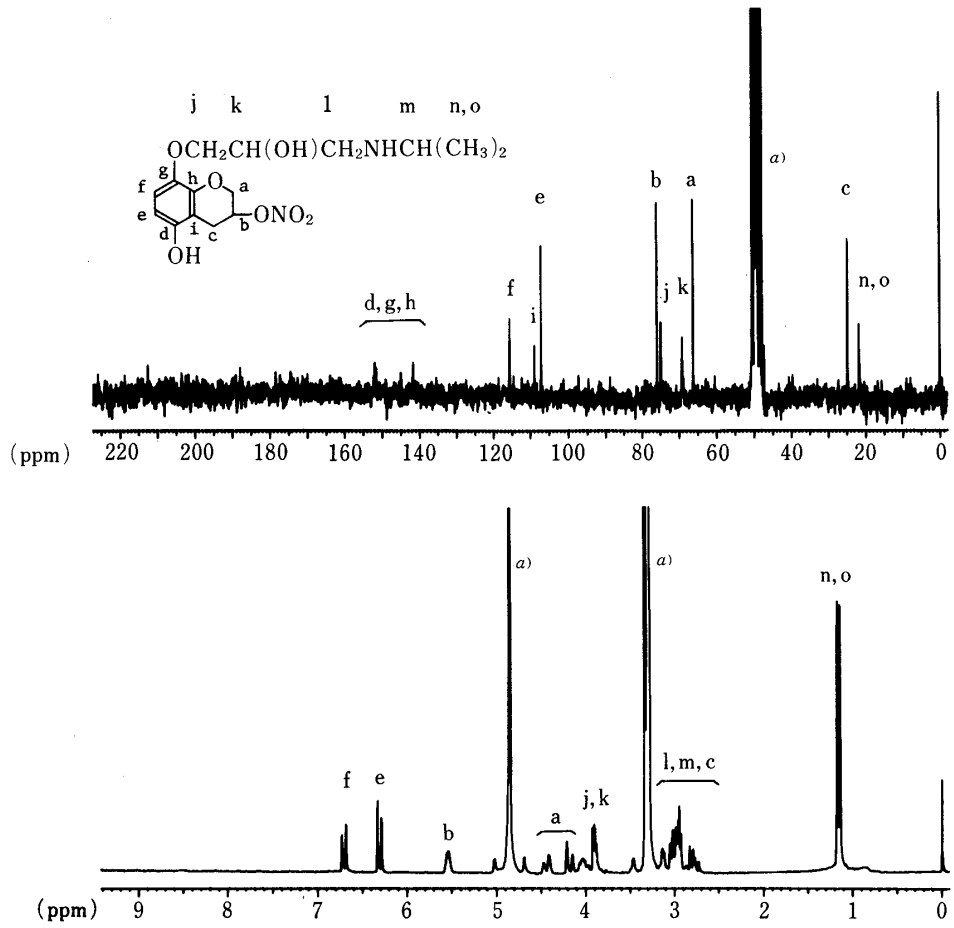


Fig. 3. ^{13}C- and ^1H-NMR Spectra of Isolated M-2

a) Solvent signals. The C_l and C_m signals in the ^{13}C-NMR spectrum are masked by the solvent resonances.

Further evidence of a denitrated structure was obtained from the NMR spectra. The signals of hydrogen and carbon at the 3-position, compared with those of NIP, were shifted upfield from δ 5.53 to approximately δ 4.15 (1H, m) and from δ 76.3 to δ 63.7, respectively. The assigned structure was confirmed by direct comparison with chemically synthesized denitro NIP.

M-4 (*N*-Deisopropyl NIP) and M-5 (*N*-Methyldeisopropyl NIP): These metabolites were present in small amounts. M-4 and M-5 each possessed a nitroxy moiety in the molecule, and the properties of their reduction products on GC-MS (TFA derivative) were identical with those of M-9 and M-10, respectively. The *Rf* values on TLC and the retention times in HPLC (Fig. 1) of M-4 and M-5 were identical with those of synthetic *N*-deisopropyl and *N*-methyldeisopropyl NIP, respectively.

