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PHARMACOKINETICS AND BIOLOGICAL FATE OF 3-(2,2,2-TRIMETHYLHYDRAZINIUM)PROPIONATE DIHYDRATE (MET-88), A NOVEL CARDIOPROTECTIVE AGENT, IN RATS

KUNIHIRO YOSHISUE, YOSHIO YAMAMOTO, KEN-ICHIRO YOSHIDA, MAYUKO SAEKI, YOSHINORI MINAMI, YOSHIO ESUMI,[1] AND YASURO KAWAGUCHI

Pharmacokinetics Research Laboratory, Tokushima Research Center, Taiho Pharmaceutical Co., Ltd., Tokushima, Japan

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ABSTRACT:

In this study, we examined the disposition, metabolism, and excretion of a novel cardioprotective agent, 3-(2,2,2-trimethylhydrazinium)propionate dihydrate (MET-88), in rats. The disposition of MET-88 after oral and i.v. administration of 2, 20, and 60 mg/kg indicated that the pharmacokinetics of MET-88 were nonlinear. The profiles of radioactive MET-88 and total radioactivity in plasma were consistent at doses of 20 and 60 mg/kg. However, at 2 mg/kg, the plasma MET-88 levels were obviously lower than the total. The excretion of radioactivity after oral administration of MET-88 indicated that increasing doses led to a shift from exhaled CO_2 to urinary excretion as the major excretion route. Major metabolites in plasma after oral administration of MET-88 were glucose, succinic acid, and 3-hydroxypropionic acid, and in vitro studies revealed that MET-88 was converted to 3-hydroxypropionic acid by γ-butyrobetaine hydroxylase (EC 1.14.11.1). An isolated liver perfusion system modified to trap CO_2 gas was used to examine the excretion pathway of MET-88. [^{14}C]CO_2 gas was decreased by the addition of iodoacetic acid, DL-fluorocitric acid, or γ-butyrobetaine to this system, and subsequent thin-layer chromatography analyses of perfusates revealed that MET-88 was first converted to 3-hydroxypropionic acid by γ-butyrobetaine hydroxylase and then was biosynthesized to glucose and metabolized to CO_2 gas via the glycolytic pathway and tricarboxylic acid cycle.

γ-Butyrobetaine hydroxylase [BBHase;[2] 4-trimethylaminobutyrate,2-oxoglutarate:oxidoreductase (3-hydroxylating), EC 1.14.11.1], which is composed of a dimer of 43,000-Da subunits (Stephane et al., 1998), catalyzes the hydroxylation of γ-butyrobetaine [GBB; 4-(*N*,*N*,*N*-trimethylammonio)butanoate] to carnitine [4-(*N*,*N*,*N*-trimethylammonio)-3-hydroxybutanoate; Lindstedt and Lindstedt, 1970] and is the final step in the biosynthesis of L-carnitine from 6-*N*-trimethyl-L-lysine (Cox and Hoppel, 1973; Tanphaichitr and Broquist, 1973). Although carnitine occurs as D- and L-isomers, L-carnitine is required for the transport of long-chain fatty acids across the inner mitochondrial membrane to the site of β-oxidation in the mitochondrial matrix (Bremer, 1962). Because all tissues, except the brain, depend on fats as an important source of fuel, the concentration of L-carnitine would be expected to play an important role in metabolism in these tissues.

In myocardial ischemia, the inhibition of carnitine-mediated *trans*-mitochondrial membrane fatty acid transport may be beneficial (Lopaschuk et al., 1988; Lopaschuk and Spafford, 1989), because it would not only prevent the accumulation of harmful fatty acid metabolism intermediates, such as long-chain acyl coenzyme A (Busselen et al., 1988), but also facilitate glucose oxidation. MET-88 was synthesized as an inhibitor of BBHase. As previously reported, MET-88 decreased tissue levels of carnitine, resulting in reduction of β-oxidation of long-chain free fatty acids in the myocardium (Simkhovich et al., 1988). MET-88 also improved ventricular remodeling and cardiac function in rats with heart failure after myocardial infarction (Hayashi et al., 1995; Aoyagi et al., 1997). However, the disposition and biological fate of MET-88 remained unclear, so we decided to investigate the disposition, metabolism, and excretion of MET-88 in male rats. Continuous isolated liver perfusion (ILP) in the manner of Miller (Ross, 1972), modified to trap the generated CO_2 gas, was used to study the excretion pathway to expired air.

Materials and Methods

Chemicals. MET-88 was supplied by the Institute of Organic Synthesis (Riga, Latvia). [^{14}C]MET-88 was synthesized by the Daiichi Pharmaceutical Co. (Tokyo, Japan). Figure 1 shows its chemical structure and positions of the ^{14}C label. Specific activity was 10.0 μCi/mg, and radiochemical purity (>95%) was determined by thin-layer chromatography (TLC; Cellulose F 0.1 mm thick; Merck, Darmstadt, Germany) using a developing solvent system of *n*-butanol/acetic acid/water (4:1:2, v/v/v). DL-Fluorocitric acid (FCA) barium

[1] Present address: Daiichi Pure Chemicals Co., Ltd., 2117 Muramatsu Tokai, Ibaraki 319-1182, Japan.

[2] Abbreviations used are: MET-88, 3-(2,2,2-trimethylhydrazinium) propionate dihydrate; BBHase, γ-butyrobetaine hydroxylase; IAA, iodoacetic acid; FCA, DL-fluorocitric acid; GBB, γ-butyrobetaine; TLC, thin-layer chromatography; TCA, tricarboxylic acid; ILP, isolated liver perfusion; RLG, radioluminography; IP, imaging plate; PSL, photostimulated luminescence; LSC, liquid scintillation counting; FAB-MS, fast atom bombardment-mass spectrometry; ESI, electrospray ionization; FDP, fructose 1,6-diphosphate; AUC, area under the plasma concentration-time curve from 0 to infinity; CL_{total}, total MET-88 body clearance; Vd_{ss}, volume of distribution at steady state; ARG, autoradioluminography.

Send reprint requests to: Dr. Kunihiro Yoshisue, Pharmacokinetics Research Laboratory, Taiho Pharmaceutical Co., Ltd., 224-2, Ebisuno, Hiraishi, Kawauchi-cho, Tokushima 771-0194, Japan. E-mail: kuni-yosisue@taiho.co.jp

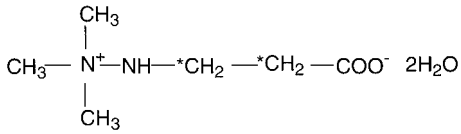


FIG. 1. *Chemical structure of MET-88, including the positions of the ^{14}C label.*

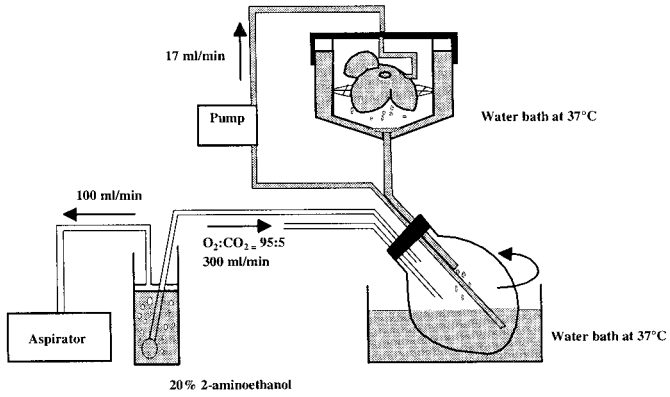


FIG. 2. *The ILP system apparatus.*

Perfusates were oxygenated by 95% O_2, 5% CO_2 gas at a flow rate of 300 ml/min in a rotary glass flask in a water bath maintained at 37°C and passed at a flow rate of 17 ml/min through the isolated liver sample mounted on a wire net in a 37°C water bath. Aspirated air was passed through a 20% 2-aminoethanol solution at a flow rate of 100 ml/min.

salt and GBB hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). Iodoacetic acid (IAA) sodium salt was obtained from Aldrich Chemical Co. (Milwaukee, WI). *p*-Bromophenyl bromide was purchased from Dojindo Chemical Co. (Kumamoto, Japan). Glucose and succinic acid were obtained from Wako Pure Chemicals Industries Ltd. (Osaka, Japan). 3-Hydroxypropionic acid was obtained from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). All reagents and solvents were commercially available guaranteed reagent grade or HPLC grade.

