The *in Vitro* Metabolism of 3-(1-Hydroxy-2-piperidinoethyl)-5-phenylisoxazole Citrate (31252-S) with Rabbit Liver Homogenate[1]

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The *in vitro* metabolism of generally labelled ^{3}H-3-(1-hydroxy-2-piperidinoethyl)-5-phenylisoxazole ([3]H-31252-S) using the $9000 \times g$ supernatant fraction of rabbit liver homogenate was studied. The metabolites were identified and determined quantitatively by reverse isotope dilution analysis. The results indicated that it was extensively metabolized and the main metabolic pathways were N-oxidation of piperidine and *p*-hydroxylation of phenyl ring. 3-{1-Hydroxy-2-(4-hydroxypiperidino)ethyl}-5-phenylisoxazole (XVII) showed slightly stronger analgesic activity than the parent compound 31252-S. All metabolites were less toxic than the parent 31252-S.

The compound 31252-S synthesized by Kanō and Adachi[3a,b] has been shown to possess analgesic, anti-inflammatory, and antitussive activities.[3b,4] The absorption, distribution and excretion of 31252-S have been investigated in rabbits[5] and rats,[6] and 3-(1-hydroxy-2-piperidinoethyl)-5-(4-hydroxyphenyl)isoxazole (XIc) has been isolated and confirmed as one of the urinary metabolites of 31252-S. In this paper we deal with *in vitro* metabolism of [3]H-31252-S with rabbit liver homogenate; the metabolites were identified and quantitatively determined. We also measured the pharmacological activities of the metabolites.

Materials and Methods

Tritium-labelled Compound

Generally labelled 3-(1-hydroxy-2-piperidinoethyl)-5-phenylisoxazole citrate ([3]H-31252-S; specific activity: 20 μCi/mg) was purchased from Sinloihi Co., Ltd. (Kanagawa, Japan). The radiochemical purity of [3]H-31252-S was greater than 95% as determined by reverse isotope dilution method.

In thin-layer radiochromatograms in the systems 1, 2, and 3, no peak other than 31252-S was detected.

Thin-Layer Chromatography (TLC)

Precoated TLC plates of Silica Gel F_{254} (E. Merck AG., 0.25 mm thick) were used for chromatographic studies. The solvent systems used were as follows:

1) $CHCl_3$–acetone–MeOH (5: 2: 3, v/v)
2) $CHCl_3$–benzene–MeOH (5: 5: 1, v/v)
3) CH_2Cl_2–MeOH (9: 1, v/v).

Radioactivity Assay

2,5-Diphenyloxazole (PPO) and 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) were purchased from E. Merck AG. Radioactivity was measured with a liquid scintillation counter (Beckman Model LS-230), and quenching correction was carried out by the external standard method. A solution of PPO (4.0 g) and POPOP (0.2 g) in toluene (to make 1 liter) was used as a scintillator. Radioactive bands on the TLC plate

1) This work was reported at the 23 rd meeting of the Kinki Branch, Pharmaceutical Society of Japan, Kyoto, November 18, 1973.
2) Location: *Fukushima-ku, Osaka, 553, Japan.*
3) *a*) H. Kanō and I. Adachi, *Shionogi Kenkyusho Nempo*, **18**, 56 (1968); *b*) H. Kano, I. Adachi, R. Kido, and K. Hirose, *J. Med. Chem.*, **10**, 411 (1967).
4) R. Kido, K. Hirose, Y. Kojima, M. Eigyo, H. Jyoyama, and H. Sato, *Shionogi Kenkyusho Nempo*, **18**, 66 (1968).
5) R. Yamamoto, J. Watanabe, and H. Okabe, *Shionogi Kenkyusho Nempo*, **18**, 78 (1968).
6) J. Watanabe, H. Okabe, K. Mizojiri, S. Takahashi, S. Hashimoto, M. Shimokata, T. Ichihashi, H. Yamada and R. Yamamoto, *Shionogi Kenkyusho Nempo*, **22**, 72 (1972).

were determined by a thin-layer radiochromatogram scanner (Aloka Model TRM-1; Japan Radiation & Medical Electronics, Inc., Tokyo, Japan).

Preparation of "9000×g Supernatant" Tissue Sample

Male albino rabbits weighing 3 to 3.5 kg were used throughout this study. The rabbit liver was removed and homogenized at 0° in ice-cold 1.15% KCl [30 ml; to 10 g (wet weight) of liver] containing nicotinamide (0.075%) with a homogenizer (Ultra Turrax; Janke und Kunkel KG.); this was centrifuged at 9000×g for 10 min at 0°, and the supernatant fraction was used.

Rate of Metabolism of ^{3}H-31252-S

^{3}H-31252-S (20 μCi) was added to the supernatant (20 ml) obtained above and the mixture was incubated at 37°. Two ml of aliquot of this mixture was pipetted out at 5, 10, 20, 30, and 60 min, and after being deproteinized by addition of ethanol, were chromatographed on silica gel plates using solvent system 1. The developed plates were analyzed with the thin-layer radiochromatogram scanner. Radioactivity was determined from peak area of the thin-layer radiochromatogram.

