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Studies on the Fate of Naproxen. II.[1] Metabolic Fate in Various Animals and Man

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The species differences in the metabolism of naproxen [(+)-6-methoxy-α-methyl-2-naphthaleneacetic acid] were investigated in 6 species of animals and man. ^{3}H-naproxen was orally administered to mice, rats, rabbits, guinea pigs, dogs and miniature pigs (OHMINI-875) and cold naproxen to human subjects, and their urinary and biliary (rats only) metabolites were separated and characterized by thin-layer chromatography, gas chromatography, high-performance liquid chromatography and gas chromatography-mass spectrometry. The excretion rates as % of dose of naproxen and its metabolites in the 24 hr urine were, in a decreasing order, miniature pig 82.5, mouse 80.4, rat 74.7, rabbit 70.3, guinea pig 48.5 and dog 23.0%. Naproxen and its metabolites, *i.e.*, 6-hydroxy-α-methyl-2-naphthaleneacetic acid (6-DMN), naproxen glucuronide, 6-DMN glucuronide and 6-DMN sulfate, were found in the urines of all species. Urinary naproxen in the rat was only 0.2% and those in the other species of animals were 4.8—13.7% of the urinary radioactivity, while in man it was 1.3% of dose. The main urinary metabolite in rat, guinea pig, dog, mouse and rabbit was 6-DMN sulfate (87.3, 46.7, 42.9, 39.9 and 36.2% of the urinary radioactivity, respectively), while those in man and miniature pig were naproxen glucuronide (25.3% of dose) and 6-DMN (78.6% of the urinary radioactivity), respectively.

The animals whose urinary metabolic profiles were the nearest to that of man were the guinea pig and mouse, while the animals that differed most from man in this regard were the rat and miniature pig.

Keywords——(+)-6-methoxy-α-methyl-2-naphthaleneacetic acid; anti-inflammatory drug; drug metabolism; urinary metabolites; species differences; high-performance liquid chromatography; gas chromatography–mass spectrometry; gas chromatography

Naproxen [(+)-6-methoxy-α-methyl-2-naphthaleneacetic acid] was synthesized by Harrison *et al.*[3] as a new nonsteroidal anti-inflammatory agent. Its analgesic and anti-inflammatory activities were observed to be superior to those of indomethacin or aspirin.[4] The prominent therapeutic efficacy of naproxen in the treatment of rheumatoid arthritis was reported by Katona *et al.*,[5a] Lussier *et al.*[5b] and Shiokawa *et al.*[5c]

We reported previously on the whole body autoradiography in the mouse and the urinary and biliary metabolites in the rat after oral administration of ^{14}C-naproxen.[1] The urinary metabolites in various animals and man after intravenous injection of ^{3}H-naproxen were investigated by Thompson *et al.*[6] The present report is concerned with the results of inves-

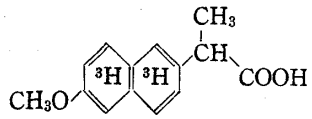
1) Part I: T. Takahashi and Y. Sato, *Radioisotopes*, **22**, 351 (1973).
2) Location: *2-2-50, Kawagishi, Toda, Saitama.*
3) I.T. Harrison, B. Lewis, P. Nelson, W. Rooks, A. Roszkowski, A. Tomolonis, and J.H. Fried, *J. Med. Chem.*, **13**, 203 (1970).
4) *a*) A.P. Roszkowski, W.H. Rooks II, A.J. Tomolonis, and L.M. Miller, *J. Pharmacol. Exp. Ther.*, **179**, 114 (1971); *b*) H. Ozawa, M. Ikeda, and N. Ito, *Oyo Yakuri*, **6**, 201 (1972).
5) *a*) G. Katona, E. Ortega, and J. Robles-Gill, *Clin. Trials J.*, **8**, 3 (1971); *b*) A. Lussier, E.J. Segre, C.V. Multz, K.L. MacCannell, S.J. Alexander, D.L.G. Howard, G. Boost, J. Varady, and W. Strauss, *Clin. Pharmcol. Ther.*, **14**, 434 (1973); *c*) Y. Shiokawa, T. Okada, and A. Sakuma, *Ryumachi*, **14**, 141 (1974).
6) G.F. Thompson and J.M. Collins, *J. Pharm. Sci.*, **62**, 937 (1973).

tigation on the species differences in the urinary metabolites of naproxen in several animal species, *i.e.*, rat, mouse, guinea pig, dog, rabbit and miniature pig (OHMINI-875), and man after oral administration of naproxen.

Experimental

Compounds——[ring-G-^{3}H]Naproxen was supplied from Syntex Research, and it was labeled on the naphthalene moiety as indicated in Chart 1. The specific radioactivity was 600 mCi/mmol, and the radiochemical purity was more than 99% when examined by thin-layer chromatography (TLC).