M-6 (DNIP Glycol): High-resolution MS analysis of the molecular ion at *m/z* 240.0984 indicated a molecular formula of $C_{12}H_{16}O_5$ (error, -1.3 mmu). The absence of a nitrogen atom and the increment of an oxygen atom compared with DNIP (M-3, $C_{15}H_{23}NO_4$) can most likely be explained by the metabolic loss of the nitroxy moiety and the isopropylamino moiety, and by the addition of a hydroxyl moiety to the side chain. In the NMR spectra, the signals of hydrogens and carbons of the isopropyl moiety were absent, in agreement with the structure arrived at by MS. The downfield positions of the H_{13} and C_{13} ($OCH_2CHOH\underline{CH_2}OH$) at δ 3.67 (2H, m) and δ 64.4, respectively, reflect the strong deshielding effect of the adjacent hydroxyl moiety. These data enabled us to assign an aryloxypropyleneglycol structure. Finally, M-6 was identified by direct comparisons of the MS and NMR spectra with those of an authentic sample.

M-7 (5-Hydroxy DNIP): The EI-MS of M-7 (Table III) showed that the molecular ion at *m/z* 297.1593 ($C_{15}H_{23}NO_5$; error, $+1.6$ mmu) as well as the fragment ions of *m/z* 253 ($M^+ - C_2H_4O$) and 182 (MH − side chain) were 16 amu heavier than the corresponding ions for M-3, indicating the presence of the intact isopropylaminopropanol side chain, the introduction of a hydroxyl moiety into the ring system and the absence of the nitroxy moiety. In the ^{1}H-NMR spectrum (Table IV), the signals due to the aromatic ring protons appeared as a doublet at δ 6.26 (1H) and a doublet at δ 6.65 (1H) which showed an *ortho*-coupling

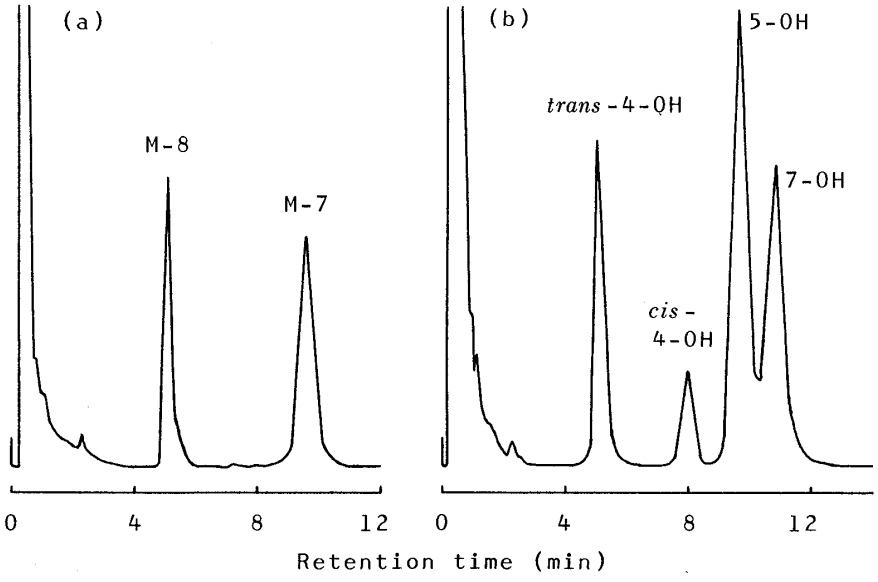


Fig. 4. GC Separation and Identification of Monohydroxy DNIP Isomers from Dog Urine (a) and Synthetic Mixtures (b) as TFA Derivatives

Conditions: column, 3% OV-17, 1 m × 3 mm i.d.; column temperature, 260 °C; carrier gas, N_2, 30 ml/min; detector, hydrogen flame ionization detecter (FID).

constant ($J=8.6$ Hz) as in the case of M-2 (Fig. 3). Gas chromatograms of the TFA derivatives of M-7 and synthetic 5- or 7-monohydroxy DNIP isomers are shown in Fig. 4. The retention time of M-7 was identical with that of the 5-hydroxy isomer and reduction product of M-2. From the above data, the metabolite (M-7) was concluded to be 5-hydroxy DNIP, and the assigned structure was confirmed by direct comparison with an authentic sample.