Identification and Quantitation of Metabolites

The residual incubation mixture obtained above was deproteinized with ethanol, then the ethanol solution was concentrated and its volume was adjusted to 5.00 ml. An aliquot (0.30 ml) of the ethanol solution was analyzed by reverse isotope dilution method.

Synthesis of the Proposed Metabolites

The following compounds were synthesized as possible metabolites: 3-(1-hydroxy-2-piperidinoethyl)-5-(2-, 3-, and 4-hydroxyphenyl)isoxazoles (XIa, XIb, XIc); 3-{1-hydroxy -2-(3- and 4-hydroxypiperidino)-ethyl}-5-phenylisoxazoles (XVI, XVII); 3-{1-hydroxy-2-(2-oxopiperidino)ethyl}-5-phenylisoxazole (XVIII); 3-{1-hydroxy-2-(N-oxidopiperidino)ethyl}-5-phenylisoxazole (XIX); 5-phenyl-3-isoxazolecarboxylic acid (XXV)[7]; piperidinoacetic acid (XXVI)[8]; (5-phenyl-3-isoxazolyl)glycollic acid (XXIV); 3-piperidinoacetyl-5-phenylisoxazole (XXVII).[3b]

All melting points were taken on a Kofler hot stage apparatus and are uncorrected. Solvents were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. IR spectra were determined with an infrared spectrophotometer (Jasco Model IRA-1; Japan Spectroscopic Co., Ltd.). Column chromatography was carried out with silica gel (Kieselgel 60; E. Merck AG.) or alumina active, neutral (Aluminiumoxid; E. Merck AG.). Preparative thin-layer chromatography was carried out on silica gel (Kieselgel GF_{254}; E. Merck AG.) or alumina (Aluminiumoxid GF_{254}; E. Merck AG.), 0.75 mm thick, activated at 110° for 60 min.

3-(1-Hydroxy-2-piperidinoethyl)-5-(2-hydroxyphenyl)isoxazole (XIa)

o-Coumaric Acid——This compound was prepared from coumarin by a method similar to that described by Dale, *et al.*[9]

o-Vinylphenol (IIa)——This compound was prepared by the method of Corson, *et al.*[10]

3-Bromoacetyl-5-(2-hydroxyphenyl)-Δ^2-isoxazoline (IIIa)——IIIa was prepared from IIa and 1-chloro-3-bromo-1-hydroxyiminoacetone by the synthesis procedure described in the previous paper.[6] IIIa was recrystallized from hexane as colorless prisms, mp 111—113°. Yield, 59%.

3-Bromoacetyl-5-(2-acetoxyphenyl)-Δ^2-isoxazoline (IVa)——IIIa was acetylated as described in the previous paper[6] for the synthesis of 3-chloroacetyl-5-(2-acetoxyphenyl)-Δ^2-isoxazoline, and IVa was recrystallized from EtOH as colorless prisms, mp 88—90°. Yield, 41%.

3-Bromoacetyl-5-(2-acetoxyphenyl)isoxazole (Va)——Va was obtained as described in the previous paper[6] for the synthesis of 3-chloroacetyl-5-(2-acetoxyphenyl)isoxazole. Va was recrystallized from EtOH as colorless prisms, mp 96—98°. Yield, 66%. IR ν_{max}^{Nujol} cm^{-1}: 1713 (aromatic α-bromoketone), 1750 (phenol acetate).

3-(1-Hydroxy-2-piperidinothyl)-5-(2-hydroxyphenyl)isoxazole (XIa)——i) From Va *via* Piperidinoketone: To a solution of Va (200 mg) in acetone (4.0 ml) was added a solution of piperidine (111 mg) in acetone (2.0 ml) under ice cooling. The mixture was stirred for 15 min at room temperature. The filtrate from the reaction mass was evaporated below 30°, the residue (piperidinoketone, Xa) was dissolved in MeOH (4.0 ml), and then $NaBH_4$ (23.6 mg) was added portionwise with stirring and cooling. The resulting solution was stirred for 1 hr at room temperature. The solution was neutralized with gl. AcOH (3 drops), and then evaporated. The residue was made alkaline with 10% aq. K_2CO_3 and extracted with CH_2Cl_2. The CH_2Cl_2 layer was separated, dried, and evaporated to leave a deep orange solid, which was submitted to preparative TLC (silica gel, solvent system 3) giving crude XIa (Rf 0.30—0.35). XIa was recrystallized from AcOEt as colorless plates, mp 202° (decomp.). Yield, 70 mg (39%). This compound was identified with the sample[6] by comparing the melting points and IR spectra.

ii) From Va *via* Bromohydrin: To a suspension of Va (250 mg) in EtOH (20 ml), $NaBH_4$ (16.7 mg) was added portionwise with stirring and cooling. After stirring for an additional 30 min, 10% aq. AcOH (2 drops)

7) A. Angeli, *Ber.*, 23, 2154 (1890).

8) H. Ley, *Ber.*, 42, 354 (1909).