Animals. Six-week-old male Sprague-Dawley strain SPF rats were purchased from Charles River Japan, Inc. (Tokyo, Japan) and were maintained on chow and water ad libitum.

Radioluminography (RLG). The components of RLG equipment that we used were an imaging plate (IP; 20 × 40 cm) for ^{14}C (Fuji Film Co., Kanagawa, Japan), a magazine cassette (Fuji Film Co.), a Fuji shield box (Fuji Film Co.), and a bioimaging analyzer system (FUJIX BAS-2000; Fuji Film Co.). The IPs were used to record radioactivity observed with TLC or microplates, and the FUJIX BAS-2000 bioimaging analyzer was used to determine the distribution of radioactivity recorded on the IP. Image analysis of the radioactivity profiles on TLC plates (Kieselgel 60F_{254}, 0.25 mm thick; Merck) was conducted after the development of two-dimensional TLC of plasma and perfusate samples with ethanol/water (3:2, v/v) as the first developing solvent system and 2-butanol/acetic acid/water (4:1:1, v/v/v) as the second developing solvent system. RLG was also used to determine the radioactivity on IPs.

Pharmacokinetic Studies. Oral doses of 2, 20, and 60 mg/kg [^{14}C]MET-88 (1.85 kBq/kg) were administered to rats ($n = 3$) in aqueous solution by gavage using steel ball-tipped feeding needles. Different groups of animals ($n = 3$) received the same doses dissolved in isotonic saline via bolus injection through a lateral tail vein. Blood samples (0.1–0.2 ml) were collected from orally administered animals at 0.25, 0.5, 1, 2, 3, 4, 5, 8, 12, and 24 h after dosing and from intravenously administered animals at 1 and 3 min and 0.25, 0.5, 1, 2, 3, 4, 5, 8, 12, and 24 h after dosing. Plasma was obtained from each sample by centrifugation and stored for pharmacokinetic studies. Plasma concentrations of [^{14}C]MET-88 were determined as follows: plasma samples were deproteinized by methanol, applied to TLC plates (Kieselgel 60F_{254}, 0.25 mm thick; Merck), and developed with a solvent system of ethanol/water (3:2, v/v). For preparation of a standard curve, varying concentrations of [^{14}C]MET-88 dissolved in methanol were developed on the same TLC plates, and [^{14}C]MET-88

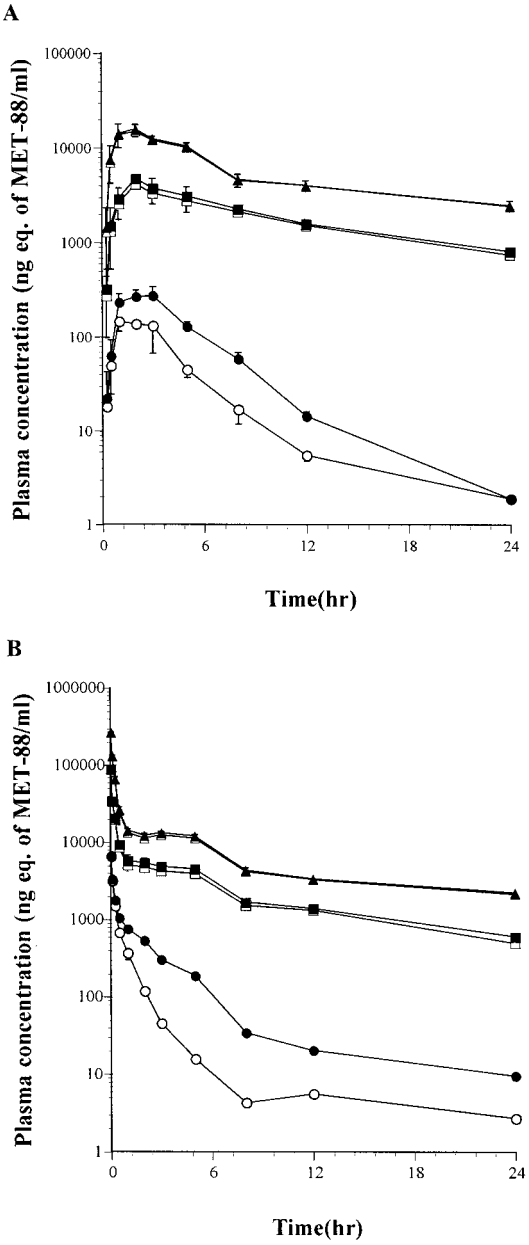


FIG. 3. *Comparative plasma concentration profile of total radioactivity (■, ●, ▲) and MET-88 (□, ○, △) in rats after oral (A) and i.v. (B) administration of 2 (●, ○), 20 (■, □), and 60 mg/kg (▲, △) [^{14}C]MET-88.*

Each point represents the mean and S.D. for three animals.

radioactivity in plasma samples and standards was detected by photostimulated luminescence (PSL) using the RLG system after overnight contact with an IP. Plasma [^{14}C]MET-88 concentrations were calculated according to the standard curve. Pharmacokinetic parameters of MET-88 were determined by noncompartmental analyses of the concentration-time data for MET-88 using WinNonlin computer software (Scientific Consulting, Inc.). Oral bioavailability was calculated as bioavailability = $AUC_{oral}/dose_{oral} \times AUC_{i.v.}/dose_{i.v.} \times 100$.