M-8 (4-Hydroxy DNIP): The direct MS (Table III) and GC-MS (tetra TFA derivative, Table II) of M-8 were practically identical with those of M-7 except for the relative intensities of the fragment ions. These data indicated that M-8 could be a positional isomer of M-7. The NMR spectrum of M-8 (Fig. 5) was entirely different from that of M-7. The absence of a hydroxyl-substituted pattern for the aromatic protons indicated that M-8 was an aliphatic hydroxylated derivative. The signals of hydrogen and carbon at the 4-position of the 3,4-dihydro-2*H*-pyran ring were shifted downfield and appeared at δ 4.44 (1H, d, $J=4.2$ Hz) and δ 68.7, respectively. From these results, M-8 was considered to be 4-hydroxy DNIP. With respect to the configuration of the 3,4-diol structure (M-8), *trans* conformation can be assumed on the basis of the following results: vicinal coupling constants (J) of *trans* and *cis* isomers of a structurally related chroman derivative were $J_{3,4}=4$ Hz and $J_{3,4}=3$ Hz, respectively, as reported by Lap,[11] and the coupling constant of M-8 ($J_{3,4}=4.2$ Hz) was similar to the former. *trans*- and *cis*-hydroxy DNIP were synthesized selectively and their NMR spectra were compared with those of M-8. M-8 was found to be identical with the synthetic *trans*-diol, but distinctly different from the synthetic *cis*-diol. The proton at the 4-position in the *trans*-diol (δ 4.44, $J=4.2$ Hz) gave a signal at higher field and a larger J value

