extensive investigations are required to determine its practical therapeutic application.

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Biopharmaceutical Studies on Aminoethanesulfonylphenetidine and Related Compounds II

SHUN-ICHI NAITO and KAZUO FUKUI

Abstract □ The binding ratio of taurinophenetidine or nicotinoyltaurinophenetidine with serum protein in rabbits varies according to experimental conditions in dialysis, but the averaged binding ratios of taurinophenetidine and its nicotinate were roughly 40–50% at high doses. Taurinophenetidine and its nicotinate are scarcely excreted in rat feces and in rat and rabbit bile. The amount of both compounds distributed in organs of mice and rats is very small. It was observed that taurinophenetidine has some analgesic and antipyretic activities, and nicotinoyltaurinophenetidine reveals some analgesic and anti-inflammatory activities but no antipyretic action.

Keyphrases □ Aminoethanesulfonylphenetidine, related compounds—biopharmaceutical studies □ Metabolites, taurinophenetidine and derivatives—determination □ Taurinophenetidine and derivatives—distribution, animal organs □ Serum protein binding—taurinophenetidine and derivatives

In a previous work (1), blood levels of aminoethanesulfonylphenetidine (taurinophenetidine) in rabbits were determined and binding of taurinophenetidine with rabbit serum was found to be about 67% of the total amount administered *in vitro* and nil *in vivo*. Metabolites of taurinophenetidine in rabbit urine are separated into four compounds: *p*-aminophenol, *p*-acetamidophenol, *p*-phenetidine, and unchanged taurinophenetidine. It was also recognized that glucuronide in urine of rabbits ingesting taurinophenetidine is the conjugated form of *p*-aminophenol.

The present paper deals with the results obtained from biopharmaceutical studies and with some of the pharmacology on two compounds of *p*-phenetidine derivatives, taurinophenetidine and nicotinoylaminoethanesulfonylaminophenetidine (hereafter abbreviated as nicotinoyltaurinophenetidine).

EXPERIMENTAL

Metabolites of Taurinophenetidine in Blood—Plasma from 10 male mice (dd strain, average weight 20 g., taken at the time of peak blood levels 1.5 hr. after oral administration of 2500 mg./kg. of taurinophenetidine) or plasma from three female rats (Wistar strain, average weight 200 g., taken at the time of peak blood levels 1.5 hr. after oral ingestion of 2000 mg./kg. of taurinophenetidine) was treated by the method described in the previous paper (1).

Metabolites of Nicotinoyltaurinophenetidine in Blood—Plasma from two female rabbits (average weight 2.3 kg., taken at the time of peak blood levels 1.5 hr. after oral ingestion of 1000 mg./kg. of nicotinoyltaurinophenetidine) was treated by the same method (1). Methylene dichloride was used instead of chloroform for extraction.

TLC for separation of the metabolites was carried out under the following conditions: solvent, benzene–acetone–ethyl acetate (1:1:2); adsorbent, diatomite (Kieselgel G); color developer, 0.4% potassium ferricyanide solution followed by spraying with 1% ferric nitrate in 0.7 *N* nitric acid; R_f values in plasma of rabbits ingested the chemical, 0.33; R_f values in control plasma, nil; and R_f value of nicotinoyltaurinophenetidine, 0.33.

Determination of Acetophenetidin in Blood—Four female rabbits (average weight 3.0 kg.) were used. After oral administration of 250 mg./kg. of acetophenetidin, blood samples were taken according to the sampling schedule. To assay the free form of acetophenetidin, a mixture of 1 ml. of plasma and 3 ml. of water was shaken with 7 ml. of chloroform for 15 min. After centrifugation and filtration, a 3-ml. aliquot of the chloroform layer was evaporated to dryness on a water bath, and the residue was treated by the same method as previously reported for the determination of isopropylantipyrine (2).

A mixture of 1 ml. of plasma and 1 ml. of 5 *N* HCl was incubated at 37 ± 2° for 1.5 hr., and 2 ml. of water was added to this mixture. The plasma was treated with the acid due to the consideration that the binding of a chemical with plasma protein can possibly be prohibited by the action of hydrochloric acid. This mixture was shaken with 7 ml. of chloroform and treated by the same procedure as described for determination of total acetophenetidin.