Excretion Studies. Rats administered [^{14}C]MET-88 (1.85 kBq/kg) orally by gavage at a dosages of 2, 20, and 100 mg/kg were kept in individual metabolic cages (Sugiyamagen IRIKI Ltd., Tokyo, Japan). Urine and feces samples were collected, and expired $^{14}CO_2$ air was trapped by use of a solution of 20% 2-aminoethanol (500 ml) at room temperature at 72 h after dosing. Air flow through the cages was 500 ml/min. Trapped [^{14}C]CO_2 was measured by adding aliquots of 20% 2-aminoethanol solution to 13 ml of HIONIC-FLOUR (Packard, Meriden, CT) liquid scintillation cocktail. Urine samples were pre-

TABLE 1

Pharmacokinetic parameters and dose proportionality of MET-88 after oral and i.v. administration to male rats at doses of 2 to 60 mg/kg

Dose	AUC	Dose-Normalized AUC	Terminal $t_{1/2}$[a]	Vd_{ss}	CL_{total}	Bioavailability
mg/kg	*mg · h/ml*		*h*	*l/kg*	*l/h/kg*	*%*
Oral administration						
2	0.71 ± 0.07	0.36 ± 0.04[b,c]	8.06 ± 1.25			38.2 ± 3.4[b,c]
20	54.74 ± 5.48	2.74 ± 0.27[b]	11.08 ± 0.96			85.5 ± 15.0[b]
60	187.03 ± 29.68	3.12 ± 0.49[c]	16.19 ± 4.63			81.8 ± 17.3[c]
i.v. administration						
2	1.95 ± 0.09	0.97 ± 0.05	9.81 ± 3.65	3.83 ± 1.44	1.03 ± 0.05[b,c]	
20	66.74 ± 11.01	3.34 ± 0.55	9.35 ± 1.71	2.62 ± 0.26	0.31 ± 0.05[b]	
60	231.27 ± 29.91	3.85 ± 0.50	18.14 ± 4.09	4.30 ± 0.51	0.26 ± 0.03[c]	

[a] Terminal $t_{1/2}$, values for half-lives were calculated over the following intervals: 12–24 h at a dose of 2 mg/kg and 8–24 h at doses of 20 and 60 mg/kg.
[b,c] Dose-normalized AUC, terminal $t_{1/2}$, CL_{total}, and bioavailability were evaluated by Tukey's multiple comparison test. Groups with the same superscript letters were judged to be different at $P < .01$.
Results are represented as mean ± S.D. ($n = 3$ per group).

TABLE 2

Excretion of radioactivity in urine, feces, and expired air after oral administration of [^{14}C]MET-88 at 2, 20, and 100 mg/kg to male rats

	Percentage of Dose		
	2 mg/kg	20 mg/kg	100 mg/kg
Urine	26.0 ± 2.3	25.9 ± 3.1	54.3 ± 4.4
Feces	12.1 ± 6.2	21.0 ± 5.6	13.2 ± 0.7
Expired air	51.0 ± 2.8	40.8 ± 8.5	19.5 ± 1.6
Carcass	6.3 ± 0.7	7.1 ± 1.2	7.6 ± 0.4[a]
Total	95.4 ± 2.0	94.7 ± 7.9	91.0 ± 2.5[a]

[a] 0–168 h.
Values, given in percentage of administered radioactivity, are the mean ± S.D. of four animals.

pared for direct counting with ACS-II (Amersham, Arlington Heights, IL) liquid scintillation cocktail. Feces and carcasses were weighed and homogenized in water with a Polytron (KINEMATICA GmbH, Littau, Switzerland), and aliquots (~1 ml) were solubilized with 1 ml of Soluene-350 (Packard)/2-propanol (1:1), to which we added 0.3 ml of 30% hydrogen peroxide solution for decolorization. Solubilized samples were added to 13 ml of HIONIC-FLOUR (Packard) liquid scintillation cocktail. Radioactivities of prepared cocktails of urine, feces, carcasses, and 20% 2-aminoethanol-trapped expired air samples were measured by liquid scintillation counting (LSC) in a Packard Tri-Carb Liquid Scintillation Spectrometer (model 460), and quenching was corrected automatically by means of an external standard.

Isolation and Identification of Metabolites. The metabolites M-1 and M-2 were isolated from pooled solutions after liver perfusion, whereas M-3 was isolated from pooled solutions after incubation in vitro. Each solution was first extracted with acetone or ethanol and shaken in a separatory flask with ethyl acetate or *n*-hexane after reduction by evaporation. Aqueous phases, containing MET-88, M-1, M-2, or M-3, were percolated through Amberlite XAD-2 resin and passed through cation-exchange resin AG50W-X8 (pH 3.0, pyridine form) to remove cationic compounds, such as MET-88. The passed fractions were then applied to anion exchange resin AG1-X2. M-1 was washed through with water, and M-2 (from perfusate samples) or M-3 (from in vitro samples) was eluted with 10% acetic acid. All eluates were freeze-dried, dissolved in water, and percolated through a chelate column of Chelex 100 resin. These procedures were repeated for each fraction for purification.

Characterization of metabolites was performed by fast atom bombardment-mass spectrometry (FAB-MS), ^{1}H NMR, and ^{13}C NMR spectroscopy. Liquid chromatography-mass spectrometry (LC-MS) was performed to identify *p*-bromophenacyl bromide-derivative M-3. A JMS DX-303 mass spectrometer equipped with a DA5000 data system (JEOL, Tokyo, Japan) was used for the measurement of FAB-MS. Spectra were obtained with both positive and negative ion detections. Dimethyl sulfoxide or water was used as the solvent, and *m*-nitrobenzylalcohol or dithiodiethanol was used as a matrix for the FAB target. A JNM GSX-400 NMR spectrometer (JEOL) was used to obtain NMR spectra. ^{1}H NMR and ^{13}C NMR spectra were measured at 400 and 100 MHz, respectively. Dimethyl sulfoxide-d_6 or deuterium oxide was used as solvent, and tetramethylsilane or 3-(trimethylsilyl)propionic acid-d_4, respectively, was used as an internal reference. A TSQ-7000 mass spectrometer (Finnigan MAT, San Jose, CA) linked with an HP-1050 HPLC system (Hewlett-Packard, Palo Alto, CA) was used for LC-MS analysis and operated in the electrospray ionization (ESI) mode. Previously fractionated reaction mixtures of M-3 were chromatographed on 150 × 2.1 mm Inertsil ODS-2 columns (GL Sciences, Tokyo, Japan) using gradient elution with water and acetonitrile (isocratic steps of 10% acetonitrile for 5 min, followed by a linear gradient to 100% acetonitrile for 30 min).

In Vitro Studies. Hepatic cytosol (40–70% ammonium sulfate fractionized cytosol) from rat was prepared essentially according to a previously reported method (Lindstedt, 1967) and had a protein concentration of 10 mg/ml, which was determined according to Bradford (1976) by use of a Bio-Rad (Hercules, CA) protein assay kit with a BSA standard.

In vitro formation of 3-hydroxypropionic acid from MET-88 was started by adding the hepatic cytosol (8 mg protein) to the incubation mixture (5 μM [^{14}C]MET-88, 100 μmol potassium chloride, 230 μmol nicotinamide, 50 μmol sodium ascorbate, 4 μmol ferrous sulfate, 4 mg catalase, NADPH-isocitrate dehydrogenase system [1 μmol NADPH, 20 μmol sodium DL-isocitrate, 0.1 mg isocitrate dehydrogenase, 6.5 μmol magnesium chloride], and 20 mM potassium phosphate buffer, pH 6.8) in a total volume of 2 ml. Incubations were carried out at 37°C and stopped by the addition of 6 ml of methanol. Enzyme activity was determined by use of the RLG system after the development of extracted solutions on TLC plates (Kieselgel 60F_{254}, 0.25 mm thick; Merck) with a solvent system of 2-butanol/acetic acid/water (4:1:1, v/v/v). To investigate the effect of GBB on MET-88 metabolism, 25, 50, 250, or 500 μM GBB was added to the reaction system, and the amount of ^{14}C was determined.

Perfusion Studies. The continuous ILP system, in the manner of Miller (Ross, 1972), is depicted in Fig. 2. Liver samples were perfused with 37°C oxygenated (95% O_2, 5% CO_2, 300 ml/min) Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 1 mg/ml glucose, 0.5% BSA, 0.03% $CaCl_2$, and 0.02 mg/ml [^{14}C]MET-88 at a flow rate of approximately 17 ml/min, and aspirated air at a rate of 100 ml/min was passed through 20% 2-aminoethanol solution (500 ml) to trap [^{14}C]CO_2 gas.