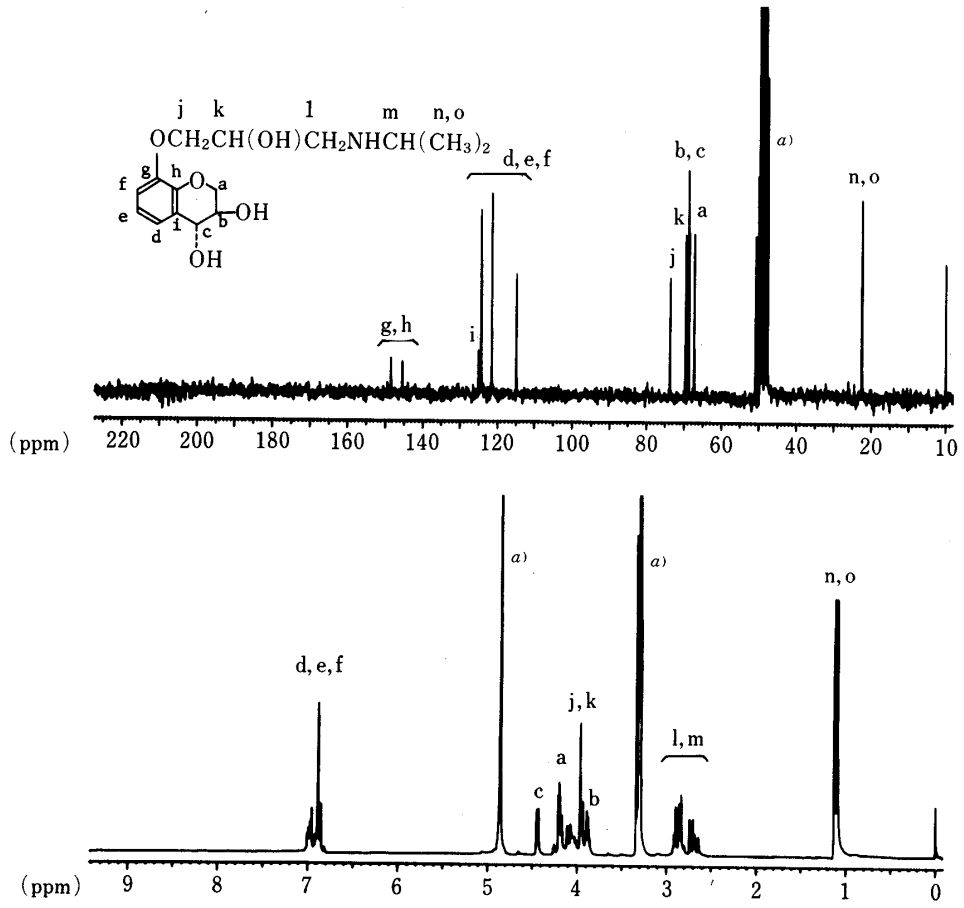


Fig. 5. ^{13}C- and ^{1}H-NMR Spectra of the Isolated M-8

a) Solvent signals. The C_l and C_m signals in the ^{13}C-NMR spectrum are masked by the solvent resonances.

than the *cis* isomer (δ 4.68, $J=3.4$ Hz). Additional evidence for the structure was obtained from GC of the TFA derivative. The synthetic *trans* and *cis* isomers were separated from each other on the chromatogram, and the retention time of isolated M-8 was identical with that of the *trans* isomer (Fig. 4).

M-9 (*N*-Deisopropyl DNIP) and M-10 (*N*-Methyldeisopropyl DNIP): In the MS of M-9 (Table II), the tri-TFA derivative showed the molecular ion at *m/z* 527.0602 ($C_{18}H_{14}F_9NO_7$; error, -2.4 mmu) and a fragment ion derived from the side chain at *m/z* 266, which corresponded to the loss of 42 amu (C_3H_6) from DNIP (M-3) possessing the isopropylamino-propanol side chain. On the other hand, the MS of M-10 (Table II) showed that the molecular ion at *m/z* 541.0758 ($C_{19}H_{16}F_9NO_7$; error, -2.5 mmu) and a fragment ion of the side chain at *m/z* 280 were 14 amu (CH_2) heavier than the corresponding ions of M-9. These data suggested that M-10 was derived by N-methylation of the primary amine in M-9. M-9 and M-10 were shown to be identical with authentic samples of chemically synthesized *N*-deisopropyl and *N*-methyldeisopropyl derivatives of DNIP, respectively, by comparison of the MS.

M-11 (DNIP Acetic Acid): The MS (Table II) of the methylester-TFA derivative showed the molecular ion at *m/z* 334.0649 ($C_{14}H_{13}F_3O_6$; error, -1.4 mmu) together with fragment ions at 220 ($M^+ - CF_3COOH$) and 147 (*m/z* 220 − side chain, CH_2COOCH_3). These data suggested the presence of an acetic acid moiety. An authentic sample of aryloxyacetic acid had the same GC-MS properties as M-11.

M-12 (DNIP Lactic Acid): The MS (Table II) of the methylester-TFA derivative exhibited the molecular ion at *m/z* 460 and an aryl ion (MH − side chain) at *m/z* 262, indicating that the ring system was identical with that in M-3, -6, -9 and -10. The characteristic side chain fragment ion of *m/z* 199.0207 ($C_6H_6F_3O_4$; error, -1.1 mmu) presumably arises from cleavage at the ether linkage, followed by loss of CO to give the ion at *m/z* 171.0267 ($C_5H_6F_3O_3$; error, -0.2 mmu), suggesting the presence of a lactic acid moiety, as reported by Hoffmann.[12] The GC and MS properties of M-12 were identical with those of the

TABLE V. Quantitative Determination of the Urinary Metabolites of NIP in Dog and Human after Single Oral Administration