9) W.J. Dale and H.E. Hennis, *J. Am. Chem. Soc.*, 80, 3645 (1958).

10) B.B. Corson, W.J. Heintzelman, L.H. Schwartzman, H.E. Tiefenthal, R.J. Lokken, J.E. Nickels, G.R. Atwood, and F.J. Pavlik, *J. Org. Chem.*, 23, 544 (1958).

was added to the reaction mixture and the solution was evaporated to give a colorless oil (bromohydrin, XIIa). To the residual oil was added a solution of piperidine (264 mg) in benzene (15 ml). The resulting solution was refluxed for 3 hr, and then evaporated giving colorless crystals. The crystals were submitted to preparative TLC (silica gel, solvent system 3) giving colorless crystals, which were recrystallized from AcOEt to give XIa as colorless plates, mp 202° (decomp.). Yield, 46.3 mg (21%). This compound was identical with the sample obtained above.

3-(1-Hydroxy-2-piperidinoethyl)-5-(3-hydroxyphenyl)isoxazole (XIb)

Methyl *m*-Hydroxybenzoylpyruvate (VIa)——To a solution of Na(0.34 g) in abs. MeOH (5.0 ml) were added a solution of *m*-hydroxyacetophenone (1.0 g) in abs. MeOH (2.0 ml) and diethyl oxalate (2.0 ml) at the same time. The temperature of the mixture was allowed to rise to *ca.* 40°. The resulting solution was heated at 50° while being stirred for 1 hr and then evaporated. The residual oil was poured into crushed ice; on adding 10% aq. H_2SO_4 (50 g) with stirring at 20—25° yellow crystals formed. The precipitated crystals were collected by filtration, then washed with H_2O. The crystalline product was recrystallized from MeOH as yellow needles, mp 150—151°. Yield, 670 mg (41%). IR ν_{max}^{Nujol} cm^{-1}: 3372 (OH), 1706 (C=O).

Methyl 5-(3-Hydroxyphenyl)-3-isoxazolecarboxylate (VIIa)——To a solution of VIa (13.3 g) in MeOH (50 ml) was added a suspension of NH_2OH-$1/2H_2SO_4$ (8.8 g) in H_2O (1.3 ml) and MeOH (13 ml). The mixture was refluxed on a water bath for 15 min and then evaporated. The residue was poured on cracked ice to precipitate crystals which were filtered, and then washed with H_2O. The crystals were recrystallized from acetone as colorless scales, mp 220—221°. Yield, 12 g (91%). IR ν_{max}^{Nujol} cm^{-1}: 1732 (C=O). *Anal.* Calcd. for $C_{11}H_9O_4N$: C, 60.27; H, 4.14; N, 6.39. Found: C, 60.26; H, 4.23; N, 6.55.

3-Acetyl-5-(3-hydroxyphenyl)isoxazole (VIIIa)——To a solution of commercial CH_3MgBr (3 moles/liter in ether, 111 ml) in abs. THF (70 ml) was added Et_3N (167 g) at room temperature in N_2 atmosphere, and then a solution of VIIa (14.5 g) in abs. THF (700 ml) was added dropwise at 20°. The mixture was heated at 50° for 4 hr while being stirred. Next, 6N HCl (600 ml) was added to the reaction mixture below 25°, which was allowed to stand overnight and then evaporated to dryness *in vacuo*. To the crystalline residue was added 2N HCl (270 ml), and the resulting solution was extracted with AcOEt. The extract was washed successively with 5% aq. $NaHCO_3$ and H_2O, then dried and evaporated giving a colorless solid, which was recrystallized from MeOH as colorless prisms, mp 222—223°. Yield, 7.95 g (60%). *Anal.* Calcd. for $C_{11}H_9O_3N$: C, 65.02; H, 4.46; N, 6.89. Found: C, 64.98; H, 4.55; N, 6.87.

3-Acetyl-5-(3-acetoxyphenyl)isoxazole (IXa)——A mixture of VIIIa (0.60 g) and AcONa (0.60 g) in Ac_2O (4.0 ml) was heated in an oil bath at 110° for 2 hr while being stirred. The reaction mixture was evaporated, and the residual oil was poured into crushed ice, and then extracted with CH_2Cl_2. The CH_2Cl_2 layer was separated, dried and evaporated to give colorless crystals. The crystals were recrystallized from MeOH as colorless prisms, mp 107—108°. Yield, 0.55 g (76%).

3-Bromoacetyl-5-(3-acetoxyphenyl)isoxazole (Vc)——To a solution of IXa (1.50 g) in CCl_4 (18 ml) was added a solution of Br_2 (0.38 ml) in CCl_4 (6.0 ml) at room temperature over a period of 4 hr. After being maintained at the temperature for 2 hr, the resulting colorless solution was treated with 5% aq. $NaHCO_3$, and the CCl_4 layer was separated, dried and evaporated. The crystalline residue was recrystallized from MeOH giving colorless needles, mp 95—96°. Yield, 1.59 g (80%).