6-Hydroxy-α-methyl-2-naphthaleneacetic acid (6-DMN), [ring-G-^{3}H] 6-DMN and N-(2-(6-methoxy-2-naphthyl)-propionyl)-glycine (Nap-Gly) were synthesized by the methods of Fried *et al.*[7] and Thompson *et al.*,[6] and used as the reference compounds for identification of the metabolites of naproxen. The optical property of these reference compounds was not considered in this experiment. The radiochemical purity of ^{3}H-6-DMN was more than 98.5% when examined by TLC. 6-Methoxy-2-naphthylacetic acid (6-MNA) supplied from Syntex Research was used as the internal standard for the quantitative analysis of the human urinary metabolites by gas chromatography (GC).



[ring-G-^{3}H naproxen]

Chart 1. Chemical Structure and Labeled Position of ^{3}H-Naproxen

Animal Experiments——Five male Wistar rats (*ca.* 200 g), 6 male ddY mice (*ca.* 20 g), 5 male Hartley guinea pigs (*ca.* 300 g), 2 male beagle dogs (*ca.* 11 kg), 4 male white rabbits (*ca.* 3.5 kg) and 2 mal OHMINI-875 miniature pigs (*ca.* 12 and 14 kg) were used. The animals were fasted for 16 hr prior to the start of the experiments while water was allowed *ad libitum*. ^{3}H-Naproxen dissolved in 0.1 M phosphate buffer (pH 8.1) was administered orally to the animals other than the miniature pigs through a stomach tube in a dose of 10 mg/kg. The miniature pigs were given the same dose of ^{3}H-naproxen in a small portion of pasty chow. The animals were kept in metabolism cages and urine was collected for 24 hr after the administration of the drug. In a similar manner as above, ^{3}H-naproxen was also given orally to 5 male Wistar rats in a dose of 30 mg/kg and the 24-hr urine was collected. For a biliary excretion experiment, 5 male Wister rats were anesthetized with urethane (1 g/kg) and a polyethylene tube was inserted into the common bile duct. Then ^{3}H-naproxen was administered orally in a dose of 30 mg/kg. The bile was collected for 24 hr.

Measurement of Radioactivity——The radioactivity in the samples of urine and AcOEt extracts was measured with a liquid scintillation spectrometer (Aloka LSC-652, equipped with an automatic quenching monitor system) in 15 ml of naphthalene–dioxane scintillator (naphthalene 35 g, PPO 5 g, dimethyl-POPOP 0.3 g, dioxane 730 ml, toluene 135 ml and MeOH 35 ml). Radioactive zones on TLC-plates were scraped into counting vials and extracted with 2 ml of MeOH; then 15 ml naphthalene–dioxane scintillator was added to the vials and the radioactivity was measured.

Influence of pH on the Extraction of Metabolites——Five rats were orally administered 30 mg/kg of ^{3}H-naproxen and the 24-hr urine was collected. Several aliquots of the pooled rat urine were diluted with 3 volumes of water and adjusted to various pH from 1.0 to 6.5 with 1 N HCl and then the solutions were extracted with 2 volumes of AcOEt 3 times. The combined AcOEt layers were washed with water and the radioactivity in the organic phase was determined. The unchanged naproxen and its metabolites in the AcOEt fraction were determined by TLC with the solvent system A as described later. The extraction recoveries of ^{3}H-naproxen and ^{3}H-6-DMN were determined by adding these compounds to samples of 4-times diluted normal rat urine and extracting them with AcOEt at various pH (1.0—7.0) in the same way as described above.

Separation of Metabolites from the Animal Urines and Rat Bile——The unchanged naproxen and its metabolites in the animal urines and rat bile were separated according to the following procedure.

Fat-soluble Metabolites: The 24-hr urine or bile was diluted with 3 volumes of water and extracted with 2 volumes of AcOEt 3 times at pH 6.0—6.5. The organic layer was washed with water. This fraction was termed F-A.

Water-soluble Metabolites: The residual aqueous layer was diluted with 24 volumes of 0.3 M acetate buffer (pH 4.8). The solution was incubated with β-glucuronidase (Sigma Chemical Co., Type B-1, Bovine Liver; 1000 units/10 μg naproxen calcd. from radioactivity) for 24 hr at 37° and extracted 3 times with 2 volumes of AcOEt. The aqueous layer was incubated once more with fresh enzyme and extracted in the same way. The combined organic layers were washed with water (F-B). The residual aqueous layer was further diluted with an equal volume of the same buffer. The solution was incubated with aryl sulfatase (Sigma Chemical Co., Type II; 10 units/5 μg 6-DMN calcd. from radioactivity) for 24 hr at 37°, and then extracted 3 times with 2 volumes of AcOEt. The aqueous layer was incubated 2 more times with fresh enzyme and extracted with AcOEt. The AcOEt layers were combined and washed with water (F-C).

7) J.H. Fried and I.T. Harrison, S. African Patent 6804378 (1968) [*C.A.*, **71**, 91163*k* (1969)].