Metabolites	Urinary excretion (% of dose)					
	Dog (10 mg/kg)			Human (12 mg)		
	F[a]	C[b]	F+C	F	C	F+C
M-1 (NIP)	5.6	7.6	13.2	7.0	10.3	17.3
M-2 (5-Hydroxy NIP)	1.7	2.1	3.8	n.d.[c]	n.d.	n.d.
M-3 (DNIP)	5.9	3.8	9.7	18.5	6.2	24.7
M-4 (*N*-Deisopropyl NIP)	3.3	1.8	5.1	3.8	0.8	4.6
M-5 (*N*-Methyl M-4)	0.9	0.3	1.2	n.d.	n.d.	n.d.
M-6 (DNIP glycol)	3.2	1.8	5.0	n.d.	n.d.	n.d.
M-7 (5-Hydroxy DNIP)	4.1	3.1	7.2	n.d.	n.d.	n.d.
M-8 (4-Hydroxy DNIP)	2.0	0.7	2.7	4.3	n.d.	4.3
M-9 (*N*-Deisopropyl DNIP)	3.3	1.2	4.5	1.2	0.9	2.1
M-10 (*N*-Methyl M-9)	1.5	0.4	1.9	n.d.	n.d.	n.d.
M-11 (DNIP acetic acid)	1.0	n.d.	1.0	0.5	n.d.	0.5
M-12 (DNIP lactic acid)	4.7	n.d.	4.7	1.5	n.d.	1.5
M-13 (4-Hydroxy NIP)	0.4	0.3	0.7	5.6	2.8	8.4
Total	37.6	23.1	60.7	42.4	21.0	63.4

a) Free metabolites. *b*) Conjugated metabolites. *c*) Not detected.

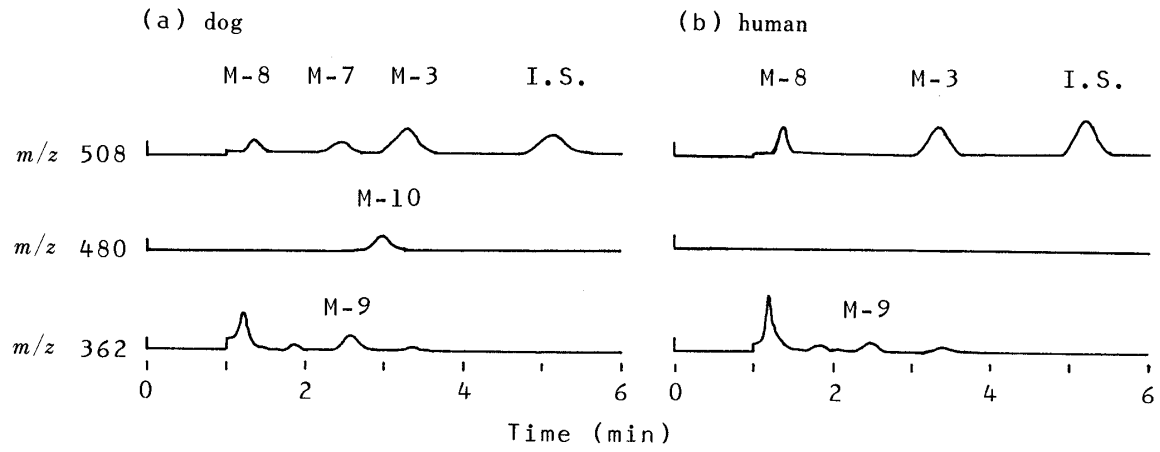


Fig. 6. Selected Ion Recordings of HFB Derivatives of Urinary Basic Extracts from Dog (a) and Human (b)

GC conditions: column, 3% OV-17, 1 m × 2 mm i.d.; column temperature, 190 °C. I.S.: internal standard (see the text).

synthetically prepared lactic acid derivative.