Determination of Nicotinoyltaurinophenetidine in Blood—Each group consisted of four female rabbits (average weight 2.3 kg.).

Table I—Experimental Conditions in Distribution of Taurinophenetidine in Animal Organs[a]

Organ	Animal	A, g. ($MV \pm SE$)	B, g.	C, ml.	D, ml.	E, ml.	F, ml.
Heart	Mouse	0.11 ± 0.0	0.3	0.5	0.5	3	2
	Rat	0.7 ± 0.0	2	2	2	5	3
	Rabbit	6.1 ± 0.4	5	3	3	5	3
Lung	Mouse	0.15 ± 0.0	0.5	0.5	0.5	3	2
	Rat	1.2 ± 0.1	3	2	2	5	3
	Rabbit	9.8 ± 0.5	5	3	3	5	3
Liver	Mouse	1.1 ± 0.4	5	5	5	5	3
	Rat	7.6 ± 1.3	15	8	8	10	7
	Rabbit	75.2 ± 8.3	25	10	10	10	7
Kidney	Mouse	0.3 ± 0.2	0.5	0.5	0.5	3	2
	Rat	1.8 ± 0.1	5	3	3	5	3
	Rabbit	14.7 ± 1.4	5	3	3	5	3
Brain	Mouse	0.29 ± 0.1	0.5	0.5	0.5	3	2
	Rat	1.4 ± 0.1	4	3	3	5	3
	Rabbit	6.4 ± 0.2	5	3	3	5	3

[a] A = weight of each organ in animals used; B = weight of the sample of organ used for homogenation; C = volume of 5 *N* HCl; D = volume of supernatant pipeted after centrifugation; E = volume of methylene dichloride for extraction; F = volume of methylene dichloride used for evaporation after extraction; and $MV \pm SE$ = mean value ± standard error.

After oral administration of nicotinoyltaurinophenetidine, blood samples were taken according to the sampling schedule. One milliliter of plasma was treated with cyanogen bromide, using the procedure for determination of guaiacol glycerol ether mononicotinate with ammonia buffer (3). A mixture of 0.9 ml. of normal rabbit plasma and 0.1 ml. of 99.5% ethanolic solution of nicotinoyltaurinophenetidine of known concentration, such as 20, 40, 60, and 80 meg./ml., was treated as described previously to prepare a calibration curve.

Binding of Taurinophenetidine with Rabbit Serum Protein *In Vivo* by Equilibrium Dialysis—The experimental design followed the method of a previous report (4). Taurinophenetidine in the solution outside the bag was determined as free taurinophenetidine by the modified Folin method (1). To assay total taurinophenetidine, blood for dialysis was obtained from the supernatant from centrifugation of a mixture of 1 ml. of plasma and 1 ml. of 5 *N* HCl incubated at 37 ± 2° for 1 hr. and was then treated as described previously.

The stability of taurinophenetidine in phosphate buffer (pH 7.4) was determined for 72 hr.; no decomposition of the compound was observed under the experimental conditions for equilibrium dialysis. A plasma sample was obtained from male rabbits (average weight 2.5 kg.) receiving 200, 280, and 360 mg./kg. of taurinophenetidine; each group consisted of four rabbits.

Binding of Nicotinoyltaurinophenetidine with Rabbit Serum Protein *In Vivo* by Equilibrium Dialysis—The experimental design followed the method of a previous report (4). To assay the compound, a mixture of 0.5 ml. of the solution outside the bag and 1 ml. of phosphate buffer (pH 7.4) was treated with cyanogen bromide, using the same procedure as for the determination of nicotinoyltaurinophenetidine in blood.

The stability of nicotinoyltaurinophenetidine in phosphate buffer (pH 7.4) was determined; no decomposition was observed for 72 hr. A plasma sample was obtained from male rabbits (average weight 2.5 kg.) receiving 240, 320, and 400 mg./kg. of nicotinoyltaurinophenetidine; each group consisted of four rabbits.