Identification of Metabolites——The metabolites in the AcOEt fractions (F-A, F-B and F-C) were separated and purified by TLC and/or high-performance liquid chromatography (HPLC), and identified by TLC, HPLC, GC and gas chromatography–mass spectrometry (GC–MS). The optical property of these metabolites was not examined in this experiment. The urinary metabolites in man were characterized by GC and GC-MS after separation and derivatization as described later.

TLC: Thin-layer plates coated with silica gel GF_{254} (Merck) in 0.25 mm thickness were activated at 110° for 30 min. Three solvent systems were used, *i.e.*, (A) benzene–tetrahydrofuran–acetic acid (25: 3: 3, v/v), (B) benzene–MeOH–acetic acid (90: 16: 8, v/v) and (C) $CHCl_3$–MeOH (10: 1, v/v). Spots on the plates were detected with a UV-lamp (2536 Å). A radio-chromatogram scanner (Aloka TRM-1B) was used for the detection of radioactive compounds. The presence of the phenolic hydroxyl group was examined with Folin-Ciocalteu's and Pauly's reagents.

HPLC: A Waters Model 660 solvent programmer equipped with two Model 6000 solvent delivery systems was used for 30min linear gradient elution from 10% MeOH–90% 0.02 M KCl to 50% MeOH–50% H_2O. The flow rate was set at 2.0 ml/min. A Waters μBondapak packed column (30 cm × 4 mm i.d.) was used, and the column effluents were monitored with Varian Model 635 UV-Visible spectrophotometer operating at 230 nm.

Each AcOEt extract or urine sample was filtered through a membrane filter (0.45 μm) and injected alone or together with reference compounds by means of Waters Model U6K injector.

GC: A Shimadzu GC-4BMPF gas chromatograph equipped with a flame ionization detector (FID) was employed. The column was a 2 m × 3 mm i.d. glass tube packed with 10% OV-101 on Chromosorb W, 100—120 mesh (Applied Science Labs., State College, Pa., USA). The temperature of the column oven was maintained at 220° and those of the injection port and the detector were both 250°. The flow rate of the carrier gas (nitrogen) was 40 ml/min.

Naproxen and its metabolites extracted with AcOEt were derivatized for GC analysis as follows. The carboxyl group of the compounds was esterified with 500 μl of 4% HCl–MeOH in the presence of 50 μl of acetone dimethylacetal at 5° overnight. After the mixture was evaporated to dryness under reduced pressure, the hydroxyl group of the residual compounds was trimethylsilylated with 200 μl of a mixture of N,O-bis (trimethylsilyl)-trifluoroacetamide and N-trimethylsilyl-imidazole (1: 1, v/v) at room temperature for more than 1 hr. Similar GC conditions and sample preparation method were used for the determination of urinary metabolites in man.

GC–MS: A Shimadzu LKB-9000 GC–MS system was employed. The column was 60 cm × 3 mm i.d. glass tube packed with 10% OV-101 on Gas Chrom Q (100—120 mesh). The temperature of the column oven was maintained at 180°. The temperatures of the injection port and the separator were both 250°, and that of the ionization source was kept at 270°. The flow rate of the carrier gas (helium) was 30 ml/min. Ionization energy and trap current were 70 eV and 60 μA, respectively.

The samples were prepared in the same way as for the GC analysis.

Quantitative Determination of Metabolites from the Animal Urine and Rat Bile——^{3}H-Naproxen and its metabolites in the fractions (F-A, F-B and F-C) separated from the animal urines and the rat bile according to the procedure described above were determined.

The AcOEt layers were subjected to TLC together with the nonlabeled reference compounds (naproxen, 6-DMN and Nap-Gly). After being developed and dried, the chromatograms were examined under the UV-lamp (2536 Å) and with the radio-chromatogram scanner. Then the zones of naproxen, 6-DMN, and Nap-Gly were scraped into counting vials and extracted with 2 ml of MeOH. The radioactivity was measured with the liquid scintillation spectrometer as described above.

Separation and Quantitative Determination of Urinary Metabolites in Man——Three healthy male volunteers, ranging in weight from 53 to 61 kg, were given a single dose of two 100 mg tablets of naproxen, and urine was collected for 24 hr. In order to determine non-conjugated metabolites, 3 ml of a urine sample was diluted with 2 ml of water and 6-MNA was added as internal standard (15 μg in 150 μl of MeOH). Then the solution was extracted 3 times with 10 ml of AcOEt at pH 6.0. The combined AcOEt extracts were washed with water, and extracted twice with 7 ml of 5% $NaHCO_3$. The alkaline aqueous phase was adjusted to pH 3.0 with 6 N HCl and extracted twice with 10 ml of AcOEt. After evaporation of the AcOEt extract, the residual compounds were treated as described above for GC analysis. To determine conjugated metabolites, 0.5 ml of urine was diluted with 4.5 ml of 0.2 M acetate buffer (pH 4.8), and to this solution was added β-glucuronidase (the same enzyme lot as used in the animal experiments, 3000 units). The solution was incubated for 16 hr at 37°, and extracted twice with 10 ml of AcOEt at pH 4.8 after addition of the internal standard. Other conjugated metabolites in the residual aqueous layer were incubated in 2 N HCl for 16 hr at 37°. The free naproxen and 6-DMN formed were extracted with AcOEt at pH 6.0 along with the internal standard added previously. These compounds extracted after enzymatic or acid-catalyzed hydrolysis were analyzed by GC as described above. Since the AcOEt extract after the treatment with β-glucuronidase contained the non-conjugated naproxen and 6-DMN which were originally present in the urine, the quantities of glucuronides were calculated by substracting the quantities of these non-conjugated metabolites.