Quantitative Determination of Urinary Metabolites——The metabolites in 48 h urine were determined by SIM after oral administration of NIP (10 mg/kg) to dogs, and the results are shown in Table V. As shown in Fig. 6, the heptafluorobutyryl derivatives (HFB) of the metabolites were separated satisfactorily on a 3% OV-17 column and could be detected selectively and sensitively. A small amount of 4-hydroxy NIP (M-13) was newly detected by SIM. The urinary excretion of unchanged NIP was less than 6% of the dose, and the amount of its glucuronide was 7.6%. The major metabolites were DNIP (M-3) and 5-hydroxy DNIP (M-7), which accounted for about 7—9% of the dose, as estimated by summation of the free and conjugated metabolites.

Human

Since the isolation of the human urinary metabolites could not be accomplished on account of the small dosage employed, identification was carried out by comparison of the mass spectral fragmentations of the TFA or HFB derivatives with those of samples isolated from dog urine or synthesized. The parent drug and 7 metabolites (M-3, -4, -8, -9, -11, -12 and -13) were identified in human urine, but the 5-hydroxy metabolites (M-2 and -7), *N*-methyldeisopropyl metabolites (M-5 and -10) and DNIP glycol (M-6) were not detected, as shown in Fig. 6. Table V lists the mean quantities of metabolites in the 0—24 h urinary fraction after oral administration of NIP at the dose of 12 mg. It was found that 7.0% of the dose was excreted as unchanged NIP in the urine, and the amount of its glucuronide was 10.3%. The other major metabolites were DNIP (M-3) and 4-hydroxy NIP, which accounted for 24.7% and 8.4% of the dose, respectively, as estimated by summation of the free and conjugated metabolites.

Discussion

NIP has three structural characteristics, a nitroxy moiety, an isopropylaminopropanol side chain and a 3,4-dihydro-2*H*-1-benzopyran ring, in the molecule. The parent drug and 11 metabolites were isolated from the urine of dog, and their structures deduced from spectral examination in comparison with chemically synthesized compounds. On the other hand, only 7 metabolites of the above 11 were identified in human urine. Urinary excretion of unchanged NIP was less than 7% of the dose in both species, suggesting extensive metabolism. A