3-(1-Hydroxy-2-piperidinoethyl)-5-(3-hydroxyphenyl)isoxazole (XIb)——i) This compound was obtained from piperidinoketone (yield, 17%) or bromohydrin XXIb (yield, 43%) by the same procedures as for the synthesis of XIa. Recrystallization from AcOEt gave colorless needles, mp 156—157°. TLC (silica gel, solvent system 3, *Rf* 0.25—0.30). *Anal.* Calcd. for $C_{16}H_{20}O_3N_2$: C, 66.64; H, 6.99; N, 9.72. Found: C, 66.59; H, 6.73; N, 9.78. The compounds obtained by both methods had the same melting point and showed no depression on admixture.

ii) From XIIb *via* Epoxide XIIIa: To a solution of XIIb (3.70 g) in MeOH (40 ml) was added a solution of KOH in MeOH (10%, 14.6 ml) dropwise with stirring at 20°. After stirring for an additional 1 hr at 20°, the reaction mixture was neutralized with gl. AcOH, and then evaporated. The residue was extracted with hot AcOEt, and the solution was evaporated. Piperidine (5.2 ml) was added to the crystalline residue 3-oxirano-5-(3-hydroxyphenyl)isoxazole (XIIIa) and the resulting mixture was warmed at 80° for 0.5 hr. The reaction mixture was evaporated, and the residue was subjected to column chromatography (silica gel) with CH_2Cl_2-MeOH (30: 1, v/v) to give a colorless solid. The solid was further submitted to column chromatography (alumina, activity II) with AcOEt to give an unidentified product (*ca.* 1.0 g), followed by that with CH_2-Cl_2-MeOH (20: 1, v/v) to give XIb as colorless crystals. XIb was recrystallized from benzene as colorless needles, mp 156—157°. Yield, 730 mg (20%). This compound was identical with the sample obtained above.

3-(1-Hydroxy-2-piperidinoethyl)-5-(4-hydroxyphenyl)isoxazole (XIc)

***p*-Vinylphenol (IIb)**——In a 100-ml round-bottomed flask connected *via* a Liebig condenser to an ice-cooled receiver containing hydroquinone (0.2 g), copper powder (1.2 g) was immersed in quinoline (2.0 ml) and heated in an oil bath to 220—230° under diminished pressure (5 mmHg). A solution of *p*-coumaric acid[11] (7.6 g) in quinoline (38 g) was added dropwise to the boiling mixture under distillation (85—105°/5 mmHg).

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11) C.G. Overberger, E.J. Luhrs, and P.K. Clein, *J. Am. Chem. Soc.*, **72**, 1200 (1950).

The distillate was poured into a mixture of ether (14 ml) and crushed ice (18 g). Next, 3N H_2SO_4 (150 ml) was added to the mixture and the ethereal layer was separated. The aqueous layer was extracted with ether (20 ml × 1, 5 ml × 2). The combined ethereal layer was washed with H_2O, dried, and evaporated leaving a colorless solid (2.1 g). Recrystallization from hexane gave colorless prisms [1.4 g, p-vinylphenol (IIb)], mp 66—69°.

3-Bromoacetyl-5-(4-hydroxyphenyl)-Δ^2-isoxazoline (IIIb)——IIIb was obtained by a procedure similar to that for the synthesis of 3-bromoacetyl-5-(2-hydroxyphenyl)-Δ^2-isoxazoline (IIIa) described above with IIb (200 mg) and 1-chloro-3-bromo-1-hydroxyiminoacetone (364 mg) in isopropyl alcohol (3.0 ml). IIIb was recrystallized from hexane-ether as colorless prisms, mp 118—119°. Yield, 178 mg (38%). IR ν_{max}^{Nujol} cm^{-1}: 1697 (C=O), 3340 (OH).

3-Bromoacetyl-5-(4-acetoxyphenyl)-Δ^2-isoxazoline (IVb)——IVb was obtained from IIIb by a procedure similar to that described for IVa from IIIa. IVb was recrystallized from EtOH as colorless prisms (yield, 84%), mp 82—84°. IR ν_{max}^{Nujol} cm^{-1}: 1698 (C=O), 1743 (O-acetate).

Methyl p-Hydroxybenzoylpyruvate (VIb)——Using a procedure similar to that for the synthesis of VIa, VIb was obtained from the corresponding p-hydroxyacetophenone. VIb was recrystallized from CHCl$_3$–hexane as yellow prisms (yield, 65%), mp 134—136°. IR ν_{max}^{Nujol} cm^{-1}: 3404 (OH), 1722 (C=O).

Methyl 5-(4-Hydroxyphenyl)-3-isoxazolecarboxylate (VIIb)——Using a procedure similar to that for the synthesis of VIIa described above, VIIb was obtained from VIb. Recrystallization from MeOH gave colorless prisms (yield, 70%), mp 183—186°. IR ν_{max}^{Nujol} cm^{-1}: 1727 (C=O).

3-Acetyl-5-(4-hydroxyphenyl)isoxazole (VIIIb)——VIIb was treated with CH$_3$MgBr as described for the synthesis of VIIIa, except that the reaction was maintained at 20° for 7 hr. VIIIb was recrystallized from MeOH as colorless prisms (yield, 49%), mp 207—209°.