Results

Influence of pH on the Extraction of the Metabolites

The ratio of naproxen and its metabolites extracted with AcOEt at various pH from the rat urine are shown in Fig. 1. At pH 6.0—6.5 less than 5% of the urinary radioactivity was extracted, but the extractable radioactivity increased with lowering pH and became nearly 80% at pH 1.0. When determined by TLC, the relative amount of naproxen extracted with AcOEt was always less than 0.2%, while that of 6-DMN increased with lowering pH from ca. 4% at pH 6.5 to *ca.* 75% at pH 1.0 and accounted for almost all extractable radioactivities at pH 6.0—6.5 and pH 1.0. The ratios of conjugated metabolites, which were extracted with AcOEt and retained at origin on TLC, was insignificant at pH 6.0 or above, but increased gradually as the pH was lowered from 4 to 2 and again decreased at pH 1.0. When ^{3}H-naproxen and ^{3}H-6-DMN were added to the normal urine of the rat, their recoveries by the AcOEt extraction were quantitative at any pH below 6.5. From these results, it was concluded that at acidic pH 6-DMN was released from its conjugates by acid-catalyzed hydrolysis and extracted with AcOEt.

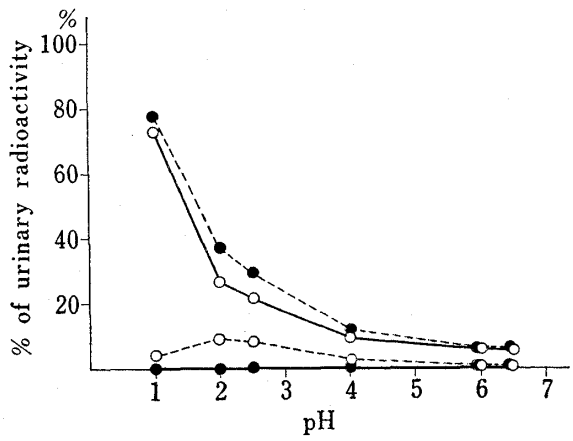


Fig. 1. Extraction Ratios of Naroxen, 6-DMN and Other Metabolites in the Rat Urine after Oral Administration of ^{3}H-Naproxen (30 mg/kg)

---●---: total extractable radioactivity, —○—: 6-DMN, ---○---: conjugates,[a] —●—: naproxen.
a) These compounds retained at origine on thin-layer chromatograms.

Urinary Excretion in the Animals and Biliary Excretion in Rats

The excretion of radioactivity in the 24-hr urine after oral administration of ^{3}H-naproxen (10 mg/kg) was estimated in the 6 animal species. Miniature pigs and mice excreted 82.5% and 80.4% of the given radioactivity, respectively, which were the highest percentages among the 6 species. The excretion ratios in rats, rabbits, guinea pigs and dogs were 74.7%, 70.3 %, 48.5% and 23%, respectively. After oral administration of ^{3}H-naproxen to rats in a dose of 30 mg/kg, the excreted radioactivities in the 24-hr urine and bile were 76.0% and 48.0% of dose, respectively.

Table I. *Rf* Values and Retention Times (t_R) of the Authentic Compounds by Thin-Layer Chromatography (TLC) and High-performance Liquid Chromatography (HPLC)

Compound	TLC *Rf* value			HPLC[c]
	t_R Solvent A[b]	Solvent B[b]	Solvent C[b]	t_R (min)
Naproxen	0.63	0.41	0.37	30.0
6-DMN	0.52	0.20	0.12	20.5
Nap-Gly	0.36	0.19	0.04	—
6-DMN sulfate[a]	—	—	—	14.0

a) This compound was isolated by HPLC from the rat urine after administration of naproxen.
b) Solvent A: benzene: THF: AcOH (25: 3: 3), solvent B: benzene: MeOH: AcOH (90: 16: 8), solvent C: $CHCl_3$: MeOH (10: 1)
c) Conditions are described in the Experimental of the text.