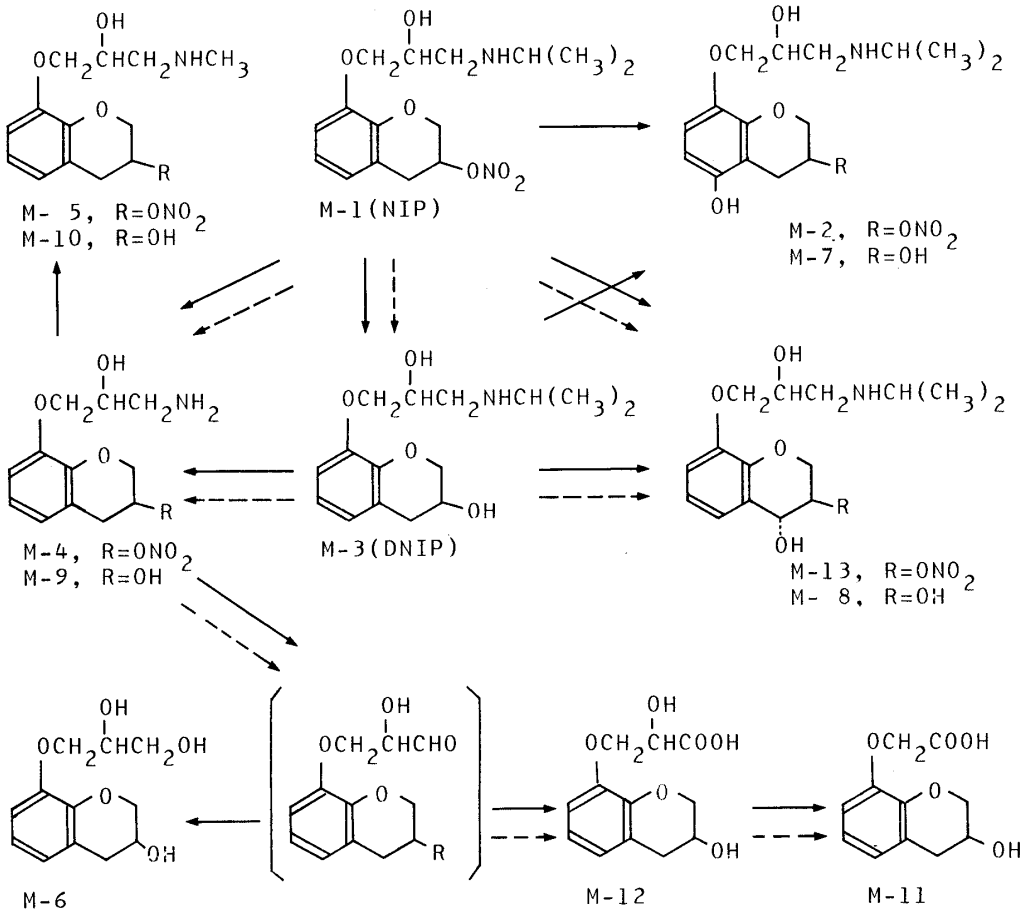


Fig. 7. Possible Metabolic Pathways of NIP in the Dog and Human

——: dog, – – –: human.

summary of the proposed metabolic pathways of NIP in dog and human is shown in Fig. 7.

NIP is metabolized by four distinct pathways: denitration, hydroxylation of the ring system, degradation of the side chain and glucuronidation.

The first major pathway involves denitration of NIP and various metabolites. The denitration was catalyzed by glutathione-dependent organic nitrate reductase (unpublished data), which is known to denitrate various organic nitrate compounds including glyceryl trinitrate and isosorbide dinitrate.[13] The total amount of the metabolites retaining the nitroxy moiety in dog and human was estimated to be about 30% and 50%, respectively, suggesting that the conversion to denitrated metabolites may proceed gradually. This was further substantiated by an additional investigation on the enzymatic reaction in rat liver, which showed that the denitration of NIP was about 20 times slower than that of the above nitrate drugs, as will be described in a succeeding paper.

The second pathway involves the aromatic and aliphatic hydroxylation of the 3,4-dihydro-2*H*-1-benzopyran ring, and exhibits species difference. The hydroxylation of the aromatic ring at the *para*-position to the isopropylaminopropanol side chain gives rise to 5-hydroxy NIP (M-2) and 5-hydroxy DNIP (M-7). It seems likely that 5-hydroxylated metabolites are formed *via* an intermediate 5,6-arene oxide, followed by opening of the oxirane ring at the 6-position to a keto tautomer and enolization, as in the hydroxylation of the aryloxy beta-blocking drugs, *e.g.*, propranolol[14,15] and oxprenolol.[16] Other aromatic hydroxylated metabolites were not detected in the urine, and it can be considered that the stability of the intermediate carbonium ion or steric hindrance of the side chain contributes to