To investigate the excretory pathway of MET-88 to expired air, we first performed ILP for 45 min to equilibrate the system and then added the inhibitor (2.5 mg of IAA or FCA) to each ILP system and continued the perfusion for an additional 135 min. Five-milliliter aliquots of 20% 2-aminoethanol were collected at intervals of 15 min throughout the perfusion. After 180 min, the perfused livers were homogenized in 0.6 M cold perchloric acid and centrifuged at 12,000*g* for 10 min. The resultant supernatants were used for spectrophotometric measurement of fructose 1,6-diphosphate and citrate concentrations (Bergmeyer, 1988).

To investigate the participation of BHHase in the excretion of MET-88 to expired air, we added 110 mg of GBB (100-fold over the molecular amount of MET-88) to the ILP system and collected 3-ml aliquots of 20% 2-aminoethanol-trapped [^{14}C]CO_2 gas after 120 min. A control experiment without GBB was performed in the same manner.

Perfusates were developed on TLC plates (Kieselgel 60F_{254}, 0.25 mm thick; Merck) with a solvent system of 2-butanol/acetic acid/water (4:1:1, v/v/v), and bioimaging analyses and determination of concentrations of MET-88 and its

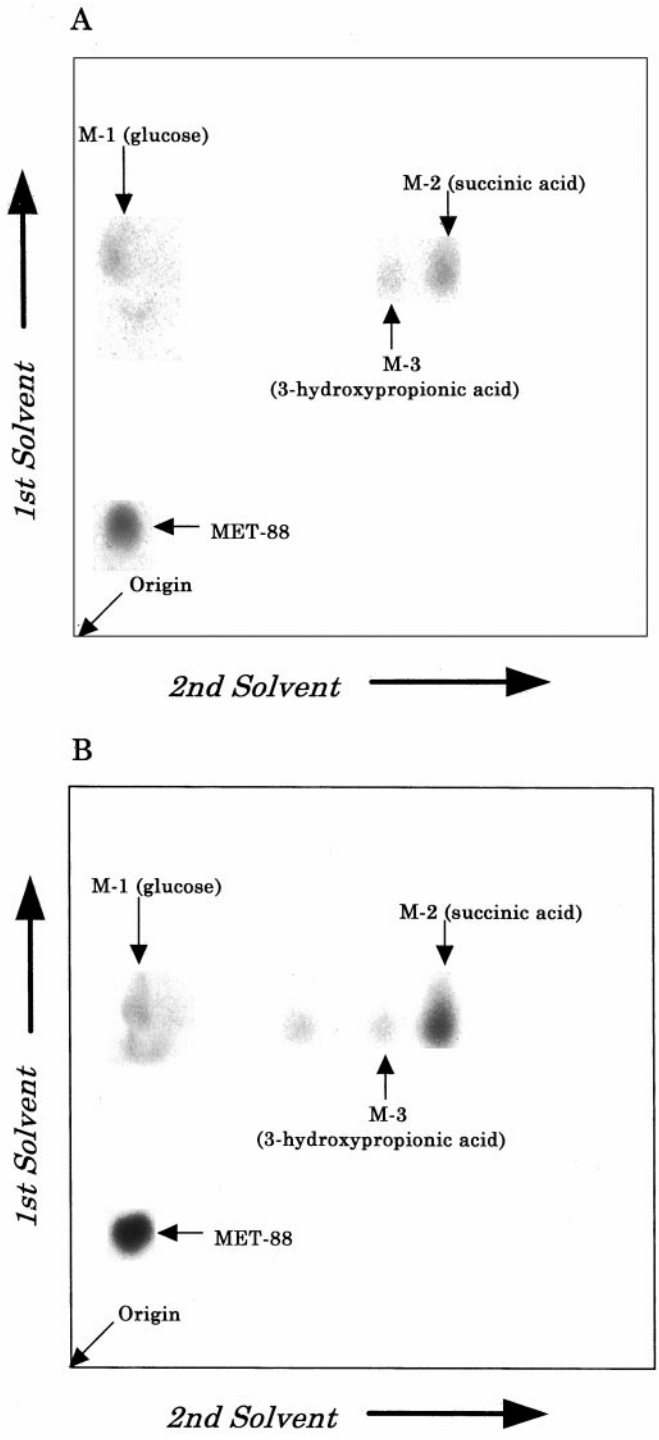


FIG. 4. *Two-dimensional TLC-ARG of plasma (A) and perfusate (B) samples after development using ethanol:water (3:2, v/v) as the first solvent system and 2-butanol/acetic acid/water (4:1:1, v/v/v) as the second one.*

A, rat plasma sample taken 2 h after the oral administration of 2 mg/kg MET-88. B, perfusate samples taken 4 h after the perfusion of isolated liver perfusion with 0.02 mg/ml MET-88.

metabolites were performed by RLG. Liver viability was assessed by using reverse phase HPLC (LC-4A; Shimadzu Co., Kyoto, Japan) to measure concentrations of ATP in the liver after perfusion (Bedford and Chiong, 1984). Using this parameter, we found liver viability to be equivalent in all groups (data not shown).

Statistical Analysis. Data storage and statistical analyses were performed with the Statistical Analysis System (SAS Institute Inc., SAS Proprietary Software, Cary, NC) software. Comparisons between groups were made with Dunnett's multiple range test or Tukey's multiple comparison test.

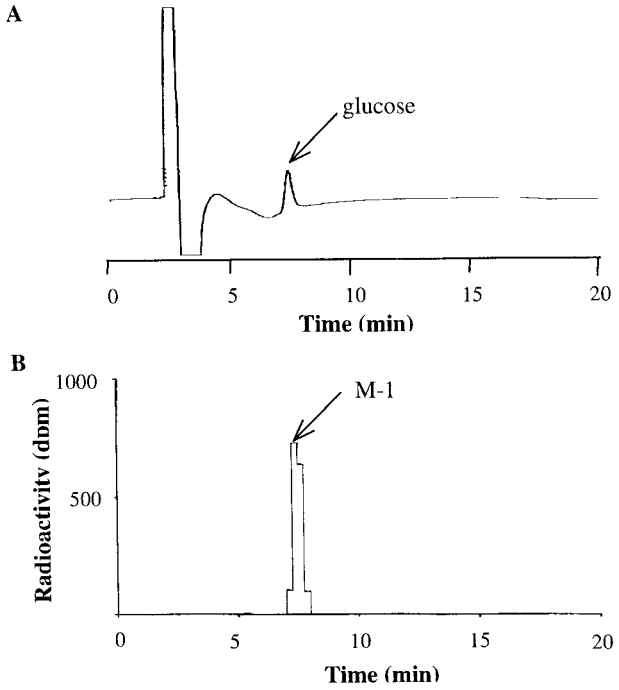


FIG. 5. *High-performance liquid chromatograms of authentic glucose (A) and partially purified M-1 (B) prepared from liver perfused with [^{14}C]MET-88.*

HPLC was performed with an LC-6 dual pump system with an RID-2A differential refractive index detector (Shimadzu). HPLC separation was achieved with an Asahipak NH_2P-50 column (25 cm × 4.6 mm i.d.; Asahi Chemical Industry Co. Ltd., Tokyo, Japan) and 1.0 ml/min flow rate of 70% (v/v) acetonitrile in distilled water. The HPLC eluate was fraction-collected into vials, and the radioactive content of each fraction was determined by LSC after the addition of ACS-II (Amersham) liquid scintillation cocktail.