Chromatography of Authentic Compounds

Table I shows the *Rf* values of authentic naproxen, 6-DMN and Nap–Gly in TLC developed with three solvent systems. The retention times (t_R) of authentic naproxen, 6-DMN and 6-DMN sulfate (this compound was isolated by HPLC from rat urine after administration of naproxen) in HPLC are also listed in Table I. In GC, the t_R of authentic compounds, *i.e.*, methyl esters of 6-MNA and naproxen, and TMS derivative of 6-DMN methyl ester were 10.2, 11.3 and 15.4 min, respectively.

Characterization of Metabolites of Naproxen in the Animals

Fat-soluble Metabolites——When the fraction F–A was subjected to TLC with solvent system A, radioactivity was detected mainly at *Rf* 0.63 and 0.52, and slightly at 0.36. These radioactive compounds were named M–I, M–II and M–III, respectively. Weak radioactivity was found also at origin.

The *Rf* values of M–I in TLC with three solvent systems and t_R of M–I or M–I methyl ester in HPLC or GC were found to be identical with those of naproxen or its methyl ester. The identity was confirmed by co-chromatographies with authetic naproxen and its methyl ester. The mass spectrum of M–I methyl ester gave two main peaks at *m/e* 244 (M^+) and *m/e* 185 (base, $[M-COOCH_3]^+$) corresponding to those of naproxen methyl ester, and the fragment ion peak pattern was identical to that of authentic compound. From these results M–I was identified to be naproxen. In addition, the identity was also confirmed by the isotope dilution method.

M–II showed positive color reaction with Pauly's and Folin–Ciocalteu's reagents, suggesting the presence of phenolic hydroxyl group. The *Rf* values of M–II in TLC and t_R of M–II or TMS derivative of M–II methyl ester in HPLC or GC were identical with those of 6-DMN or TMS derivative of 6-DMN methyl ester. On the mass spectrum of TMS derivative of M–II methyl ester, two main peaks at *m/e* 302 (M^+) and *m/e* 243 (base, $[M-COOCH_3]^+$) and the other fragment ion peaks corresponded to those of authentic 6-DMN derivative. These results prove that M–II is identical with 6-DMN.

M–III was presumed to be a glycine conjugate of naproxen (Nap–Gly) from the *Rf* values in TLC, but its quantity was not enough for identification.

The radioactive substance at origin in TLC with solvent system A was also retained at origin when re-chromatographed with solvent system B and C. This radioactive material was extracted from the silica gel of the TLC plates with MeOH and treated with 2 N HCl or β-glucuronidase. The products formed were identified as naproxen and 6-DMN by TLC with the three solvent syetms. These results indicated that a part of the glucuronide conjugates of naproxen and 6-DMN were extracted with AcOEt and retained at origin on the thin-layer plate.

Water-soluble Metabolites——After the extraction of fat-soluble metabolites, the aqueous layer was treated with β-glucuronidase, and extracted with AcOEt. The AcOEt extract, F–B, was examined by TLC with solvent system A. The resulting chromatogram showed the same pattern as that of F–A, and the two main components at *Rf* 0.63 and 0.52 were identified as naproxen and 6-DMN, respectively, by TLC with the three solvent systems, and by HPLC, GC and GC–MS. These results showed that glucuronides of naproxen and 6-DMN were excreted as water-soluble metabolites in the urine of the animals and in the rat bile. Nap–Gly was presumably present also in this fraction (F–B), because a weak radioactivity was detected at *Rf* 0.36 on TLC with solvent system A. The radioactive material retained at origin on the thin-layer plate yielded 6-DMN on treatment with 2 N HCl or aryl sulfatase, indicating the presence of 6-DMN sulfate.

After the AcOEt layer (F–B) was removed, the aqueous layer was treated with aryl sulfatase, and the resulted hydrolyzate was extracted with AcOEt. The AcOEt layer (F–C) was examined in the same way as for F–A and F–B. Almost all the radioactivity in F–C

was confirmed to be 6-DMN, indicating that 6-DMN sulfate was present in the animal urine and rat bile. 6-DMN sulfate in the animal urines was isolated by HPLC and hydrolyzed with aryl sulfatase. The reaction mixture was then chromatographed to confirm the formation of 6-DMN by the hydrolysis.

Metabolic Profiles in the Animals

The ratios of naproxen and its metabolites to the total radioactivity excreted in the 24-hr urine or bile of rat after oral administration of 30 mg/kg of ^{3}H-naproxen are shown in Table II. The ratio of unchanged naproxen in the urine was very low, accounting for only 0.3%

Table II. Naproxen and Its Metabolites in Urine and Bile of Rats collected for 24 hr after Oral Administration of ^{3}H-Naproxen (30 mg/kg)

Compound	% of radioactivity in	
	Urine	Bile
Naproxen	0.3	39.1
6-DMN	4.2	1.2
Naproxen glucuronide	1.5	12.3
6-DMN glucuronide	0.5	9.6
6-DMN sulfate	86.5	11.6
Nap-Gly	0.2	1.7
Other	7.3	24.5

The urine or bile samples of 5 rats were combined before analysis.

of the total urinary radioactivity. The ratio of demethylated naproxen, free 6-DMN, was 4.2%, but that of its sulfate conjugate was 86.5% of the urinary radioactivity. The profile of biliary metabolites of rat was considerable different from that of the urinary metabolites. The ratio of unchanged naproxen, 39.1%, was very high compared with that in the urine, while those of 6-DMN sulfate and 6-DMN glucuronide were both about 10%.