3-Acetyl-5-(4-acetoxyphenyl)isoxazole (IXb)——Using a procedure similar to that described for the synthesis of IXa, IXb was obtained from VIIIb. Recrystallization from MeOH gave colorless prisms (yield, 86%), mp 143—144°.

3-Bromoacetyl-5-(4-acetoxyphenyl)isoxazole (Vb)——i) From IVb: Vb was obtained by the procedure for synthesizing 3-chloroacetyl-5-(4-acetoxyphenyl)isoxazole described in the previous paper.[6] Vb was recrystallized from EtOH as colorless needles (yield, 61%), mp 165—167°. IR ν_{max}^{Nujol} cm^{-1}: 1720 (C=O), 1744 (O-acetate).

ii) From IXb: Using a procedure similar to that described for synthesizing Vc, Vb was obtained from IXb. Vb was recrystallized from EtOH as colorless needles (yield, 67%), mp 165—167°. This compound was identified with the one obtained above by mixed melting point and IR spectrum.

3-(1-Hydroxy-2-piperidinoethyl)-5-(4-hydroxyphenyl)isoxazole (XIc)——i) From Vb via Piperidinoketone: XIc was obtained by a procedure similar to that described for synthesis of XIa-i) from Vb. The product was submitted to preparative TLC (silica gel, solvent system 3) to give XIc (Rf 0.15—0.20), which was recrystallized from AcOEt giving colorless prisms (yield, 16%), mp 165° (decomp.). This compound was identical with the specimen[6] with respect to the melting point and IR spectrum.

ii) From Vb via Bromohydrin XIIc: XIc was obtained by a procedure similar to that described for synthesis of XIa-ii) from Vb. XIc was recrystallized from AcOEt as colorless prisms (yield, 29%), mp 164° (decomp.). This compound was identical with the specimen obtained above.

iii) From XIIc via Epoxide: XIc was obtained from XIIc via epoxide by a procedure similar to that described for synthesis of XIb-ii). XIc was recrystallized from AcOEt as colorless prisms (yield, 20%), mp 165° (decomp.). This compound was identical with the specimen obtained above.

3-Oxirano-5-phenylisoxazole (XV)——To a solution of 3-(1-hydroxy-2-bromoethyl)-5-phenylisoxazole[3b] (XIV; 1.50 g) in MeOH (150 ml) was added a solution of KOH in MeOH (10%, 4.7 ml) dropwise with stirring at 20°. After stirring for an additional 1 hr at 20°, the solution was evaporated. The residue was extracted with benzene, and the solvent was evaporated to give colorless crystals (1.05 g), mp 78—81°.

3-{1-Hydroxy-2-(3-hydroxypiperidino)ethyl}-5-phenylisoxazole (XVI)——A mixture of XV (1.05 g) and 3-hydroxypiperidine (1.13 g) was warmed at 70° for 20 min, and then submitted to preparative TLC on silica gel with solvent system 1 (Rf 0.75—0.80). The crystalline product was recrystallized from AcOEt–hexane yielding colorless prisms (0.39 g), mp 94—96°. *Anal.* Calcd. for $C_{16}H_{20}O_3N_2$: C, 66.64; H, 6.99; N, 9.72. Found: C, 66.76; H, 6.74; N, 9.57.

3-{1-Hydroxy-2-(4-hydroxypiperidino)ethyl}-5-phenylisoxazole (XVII)——i) From XV: A mixture of XV (0.58 g) and 4-hydroxypiperidine (0.63 g) was warmed at 80° under stirring for 0.5 hr. The reaction mixture was separated by preparative TLC on silica gel with solvent system 3 (Rf 0.3—0.4) to give a crystalline product (0.45 g). Recrystallization from AcOEt–hexane gave colorless prisms (0.30 g), mp 117—119°. *Anal.* Calcd. for $C_{16}H_{20}O_3N_2$: C, 66.64; H, 6.99; N, 9.72. Found: C, 66.38; H, 7.12; N, 9.58.

ii) From XIV: To a solution of XIV (268 mg) and 4-hydroxypiperidine (121 mg) in EtOH (2.0 ml) and benzene (2.0 ml) was added NaHCO$_3$ (168 mg) under stirring. The mixture was warmed to 70° under stirring for 5 hr and then evaporated. The residue was chromatographed on silica gel with benzene giving an unidentified product (72 mg) and then with CH$_2$Cl$_2$–MeOH (9: 1, v/v) yielding colorless crystals (268 mg), which were recrystallized from AcOEt as colorless prisms (180 mg), mp 117—119°. This compound was identified as the one obtained above by mixed melting point and IR spectrum.