Results

Pharmacokinetic Studies. [^{14}C]MET-88 was administered to male rats at doses of 2, 20, and 60 mg/kg oral and i.v. A representative comparison of MET-88 radioactivity and total radioactivity in plasma after oral and i.v. administration of [^{14}C]MET-88 is shown in Fig. 3. There were no differences between MET-88 and total radioactivity levels in plasma at doses of 20 and 60 mg/kg regardless of the administration route. However, at 2 mg/kg, plasma concentrations of MET-88 by both administration routes were markedly lower than the total radioactivity. A summary of the pharmacokinetic parameters of MET-88 after oral and i.v. administrations is shown in Table 1. Increases in the area under the plasma concentration-time curves from 0 to infinity (AUC) were greater than increases in dose, such that there was an almost 263- and 119-fold increase in AUC when the dose was increased from 2 to 60 mg/kg after oral and i.v. administration, respectively. The dose-normalized AUC also increased with dose for both administration routes. After i.v. administration at doses of 20 and 60 mg/kg, total body clearance (CL_{total}) values were very similar at 0.31 ± 0.05 and 0.26 ± 0.03 l/h/kg, respectively. However, at the 2 mg/kg dose, CL_{total} was significantly higher (1.03 ± 0.05 l/h/kg). In contrast, the volumes of distribution at steady state (Vd_{ss}) were not significantly different for this dose range. Bioavailability via the oral route for the 2 mg/kg dose (38.2 ± 3.4%) was significantly lower than that for the 20 and 60 mg/kg doses (85.5 ± 15.0 and 81.8 ± 17.3%, respectively).

Excretion Studies. The mean cumulative excretion of radioactivity over 72 h into urine, feces, and expired air at doses of 2, 20, and 100

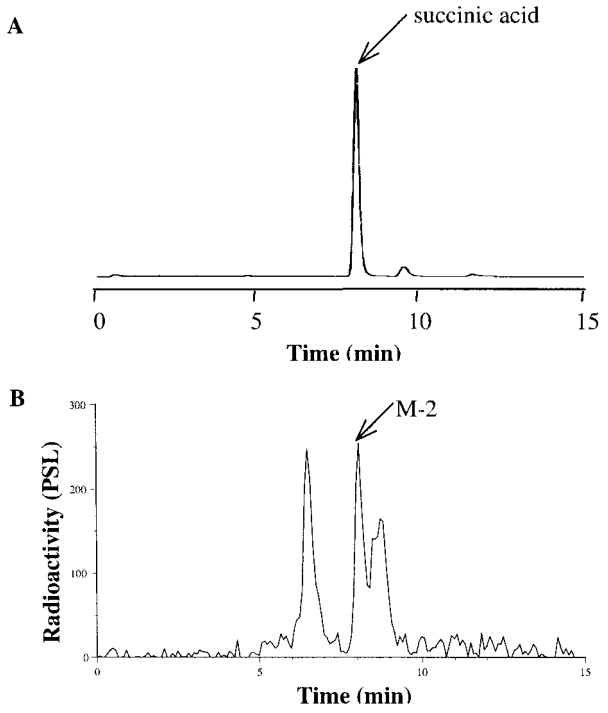


FIG. 6. *High-performance liquid chromatograms of authentic succinic acid (A) and partially purified M-2 (B) prepared from liver perfused with [^{14}C]MET-88.*

HPLC was performed with an LC-6 dual pump system with an SPD-6A variable-wavelength UV detector (Shimadzu) operated at 214 nm. HPLC separation was achieved with an organic acid column (30 cm × 7.8 mm i.d.; Waters, Tokyo, Japan) and 1.0 ml/min flow rate of 1% (w/v) phosphoric acid in distilled water. The HPLC eluate was fraction-collected into a microplate, and the radioactive content of each fraction was determined by RLG after evaporation.

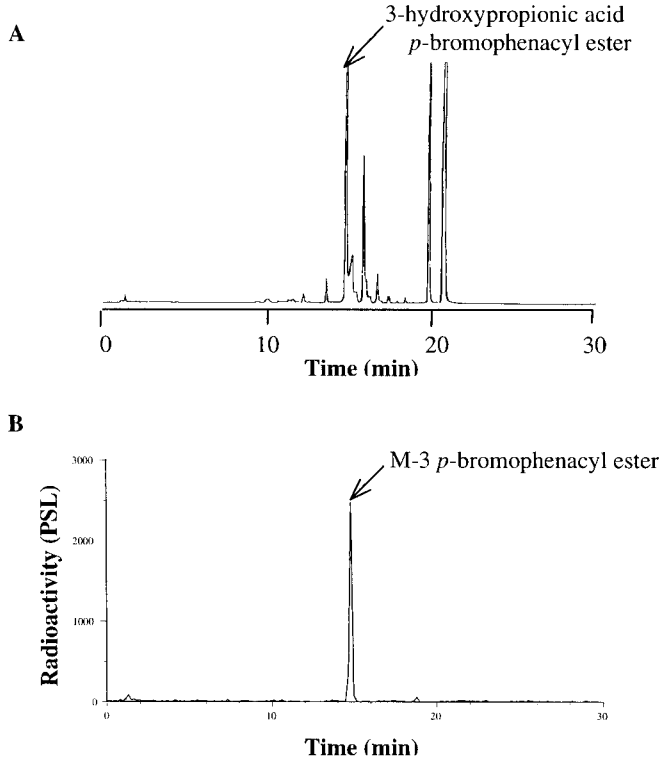


FIG. 7. *High-performance liquid chromatograms of p-bromophenacyl ester of authentic 3-hydroxypropionic acid (A) and partially purified M-3 (B) prepared from medium after incubation with [^{14}C]MET-88 in vitro.*

HPLC was performed with an LC-6 dual pump system with an SPD-6A variable-wavelength UV detector (Shimadzu) operated at 264 nm. HPLC separation was achieved with an Inertsil ODS-2 column (15 cm × 4.6 mm i.d.; GL Sciences). The mobile phase was composed of distilled water and acetonitrile (90:10–0:100 over 2–30 min) at a flow rate of 1.2 ml/min. The HPLC eluate was fraction-collected into a microplate, and the radioactive content of each fraction was determined by RLG after evaporation.