the highly selective 5-hydroxylation on the ring system. On the other hand, aliphatic hydroxylation occurs at the 4-position of the 3,4-dihydro-2*H*-pyran ring, giving rise to 4-hydroxy DNIP (M-8). The stereochemistry of this 3,4-diol metabolite was confirmed by analysis of the ^{1}H-NMR splitting patterns of the protons of the pyran ring (Table IV, Fig. 5). The large value of the long-range coupling constant $J_{2eq,4}$ (1.0 Hz) suggests the half-chair conformation of the 3,4-dihydro-2*H*-pyran ring due to the restricted rotation imposed by the benzene ring.[11] The C-3 proton shows vicinal coupling constants of $J_{2ax,3}=2.6$ Hz, $J_{2eq,3}=3.2$ Hz and $J_{4,3}=4.2$ Hz, implying an equatorial–equatorial relationship for the protons at the 3- and 4-position. Thus, the 3,4-diol metabolite is considered to have a *trans*-conformation, with both hydroxyl groups in a pseudoaxial position of a half-chair conformation. The *trans*-conformation was established by comparison of the NMR spectrum and the GC properties of the TFA derivative with those of synthesized geometrical isomers. This *trans*-diaxial conformation seemed to be formed readily because of the occurrence of dipole–dipole repulsion between the 3- and 4-substituents and repulsion between the 4-substituent and the peri-hydrogen, as discussed by Cotterill *et al.*[17] The conversion of NIP to the 3,4-diol metabolite presumably proceeds *via* formation of 4-hydroxy NIP, which was found in dog and human urine. The bulky nitroxy moiety in NIP exists in the axial conformation on the basis of the observed vicinal $J_{2ax,3}$ (1.2 Hz) and $J_{2eq,3}$ (2.9 Hz) coupling constants, and such an axial conformation is favored by the biotransformation of the *trans*-diaxial metabolite. Pronounced species difference was observed in the above hydroxylation: aromatic hydroxylation was favored in the dog whereas only alipatic hydroxylation was observed in the human. This difference is of particular interest.

In the dog, the isopropylaminopropanol side chain is metabolized through a variety of metabolic processes including oxidative dealkylation, N-methylation, and deamination. However, these pathways were minor in the human. Moreover, N-methylation, which seems likely to be catalyzed by phenylethanolamine N-methyltransferase or by nonspecific N-methyltransferase,[18] was not found in the human metabolism of NIP. Such a species difference has been commonly reported for most beta-blockers.[19] As for the conversion of NIP to oxidative deamination products (M-6, -11 and -12), it is assumed that NIP is first transformed into the primary amines (M-4 and -9) and subsequently led to an aldehyde intermediate, which then may be reduced to the glycol (M-6) or oxidized to acidic metabolites (M-11 and -12). Similar reaction sequences have been proposed for propranolol[20–24] and alprenolol.[12]

The glucuronide of NIP was also identified indirectly by treatment of the urine with β-glucuronidase and a specific inhibitor of the enzyme, saccharo-1,4-lactone. Since the amount of glucuronide was 7.6% of the dose for the dog and 10.3% for the human (Table V), glucuronidation is one of the principal pathways together with the above three pathways.

The results of radiochemical analysis of the urine and feces after oral administration of ^{14}C-NIP to rats showed that NIP was completely absorbed, but extensively metabolized through pathways similar to those in the dog, and that 60% of the dosed radioactivity was excreted *via* the bile into the feces (unpublished data). The reason for the relatively low total urinary excretion (60% of dose) in the present studies on dog and human may be biliary excretion.

The pharmacological effects of the metabolites are important for elucidation of the overall effects of a drug. NIP is chemically related to other beta-blockers, *e.g.* propranolol and alprenolol in having the isopropylaminopropanol side chain and aromatic ring system, except for the nitroxy moiety. Propranolol[3,4] and alprenolol[5] were metabolized to ring-hydroxylated compounds, which have been shown to possess significant beta-adrenergic blocking activity. As denitrated (M-3) and monohydroxylated (M-2, -7, -8 and -13) metabolites of NIP have an intact side chain, they would be expected to possess beta-

adrenergic blocking activity, even if they lack the vasoactive property which is due to the nitroxy moiety. A single oral treatment with NIP to SHR caused significant falls of both systolic and diastolic blood pressures, whereas M-3 failed to cause any appreciable change in blood pressure, but remarkably reduced the heart rate.[2)] In screens for the assessment of antihypertensive action by intravenous administration to SHR, M-2, -3, -8 and -13 all possessed only transient vasoactivity, while the heart rate was remarkably reduced by all these metabolites, as will be described in a succeeding paper. Since a remarkable antihypertensive effect was produced by repeated oral administration of M-3 to SHR, as found with other beta-blockers,[25)] metabolite M-3 and other monohydroxylated metabolites might contribute to the antihypertensive action of the parent drug.

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