3-{1-Hydroxy-2-(2-oxopiperidino)ethyl}-5-phenylisoxazole (XVIII)——i) From 31252-S Free Base: This compound was prepared using Möhrle's[12] procedure for the synthesis of 1-phenyl-2-(2-oxopiperidinyl)-ethanol. The 31252-S free base (136 mg) was added to a hot solution of Hg (OAc)$_2$ (319 mg) and EDTA·2Na·2H$_2$O (372 mg) in aq. AcOH (1%, 5.0 ml) and the solution was heated for 1.5 hr under a nitrogen stream on a water bath. Hg began to separate after a few minutes. Aqueous NaOH (5%, 5.0 ml) was added to the mixture, which was extracted with CH$_2$Cl$_2$. This CH$_2$Cl$_2$ layer was evaporated and its residue was chromatographed on silica gel with CH$_2$Cl$_2$-MeOH (9: 1, v/v) yielding colorless crystals which were recrystallized from AcOEt as colorless plates (105 mg), mp 118—119°. IR ν_{max}^{Nujol} cm^{-1}: 1604 (C=O). *Anal.* Calcd. for C$_{16}$H$_{18}$O$_3$N$_2$: C, 67.11; H, 6.34; N, 9.78. Found: C, 66.96; H, 6.47; N, 9.50.

ii) From XV: A mixture of XV (187 mg) and α-piperidone (297 mg) was heated at 110° for 26 hr, and then chromatographed on silica gel giving the starting material (2 mg) as the first fraction with benzene and then with AcOEt giving colorless crystals (165 mg), which were purified by preparative TLC (silica gel, solvent system 3) yielding crude XVIII (94 mg), *Rf* 0.5—0.7. The crude XVIII was submitted to TLC (alumina, AcOEt, *Rf* 0.35—0.50), and further purified by TLC (silica gel, AcOEt, *Rf* 0.35—0.45) giving colorless plates (11 mg), and identified with the one obtained above by IR spectrum and mixed melting point.

3-{1-Hydroxy-2-(N-oxidopiperidino)ethyl}-5-phenylisoxazole (XIX)——To a solution of 31252-S free base (4.08 g) in MeOH (90 ml) was added a solution of *m*-chloroperbenzoic acid (purity 85%, 3.81 g) in MeOH (30 ml) at 5°. This mixture was maintained at 0—5° for 0.5 hr and then evaporated below 5°. The residual oil was made alkaline with aq. Na$_2$CO$_3$ and extracted with CH$_2$Cl$_2$. The CH$_2$Cl$_2$ layer was separated, dried over Na$_2$SO$_4$, and evaporated to leave a colorless solid (4.2 g). Recrystallization from acetone gave colorless prisms (3.0 g), mp 173—174°. *Anal.* Calcd. for C$_{16}$H$_{20}$O$_3$N$_2$: C, 66.64; H, 6.99; N, 9.72. Found: C, 66.74; H, 7.08; N, 9.33.

(5-Phenyl-3-isoxazoly)glycollic Acid (XXIV)

5-Phenyl-3-isoxazolecarboxaldehyde (XXI)——A solution of 3-hydroxymethyl-5-phenylisoxazole[3b] (175 mg) and K$_2$Cr$_2$O$_7$ (176 mg) in aq. AcOH (30%, 10 ml) was added to a boiling solution of conc. H$_2$SO$_4$ (0.16 ml) in H$_2$O (1.5 ml) dropwise under steam distillation. The distillate (*ca.* 300 ml) was saturated with NaCl and extracted with ether. The ethereal layer was separated, dried, and evaporated. The crystalline residue (100 mg) was purified with preparative TLC (silica gel with benzene; *Rf* 0.50—0.60) to give colorless crystals (70 mg), mp 52—55°. IR ν_{max}^{Nujol} cm^{-1}: 1711 (C=O).

3-(1-Hydroxy-1-cyanomethyl)-5-phenylisoxazole (XXIII)——To a solution of XXI (104 mg) in aq. NaHSO$_3$ (10%, 73 mg NaHSO$_3$) which was cooled to 0° was added ether (2.0 ml) and then aq. NaCN (20%, 31 mg NaCN) dropwise under magnetically stirring. After stirring for an additional 0.5 hr at 0°, the cooled mixture was extracted with ether. The ethereal layer, after being washed with aq. NaHSO$_3$, was evaporated giving a colorless solid (117 mg), which was recrystallized from CCl$_4$ as colorless prisms (98 mg), mp 93—94°. *Anal.* Calcd. for C$_{11}$H$_8$O$_2$N$_2$: C, 65.99; H, 4.03; N, 13.99. Found: C, 66.16; H, 3.98; N, 14.03.

(5-Phenyl-3-isoxazolyl)glycollic Acid (XXIV)——A suspension of XXIII (391 mg) in 6 N HCl (2.5 ml) was refluxed for 0.5 hr and then evaporated. The colorless crystalline residue was treated with H$_2$O, and the insoluble material was collected by filtration. Recrystallization from H$_2$O gave colorless prisms (302 mg), mp 186° (decomp.). *Anal.* Calcd. for C$_{11}$H$_9$O$_4$N: C, 60.27; H, 4.14; N, 6.39. Found: C, 60.08; H, 4.15; N, 6.50.