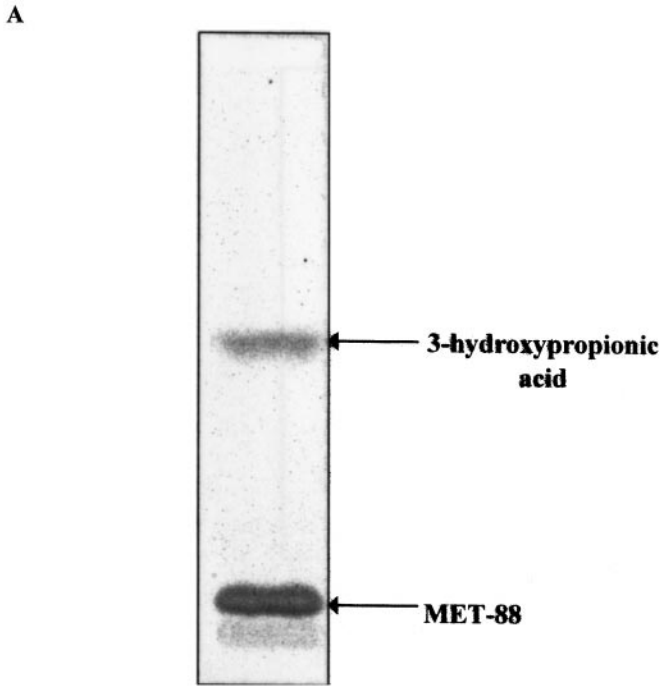
mg/kg is shown in Table 2. Fecal elimination accounted for 12.1 ± 6.2, 21.0 ± 5.6, and 13.2 ± 0.7% of the dose for the 2, 20, and 100 mg/kg doses, respectively. However, excretion at each of these doses accounted for 26.0 ± 2.3, 25.9 ± 3.1, and 54.3 ±4.4% of the dose in urine, and 51.0 ± 2.8, 40.8 ± 8.5, and 19.5 ± 1.6% of the dose in expired air, respectively. Thus, an increase in dose led to increased urinary excretion and decreased excretion in expired air. The radioactivity of carcasses at doses of 2, 20, and 100 mg/kg remained at 6.3 ± 0.7, 7.1 ± 1.2, and 11.7% of the dose, respectively, during the 72-h period.

Metabolite Profiles and Identification in Plasma and Perfusate. Figure 4 shows two-dimensional TLC-autoradioluminography (ARG) analysis of a plasma sample taken 2 h after the oral administration of 2 mg/kg [^{14}C]MET-88 and of an ILP perfusate sample taken 4 h after perfusion of the liver with 0.02 mg/ml [^{14}C]MET-88. The major radioactive components in plasma were MET-88 and three metabolites (i.e., M-1, M-2, and M-3) with the approximate percentages being 64, 13, 13, and 5, respectively. The three metabolites were also observed in perfusate samples in approximately the same proportions as in plasma.

M-1, M-2, and M-3 were identified as glucose, succinic acid, and 3-hydroxypropionic acid, respectively, by spectroscopic analysis and comparison with authentic standard samples. The molecular weights of M-1 and M-2 were determined as 180 and 118, respectively, by FAB-MS. The ^{1}H NMR and ^{13}C NMR spectra were very similar to those of authentic samples of glucose and succinic acid. Partially purified fractions containing M-1 or M-2, prepared from liver-perfused medium with [^{14}C]MET-88, were compared with authentic glucose or succinic acid by use of two different HPLC conditions (Fig. 5 or 6). The retention times of the radioactive peaks corresponding to M-1 and M-2 were identical with those of authentic glucose and succinic acid, respectively, under both HPLC conditions. In addition, the chemical identities of M-1 and M-2 were demonstrated by two TLC techniques, one using a silica gel plate (Kieselgel 60F_{254}, 0.25-mm thick; Merck) and one using a cellulose plate (Cellulose F, 0.1-mm thick; Merck), after development with several solutions in which the ^{14}C was shown to have the same R_f values as glucose (or [^{14}C]glucose; Daiichi Pharmaceutical Co.) and succinic acid, respectively.

FAB-MS and NMR analysis of isolated M-3 revealed that the main component of the sample was M-2. However, minor peaks (~8%, molar ratio) corresponding to 3-hydroxypropionic acid were detected in both FAB-MS and ^{1}H NMR spectra. Because complete separation of these components using chromatographic methods is quite difficult, the sample was transformed into its *p*-bromophenacyl derivative and subjected to LC-MS analysis. The objective HPLC peak showed an $[M-H]^-$ ion at *m/z* 285 in the negative ESI mode, which is consistent with the *p*-bromophenacyl ester of 3-hydroxypropionic acid. Furthermore, the retention time of the objective peak was identical with that of the *p*-bromophenacyl ester of authentic 3-hydroxypropionic acid (Fig. 7).

In Vitro Studies. Figure 8 shows the results of TLC-ARG on the extract of [^{14}C]MET-88 metabolites and the effect of GBB on [^{14}C]MET-88 metabolism. A radioactive spot corresponding to 3-hydroxypropionic acid was detected after 1-h incubation with hepatic



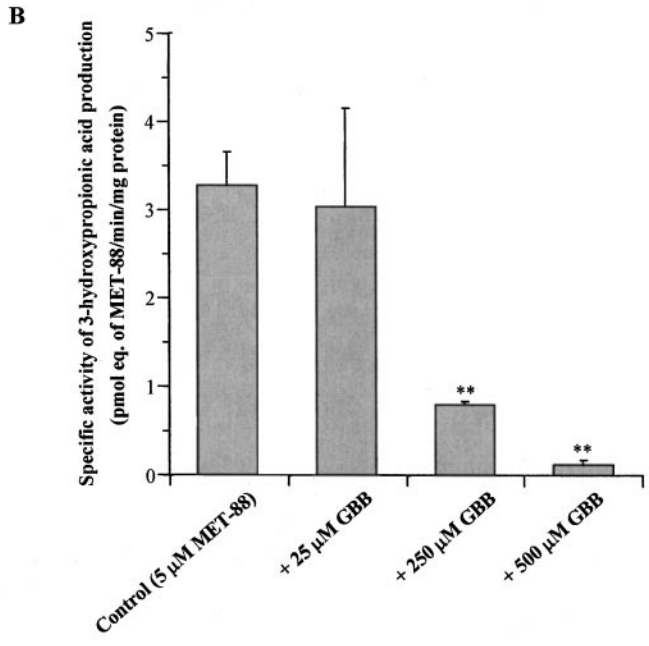


FIG. 8. *TLC-ARG of [^{14}C]MET-88 after 1-h incubation with enzyme solution (A) and effect of GBB on the [^{14}C]MET-88 metabolism (B).*

Incubated solutions were developed with 2-butanol/acetic acid/water (4:1:1, v/v/v). Reactions were performed as described in *Materials and Methods*. Enzyme activities after incubation with or without GBB ($n = 3$) were determined by use of the RLG system. Statistical comparisons between control and GBB groups were performed by Dunnett's multiple comparison test. $^{**}P < .01$ versus control.

cytosol fraction, and its specific activity was approximately 3.26 pmol eq./min/mg protein. The addition of GBB at concentrations of 25, 250, and 500 μM caused a concentration-dependent decrease in its production.