Table III. Naproxen and Its Metabolites in 24-hr Urine of Various Animals after Oral Administration of ^{3}H-Naproxen (10 mg/kg)

Compound	% of urinary radioactivity					
	Rat (5)	Mouse (5)	Guinea pig (5)	Dog (2)	Rabbit (4)	OHMINI-875 miniature pig (2)
Naproxen	0.2	9.3	10.5	13.7	9.4	4.8
6-DMN	3.4	10.4	13.8	8.0	19.4	78.6
Naproxen glucuronide	1.4	11.1	14.4	12.8	4.6	1.3
6-DMN glucuronide	0.5	19.5	7.0	6.4	17.0	2.6
6-DMN sulfate	87.3	39.9	46.7	42.9	36.2	3.1
Nap-Gly	0.1	0.2	0.2	0.2	1.6	0.2
Others	7.1	9.6	7.4	16.0	11.8	9.4

Numbers in parentheses indicate the number of animals used.
The urine of each species were combined before analysis.

Table III shows the ratios of naproxen and its metabolites excreted in the 24-hr urine of the six animal species after oral administration of ^{3}H-naproxen in a dose of 10 mg/kg. The ratio of unchanged naproxen in the rat urine was only 0.2% of the total urinary radioactivity, the lowest among the six animal species, while the corresponding ratios were 4.8% in the miniature pig and 9.3—13.7% in the other four species. In the miniature pig 6-DMN was excreted

in an extremely high proportion, 78.6%, while the ratio was only 3.4% in the rat. Urinary glucuronides of naproxen and 6-DMN in mouse urine accounted for 30.6%, the highest among the 6 species, and the ratios in the rabbit, guinea pig and beagle dog were about 20%, while those in the rat and miniature pig were only 1.9% and 3.9%, respectively. Urinary 6-DMN sulfate occupied the highest proportions, more than 35% in all the test animals except the miniature pig, which excreted this sulfate conjugate in ratio of only 3.1%. The highest percentage of 6-DMN sulfate was 87.3% shown by the rat. The compounds termed "others" in the Table III represent the radioactivity of the aqueous layer after treatment with aryl sulfatase followed by extraction with AcOEt.

High-performance liquid chromatograms of the rat and mouse 24-hr urines after oral administration of ^{3}H-naproxen are shown in Fig. 2. These chromatograms show marked differences in the urinary metabolites between the rat and mouse; in the chromatogram of the mouse urine three peaks, which were presumed to be the ester type glucuronide of

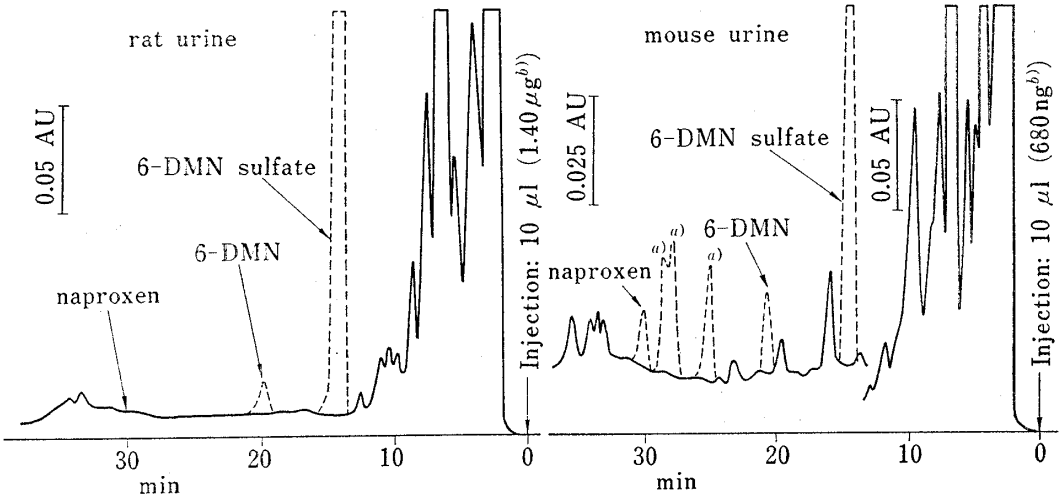


Fig. 2. High-performance Liquid Chromatograms of 24-hr Urine of the Rat and the Mouse after Oral Administration of ^{3}H-Naroxen (10 mg/kg)

The solid lines represent the chromatograms of control urines; the dotted line peaks indicate urinary naproxen and its metabolites.
a) These peaks were presumed to indicate the glucuronides of naproxen and 6-DMN.
b) These values are based on the assumption that all ^{3}H in the urine is ^{3}H-naproxen.