Results and Discussion

Rate of Metabolism of [3]H-31252-S

A time course study of the metabolism of 31252-S was carried out by thin–layer radio-chromatogram analyses. Radiochromatograms of the metabolites of 31252-S showed five radioactive peaks at *Rf* 0.03 (peak 1), 0.25 (peak 2), 0.38 (peak 3), 0.44 (peak 4), and 0.67 (peak 5) with solvent system 1. The main peak 3 was rechromatographed using solvent system 2 by topless thin–layer chromatography for 3 hr, giving three radioactive peaks, 3-1, 3-2, and 3-3, a running distance of 11 mm, 31 mm, and 52 mm from the origin, respectively. These results are shown in Fig 1 and Table I. Peaks 3-3 and 4 had one constituent each but the other peaks contained two or more constituents as shown by further chromatography. Peak 4, 31252-S, was metabolized to approximately 90% of the final amount in 10 min, and the metabolism did not proceed after 30 min. These results indicated clearly that 31252-S was rapidly metabolized to many metabolites by rabbit liver homogenate.
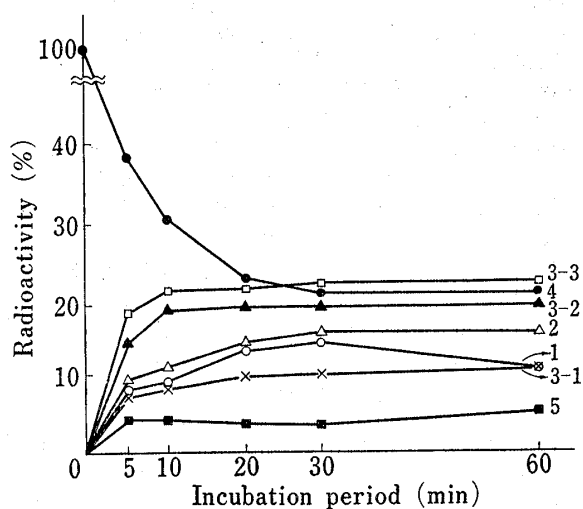
12) H. Möhrle, *Arch. Pharm.*, **297**, 474 (1964).

Fig. 1. Time Course Study on the Metabolism of ³H-31252-S with Rabbit Liver Homogenate

Numerals show peak number on the chromatogram.

Identification and Quantitation of the Metabolites

The recovered 31252-S free base (35%) and 12 possible metabolites were determined by reverse isotope dilution analysis. That is, the incubated mixture was recrystallized together with each possible metabolite repeatedly, until the specific radioactivity of the crystals attained a constant value. The results thus obtained are shown in Table I. The recovered radioactivities are shown as the percentage of incubated ³H-31252-S. The totally recovered radioactivities reached approximately 90%. The predominant metabolites of 31252-S with rabbit liver homogenate were the N-oxide (XIX; 38%) and the p-hydroxylated derivative (XIc; 26%) of 31252-S. We were unable to conclude

TABLE I. Identification and Quantitation of ³H-31252-S Metabolites

Compound		Percentage[a] formed	Rf-Values[b]
		35	0.44
	XIa	<0.5	0.38
	XIb	4	0.39
	XIc	17	0.38
	XIX	25	0.51
	XXVII[3b]	<0.5	0.83
	XVIII	4	0.70
	XVI	1	0.56
	XVII	2	0.38
	XXIV	3	0.16
	XXV[7]	3	0.12
	XXVI[8]	<0.5	0.09
		<0.5	0.52

a) condition: 37°, 60 min incubation, see Materials and Methods
b) solvent system 1

definitely whether the *o*-hydroxylated derivative XIa, 3-piperidinoacetyl-5-phenylisoxazole[3b] XXVII, piperidinoacetic acid[8] XXVI and benzoic acid were present or not because the amounts were very *small or nil*.

Metabolic Pathways of 31252-S in Rabbits

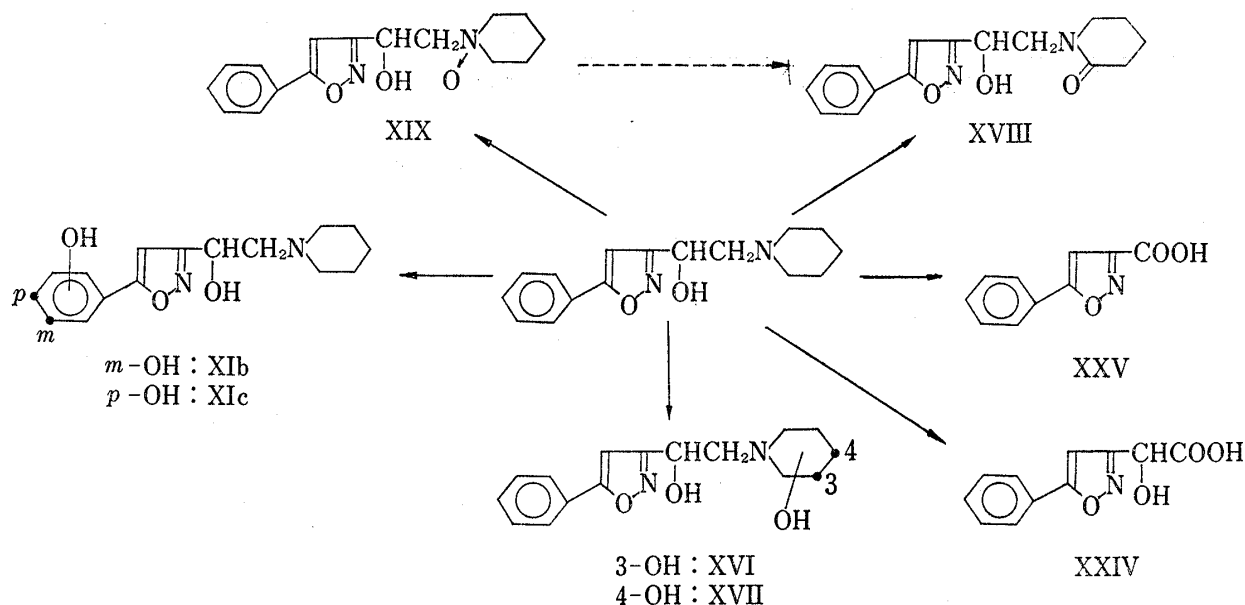The proposed metabolic pathways of 31252-S with rabbit liver homogenate are shown in Fig. 2.