Perfusion Studies. To investigate the generation pathway of [^{14}C]CO_2 from [^{14}C]MET-88, we used an in situ perfused liver technique. The comparative changes in generated [^{14}C]CO_2 from

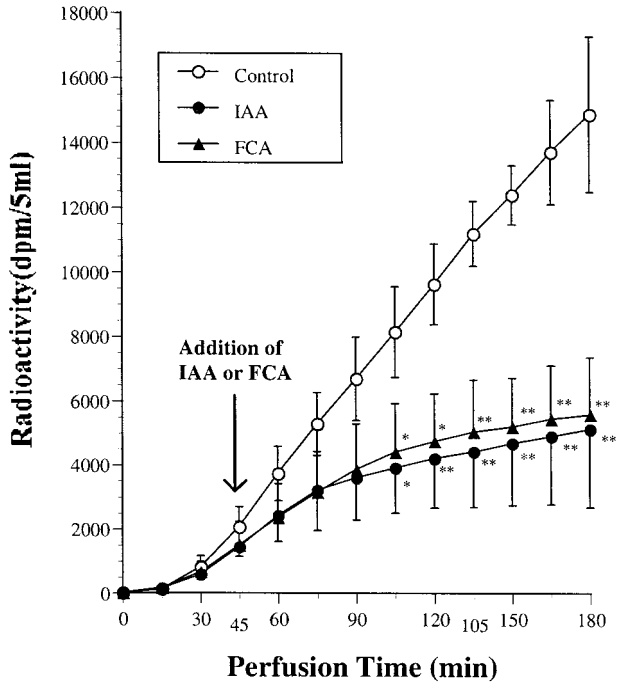


FIG. 9. *Comparative changes in generation of [^{14}C]CO_2 by isolated perfused livers.*

Livers were perfused cyclically at 37°C for 135 min after the addition of 2.5 mg of IAA or FCA. Aliquots of the 20% 2-aminoethanol solution that had trapped the [^{14}C]CO_2 gas were removed at intervals of 15 min. Results are expressed as the mean dpm/5 ml ± S.D. Statistical comparisons between control and inhibitor groups were performed by Dunnett's multiple comparison test. $^{*}P < .05$, $^{**}P < .01$ versus control.

[^{14}C]MET-88 during ILP studies are shown in Fig. 9. IAA, an inhibitor of the glycolytic pathway (Thoma and Ugurbil, 1987), or FCA, an inhibitor of the tricarboxylic acid (TCA) cycle (Kirk and Goldman, 1970), was added 45 min after initiation of the system. Compared with the control, to which only physiological saline was added, the generation of [^{14}C]CO_2 was significantly decreased by the addition of either inhibitor within 105 min. [^{14}C]CO_2 gas was not generated in the absence of the liver tissue (data not shown). The fructose 1,6-diphosphate and citrate contents in the isolated perfused liver samples after perfusion, with and without inhibitors, are shown in Fig. 10. Compared with the control, addition of IAA caused significant accumulation of fructose 1,6-diphosphate in perfused livers ($P < .01$), and FCA also led to significant accumulation of citrate in them ($P < .01$). Figure 11 shows the amounts of generated [^{14}C]CO_2 after 2-h perfusion with [^{14}C]MET-88 and GBB, IAA, or FCA. Compared with the control, the addition of an 100-fold molar excess of GBB, with respect to MET-88, led to significantly decreased [^{14}C]CO_2 generation, and the addition of IAA or FCA also decreased it. TLC-ARG analysis and concentration of MET-88 and its radioactive metabolites in perfusate after 2-h perfusion with [^{14}C]MET-88 and IAA, FCA, or GBB are shown in Fig. 12 and Table 3. Only [^{14}C]MET-88 was observed after perfusion with GBB. The concentration of MET-88, glucose, 3-hydroxypropionic acid, and succinic acid in the control perfusate was almost 57.2, 7.3, 3.1, and 7.5 nmol eq. of MET-88/ml, respectively. Compared with that of the control, the concentration of radioactive 3-hydroxypropionic acid was markedly increased (12.4 nmol eq. of MET-88/ml), and that of radioactive glucose was not detected after perfusion with IAA. After perfusion with FCA, the concentrations of radioactive glucose and succinic acid

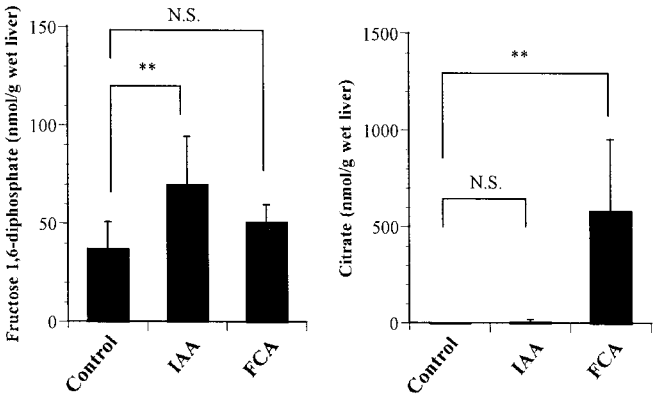


FIG. 10. *Fructose 1,6-diphosphate and citrate content in isolated perfused livers after 180-min perfusion.*

Values are the mean and S.D. (error bars) of individual samples from three rats. Statistical comparisons between control and inhibitor groups were performed using Dunnett's multiple comparison test. $^{**}P < .01$.

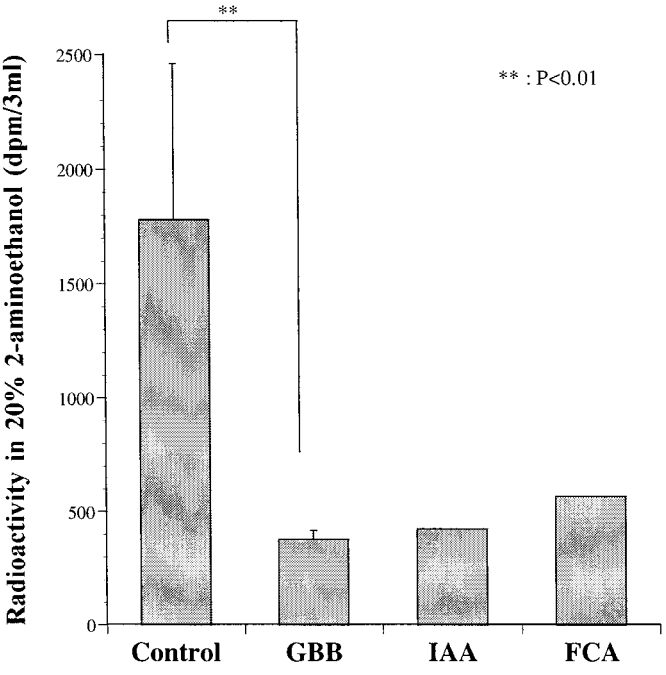


FIG. 11. *Cumulative excretion of [^{14}C] radioactivity as CO_2 after perfusion.*

Livers were perfused cyclically at 37°C for 120 min with [^{14}C]MET-88 and GBB ($n = 3$), 2.5 mg of IAA ($n = 1$), or FCA ($n = 1$), or without additional compounds (control) ($n = 3$). Statistical comparisons between the control and inhibitor groups were performed with Dunnett's multiple comparison test. $^{**}P < .01$.

in the perfusate were decreased (0.8 and 3.4 nmol eq. of MET-88/ml, respectively).