Table IV. Urinary Excretion of Naproxen and Its Metabolites in 24-hr Urine of Man after Oral Administration of Naproxen (100 mg-Tablet × 2)

Subjects (age, weight)		Naproxen or 6-DMN (% of dose)				
		Non-conjugates	Conjugates: Hydrolyzed with β-glucuronidase	Conjugates: Hydrolyzed with 2N HCl[a)]	Total	
I.T. (34 y, 63 kg)	Naproxen	1.1	30.2	4.0	35.3	58.9
	6-DMN	0.8	11.2	11.6	23.6	
A.Y. (44 y, 61 kg)	Naproxen	1.9	33.6	3.9	39.4	54.8
	6-DMN	0.5	4.5	10.4	15.4	
H.K. (52 y, 53 kg)	Naproxen	0.9	12.1	2.9	15.9	36.5
	6-DMN	1.3	9.1	10.2	20.6	
($\bar{X}\pm$SE)	Naproxen	1.3 ±0.31	25.3 ±6.7	3.60±0.35	30.2±7.3	50.1±6.9
	6-DMN	0.87±0.23	8.27±2.0	10.8 ±0.54	19.9±2.4	

a) The conjugates hydrolyzed with β-glucuronidase had been removed before hydrolysis with 2N HCl.

naproxen and both ester and ether type glucuronide of 6-DMN, are seen between the peaks of naproxen and 6-DMN, while in the chromatogram of the rat urine no such peaks are seen in the same t_R range; the ratio of peak area of 6-DMN sulfate to that of 6-DMN or naproxen is larger in the rat urine than in the mouse urine.

Urinary Metabolites in Man

The relative quantities of non-conjugated and conjugated metabolites of naproxen excreted in the 24-hr urine of three healthy male subjects after ingestion of naproxen (200 mg) are shown in Table IV. The ingested naproxen was excreted mainly as conjugates of naproxen and 6-DMN in ratios of 28.9 and 19.07%, respectively, of the dose, while non-conjugated naproxen and 6-DMN were only 1.3 and 0.87%, respectively. The major conjugate of naproxen was the glucuronide, accounting for 25.3%. As for the conjugates of 6-DMN, the glucuronide and another conjugate, presumably the sulfate, were excreted in nearly equal percentages, *i.e.*, 8.27 and 10.8%, respectively.

The urinary naproxen and 6-DMN were identified by GC and GC–MS, and the glucuronides of these compounds were characterized after enzymatic hydrolysis. 6-DMN sulfate was characterized by enzymatic hydrolysis after purification by HPLC.

Discussion

Runkel *et al.*[8] reported that the main route of excretion of ^{3}H-naproxen administered intravenously was *via* the urine in man, rat, guinea pig, rhesus monkey and mini pig, and *via* feces in dog, the latter suggesting extensive biliary involvement. We determined the urinary excretion rates after oral administration of ^{3}H-naproxen to six animal species. The results were similar to those reported by Runkel *et al.*[8]

We previously reported the urinary and biliary metabolites in the rat after oral administration of ^{14}C-naproxen.[1] In a subsequent investigation, however, new information was obtained concerning the stability of the conjugated metabolites; the results of the study on the influence of pH on the extraction of rat urinary metabolites with AcOEt indicated that the pH range suitable for extraction of unchanged naproxen and free 6-DMN was 6.0—6.5. At lower pH (pH 4—2), substantial part of conjugated metabolites and free acids formed by acid-catalyzed hydrolysis were extracted with AcOEt. Since 6-DMN sulfate, which is hydrolyzed easily with acid to release 6-DMN, was the main metabolite of naproxen in the rat urine, the most of the conjugated metabolites in the rat urine were hydrolyzed at pH 1.0, which resulted in the decrease of the ratio of extractable conjugates and in the increase of the ratio of free 6-DMN which occupied the most part of the extractable radioactivity (Fig. 1). Therefore, we re-examined the urinary and biliary metabolites in the rat after oral administration of ^{3}H-naproxen in a dose of 30 mg/kg according to the procedure described above. The profile of the metabolites (Table II) were considerably different from those reported in our previous paper.[1] Our previous extraction of non-conjugated naproxen and 6-DMN with AcOEt was performed below pH 2.0. Therefore, the discrepancy between the previous and present results mainly stem from the difference in pH during the extraction.