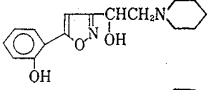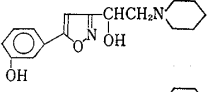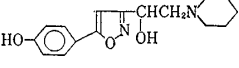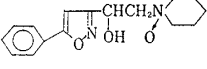Fig. 2. Proposed Metabolic Pathways of [3]H-31252-S with Rabbit Liver Homogenate
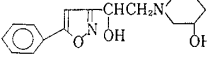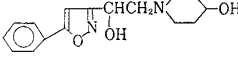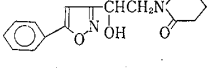
The pathways are as follows: (i) hydroxylation of the *meta* (XIb) and *para* (XIc) positions of the benzene moiety; (ii) α-oxidation (XVIII) and hydroxylation of the C-3 (XVI) and C-4 (XVII) positions of the piperidine moiety; (iii) oxidative cleavage to 5-phenyl-3-isoxazole-carboxylic acid (XXV) and (5-phenyl-3-isoxazolyl)glycolic acid (XXIV); (iv) N-oxidation of piperidine (XIX).

When the ^{3}H-labelled N-oxide (XIX) isolated from the incubation mixture of [3]H-31252-S was further treated with the liver preparation, 31252-S free base (17%), unidentified materials (32%), and unchanged N-oxide (51%) were obtained under the same conditions as the [3]H-31252-S metabolism. Thus, we found that N-oxide was in equilibrium with 31252-S, and the N-oxide seems to be an intermediary metabolite of 31252-S. In our previous experiments we had considered that 3-piperidinoacetyl-5-phenylisoxazole (XXVII) formed by oxidation of the alcohol group at the first step of the metabolism, and further underwent oxidative cleavage at the second step of the metabolism. If such a reaction had proceeded as a major metabolic pathway, the piperidinoketone XXVII would have given the same metabolites as 31252-S. To prove this hypothesis, XXVII was treated under the same conditions as that of 31252-S metabolism. Contrary to expectation, reduction took place to give 31252-S free base in 35% yield. As 31252-S was metabolized rapidly, the true yield of 31252-S from XXVII would be much higher than the observed one. Whereas the synthesized 31252-S is a racemic mixture, the 31252-S obtained by enzymatic reduction of XXVII was optically active. The optical rotation was found to be levorotatory, $[\alpha]_D^{24} = -21.1°$ (c=0.757, CHCl$_3$), but the optical purity was not determined. If the piperidinoketone XXVII were the intermediate in the metabolism of 31252-S, the 31252-S obtained from the incubation mixture should have shown optical activity, but it did not. These results denied the above hypothesis that the piperidinoketone XXVII may be an intermediate of the metabolism.

Pharmacological and Toxicological Studies

The results of analgesic action which was measured by phenylquinone-stretching method, and the acute toxicity of the metabolites of 31252-S are listed in Table II together with those of parent 31252-S for comparison. Among the metabolites listed in Table II, 3-{1-hydroxy-2-

TABLE II. Analgesic Activity and Acute Toxicity of
31252-S Metabolites (Mice, $p. o.$, mg/kg)

Compound		ED_{50}	LD_{50}
31252-S		107	612
	XIa	320(P)	—
	XIb	750(P)	>1000(P)
	XIc	450(P)	>1000(P)
	XIX	400	>1000(P)
	XVI	200(P)	750(P)
	XVII	72	645
	XVIII	650(P)	>1000(P)

(P): presumed dose

(4-hydroxypiperidino)ethyl}-5-phenylisoxazole (XVII) showed slightly stronger analgesic activity than the parent 31252-S. All metabolites were less toxic than the parent 31252-S. These results suggest that the pharmacological activities of 31252-S are based on the properties of 31252-S itself, not that of the metabolites.