Discussion

Pharmacokinetic studies were performed after the i.v. and oral administration of a novel cardioprotective drug, MET-88, to rats at doses of 2, 20, and 60 mg/kg. Pharmacokinetic parameters indicated that MET-88 had nonlinear pharmacokinetics within this dose range regardless of the administration route. The plasma total radioactivity profile was markedly higher than that of MET-88 at low i.v. and oral doses (2 mg/kg), whereas at higher doses (60 mg/kg), total and MET-88 radioactivity profiles were very similar. Plasma protein binding of MET-88 was negligible (0.0%), Vd_{ss} values were similar within

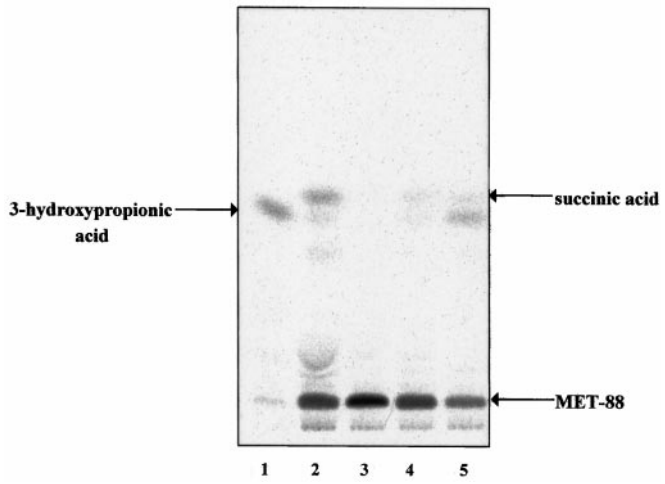


FIG. 12. *TLC-ARG of [^{14}C]MET-88 after 2-h perfusion with IAA, FCA, or GBB or without additional compounds as a control.*

Perfusates were developed with 2-butanol/acetic acid/water (4:1:1, v/v/v) as described in *Materials and Methods*. Lane 1, [^{14}C]3-hydroxypropionic acid biotransformed from [^{14}C]MET-88 in vitro; lane 2, control liver perfusate of [^{14}C]MET-88; lane 3, liver perfusate of [^{14}C]MET-88 with GBB; lane 4, liver perfusate of [^{14}C]MET-88 with FCA; and lane 5, liver perfusate of [^{14}C]MET-88 with IAA.

TABLE 3

Effect of perfusion with GBB, IAA, and FCA on the concentration of [^{14}C]MET-88 and its metabolites in perfusate after 2-h liver perfusion

	MET-88	Glucose	3-Hydroxypropionic Acid	Succinic Acid
Control	57.2 ± 0.2	7.3 ± 0.2	3.1 ± 0.0	7.5 ± 0.2
GBB	87.9 ± 0.5	N.D.	N.D.	N.D.
IAA	50.9	N.D.	12.4	6.1
FCA	74.9	0.8	2.6	3.4

[a] Each value (nmol Eq. of MET-88/ml) of control and GBB is presented as the mean ± S.D. ($n = 3$).
Each value (nmol Eq. of MET-88/ml) of IAA and FCA is result from $n = 1$.
N.D., not detected.

the dose range, and excretion studies indicated that the major excretion route of MET-88 changed from exhaled CO_2 at the low dose to urine at the high doses. We speculated that the nonlinear pharmacokinetics of MET-88 may have been related to the oxidative metabolic pathway of MET-88.

The radioactive components in plasma after the oral administration of [^{14}C]MET-88 were mostly consistent with those found in solution after liver perfusion with [^{14}C]MET-88. The metabolites were identified as glucose, succinic acid, and 3-hydroxypropionic acid. In plasma, 2 h after oral administration, MET-88 was the major compound, followed by glucose and succinic acid, whereas 3-hydroxypropionic acid was a minor compound. In vitro studies confirmed that MET-88 was converted to 3-hydroxypropionic acid by BBHase. 3-Hydroxypropionic acid was produced in the hepatic cytosol fraction that contained BBHase, required the cofactor of 2-oxoglutarate-dependent dioxygenase (Lindstedt, 1967; Lindstedt et al., 1968), and could be inhibited by excess GBB, a typical BBHase substrate. BBHase belongs to the class of enzymes known as 2-oxoglutarate-dependent dioxygenases, and it catalyzes the conversion of 2-oxoglutarate into succinate and CO_2 concomitantly with the oxygenation of the other substrate. We speculate that MET-88 is biotransformed to 3-hydroxy-MET-88, an unstable intermediate compound, by BBHase and is then converted to 3-hydroxypropionic acid, leaving trimethylhydrazine (Fig. 13).

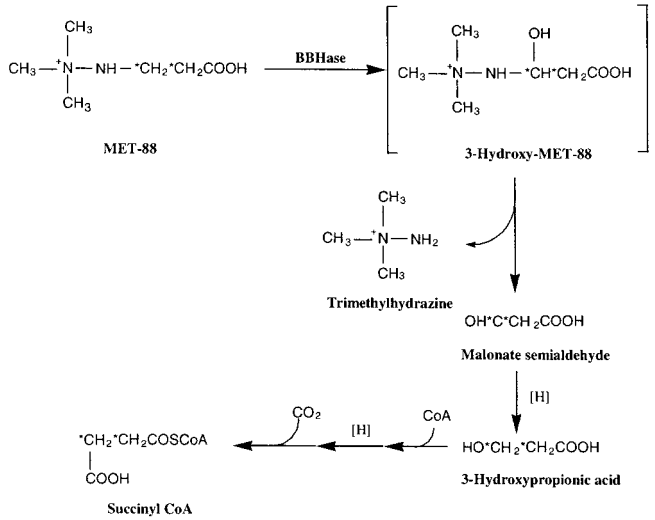


FIG. 13. *Speculated metabolic pathway for MET-88 elimination to expired air.*
*Position of the ^{14}C label.

Moreover, we examined the excretion pathway of MET-88 to CO_2 by a continuous ILP method using a modified apparatus able to trap CO_2 gas. [^{14}C]CO_2 was constantly generated from [^{14}C]MET-88 during perfusion, but no generation of [^{14}C]CO_2 was observed in the absence of liver tissue (data not shown). We estimated that the conversion of MET-88 to CO_2 in this ILP system accurately reflected the in vivo MET-88 excretion pathway to expired gas, and its excretion occurred via the glycolytic pathway and TCA cycle because the radioactivity profile in the perfusate after liver perfusion was consistent with the plasma profile after oral administration and its metabolites were intermediates of their pathway (i.e., glucose and succinic acid). IAA inhibits the enzyme activity of glyceraldehydephosphate dehydrogenase, which catalyzes the conversion of glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate and leads to the inhibition of the glycolytic pathway and the accumulation of fructose 1,6-diphosphate in tissues. FCA inhibits the enzyme activity of aconitase, which catalyzes the conversion of citrate to isocitrate via the obligatory intermediate *cis*-aconitate. The inhibition of aconitase leads to a build-up of citrate in tissues. The addition of either IAA or FCA to the ILP system significantly decreased the generation of [^{14}C]CO_2 compared with the control, to which only physiological saline had been added. In liver tissue after perfusion, citrate was significantly accumulated by the addition of FCA, and fructose 1,6-diphosphate was significantly accumulated by the addition of IAA.

The addition of a 100-fold molar excess of GBB over MET-88 to this ILP system significantly decreased [^{14}C]CO_2 production after 2 h of perfusion compared with the control production. TLC-ARG performed on solutions after perfusion with [^{14}C]MET-88 in the presence of GBB, FCA, or IAA revealed the metabolic fate and excretion pathway to expired air. The only radioactive spot was MET-88 in TLC-ARG after perfusion with GBB. Liver perfusion with FCA led to decreased radioactive glucose, succinic acid, and 3-hydroxypropionoic acid, whereas that with IAA led to the accumulation of radioactive 3-hydroxypropionic acid, decreased radioactive succinic acid, and the disappearance of radioactive glucose. Our data support the proposal that the conversion of MET-88 to 3-hydroxypropionic acid, catalyzed by BBHase via an unstable intermediate compound, was the first step in the expired [^{14}C]CO_2 gas excretion pathway, with 3-hydroxypropionic acid converted to glucose, probably via glyconeogenesis, and then excreted as CO_2 gas through the glycolytic pathway and TCA cycle.

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