Thompson *et al.*[6] separated by ion-exchange chromatography several unknown metabolites of naproxen from the rat urine including two major compounds (termed as Compound 18 and Compound 19) in ratios of 24.4 and 32.8%, respectively, of total urinary radioactivity. Our present study revealed the presence of a large amount of 6-DMN sulfate (87.3% of total urinary radioactivity) in the rat urine after oral administration of ^{3}H-naproxen. Considering these percentages, one of the two major unknown metabolites (Compound 18 and Compound 19) reported by Thompson *et al.* probably corresponds to 6-DMN sulfate.

8) R. Runkel, M. Chaplin, G. Boost, E. Segre, and E. Forchielli, *J. Pharm. Sci.*, **61**, 703 (1972).

6-DMN sulfate was found to be unstable at acidic pH and partially hydrolyzed to release 6-DMN. With aryl sulfatase it was hydrolyzed with ease in 0.2 M acetate buffer (pH 4.8) but not readily in the urine solution of usual dilutions (4 to 10-times dilution with the buffer). However, when the urine was diluted 200 times with the buffer this sulfate was hydrolyzed by the enzyme almost quantitatively. These findings suggest some inhibitors may be present in the urine. The behavior of 6-DMN sulfate resembles that of other sulfate conjugated metabolite reported by Tamura *et al.*[9a] and Chen *et al.*[9b]

The species differences in the urinary metabolites in the animals were investigated after oral administration at a dose of 10 mg/kg, since the therapeutic dose of naproxen in man is *ca.* 10 mg/kg/day. In the urines of all the animal species tested, the sum of the amounts of naproxen and its conjugates was lesss than that of the demethylated naproxen derivatives, *i.e.*, free and conjugated 6-DMN, as shown in Table III. Therefore, the demethylation activities in these animals are presumably high, especially in rat and miniature pig. 6-DMN sulfate was excreted in highest quantities in 5 species other than the miniature pig. The percentage of 6-DMN sulfate in the rat urine was especially large (87.3% of the urinary radioactivity), suggesting that the sulfate conjugation of 6-DMN occurred in the rat at a rate higher than in any other animals tested. The main urinary metabolite of naproxen in the miniature pig was 6-DMN (78.6% of the urinary radioactivity), but the percentages of the conjugated metabolites including 6-DMN sulfate were less than 4%. Therefore, it could be stated that in the miniature pig the conjugation activity was very low compared with the other test animals.

In man the metabolites of naproxen administered orally were excreted in the 24-hr urine mainly as the conjugated forms, mostly naproxen glucuronide, and very little was excreted as non-conjugated naproxen and 6-DMN (1.3 and 0.87% of dose, respectively) (Table IV).

From these urinary profiles of man and the test animals, it could be assumed that in the test animals the demethylation activity for naproxen and the sulfate conjugation activity for 6-DMN (except in the miniature pig) are markedly higher than those in man, while the glucuronide conjugation activity in the animals is lower than that in man. Segre[10] and Thompson *et al.*[6] reported the ratios of unchanged naproxen and 6-DMN to the total urinary metabolites in man after intravenous administration of naproxen were 10% and 5%, respectively, and that the animals whose urinary metabolic profiles appeared to be the most akin to that of man were the rat and the mini pig (not OHMINI-875 miniature pig). But from our results, the guinea pig and mouse could be picked up as the animals whose urinary metabolic profiles are the closest to that of man. This choice is based on the fact that the percentage of the total of non-conjugated and conjugated naproxen and that of the naproxen glucuronide in these 2 species were nearest to those in man. The rat and the OHMINI-875 miniature pig are the animals that deviate farthest from man in this regard. The difference between our results and those of Thompson *et al.* may be due to the differences in experimental methods, *i.e.*, Thompson *et al.* administered the drug intravenously and they isolated the metabolites by ion-exchange chromatography at pH 3.5—6.5.

The results of the study on the acute toxicity by Kuramoto *et al.*[11] showed that the LD_{50} of naproxen (*p.o.*) in the rat and mouse were 347 mg/kg and 825 mg/kg, while that of 6-DMN (*p.o.*) in the rat and mouse were 4196 mg/kg and 2036 mg/kg, respectively. Considering these differences in the acute toxicities of naproxen and 6-DMN between the rat and the mouse,

9) *a*) Z. Tamura, T. Tanimura, K. Samejima, T. Imanari, and C. Chen, Proceedings of the 1st Symposium of Analytical Chemistry of Biological Substance, Tokyo, Nov. 1973, p. 27; *b*) C. Chen, K. Samejima, and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **24**, 97 (1976).

10) E.J. Segre, *J. Clin. Pharmacol.*, **15**, 316 (1975).

11) *a*) M. Kuramoto, Y. Ishimura, T. Okubo, and T. Hashimoto, *Shikoku Acta Medica*, **29**, 439 (1973); *b*) M. Kuramoto, private communication.

it is very interesting that unchanged naproxen, which is more toxic to the rat than to the mouse, was excreted in the rat urine in lesser amount than in the mouse urine, while 6-DMN which showed weaker acute toxicity in the rat than in the mouse, was excreted more abundantly in the urine than in the mouse urine.

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