Separation of Metabolites of Taurinophenetidine in Mouse Urine—Pooled urine from five male mice (dd strain, average weight 20 g.), ingesting 2500 mg./kg./day of taurinophenetidine for 2 days, was shaken with methylene dichloride for 20 min. The methylene dichloride layer from centrifugation was evaporated *in vacuo* below 50°. The residue was dissolved in a small amount of ethanol and submitted to TLC. The conditions for TLC were: solvent, benzene–acetone (2:1); adsorbent, diatomite (Kieselgel G); and color developer, 0.4% potassium ferricyanide solution followed by spraying with 1% ferric nitrate in 0.7 *N* nitric acid.

Separation of Metabolites of Nicotinoyltaurinophenetidine in Rabbit Urine—About 400 ml. of urine from four male rabbits (average weight 2.2 kg.), ingesting 500 mg./kg. of nicotinoyltaurinophenetidine, was submitted to freezing evaporation. The residue was treated by the method for separation of metabolites of taurinophenetidine in urine described in the previous report (1). The conditions for TLC were: solvent, the same spots obtained from a mixed solvent of acetone–benzene–ethyl acetate (1:1:2), acetone–chloroform–ethyl acetate (1:1:2), or acetone–hexane–ethyl acetate (1:1:2); color developer, iodine vapor, 5% silver nitrate solution, or 0.4% potassium ferricyanide solution followed by spraying with 1% ferric nitrate in 0.7 *N* nitric acid; and adsorbent, diatomite (Kieselgel G).

Assay of Metabolites of Nicotinoyltaurinophenetidine in Urine—A mixture of 1 ml. of 5 *N* HCl and 200 mg. of the frozen evaporate of urine of three male rabbits (mean weight 2.5 kg.), receiving nicotinoyltaurinophenetidine (400 mg./kg.), was treated as previously reported (1) and submitted to TLC, using a mixed solvent of benzene–acetone–ethyl acetate (1:1:2). The spots with R_f values corresponding to nicotinoyltaurinophenetidine and taurinophenetidine were scratched off the glass plate of the TLC and extracted with 3 ml. of ethanol for nicotinoyltaurinophenetidine or 3 ml. of water for taurinophenetidine, using a method similar to one previously reported (1). After centrifugation, 1 ml. of the supernatant was used for assay of the corresponding metabolite. Taurinophenetidine was assayed by the modified Folin method (1), and nicotinoyltaurinophenetidine was determined by the cyanogen bromide method for the determination of nicotinoyltaurinophenetidine in blood.

Assay of Metabolites of Taurinophenetidine in Feces—To three male rats (Wistar strain, average weight 200 g.), 2000 mg./kg. of taurinophenetidine in 0.2% tragacanth solution was administered orally; their feces were collected for 4 days. As a control group, three male rats were given 0.2% tragacanth solution without the compound under the same experimental condition. Total feces for each rat were triturated with 150 ml. of water and shaken vigorously with 150 ml. of methylene dichloride for 2 hr. The supernatant from centrifugation at 12,000 r.p.m. was obtained by decantation and filtered to remove water-insoluble matter. The filtrate was stored in a separator overnight at room temperature, and the layer of methylene dichloride was evaporated *in vacuo* below 50°. A mixture of the residue and 2 ml. of 5 *N* HCl was kept in an incubator at 37 ± 2° for 1 hr. and neutralized with about 2 ml. of 5 *N* sodium carbonate solution. This nearly neutralized mixture was extracted with an adequate amount of methylene dichloride, and the solvent was evaporated *in vacuo*. The residue was dissolved in 1 ml. of methylene dichloride, and 20 μl. of this solution was submitted to TLC as described for the assay of metabolites of taurinophenetidine in urine (1).

Distribution of Taurinophenetidine in Animal Organs—Taurinophenetidine used in the experiments was screened through 100 mesh.

Mice—To five male mice (dd strain, average weight 15 g.), 2500 mg./kg. of taurinophenetidine in 0.2% tragacanth solution was administered orally.

Rats—To three female rats (Wistar strain, average weight 200 g.), 2000 mg./kg. of taurinophenetidine in 0.2% tragacanth solution was given orally.

Rabbits—To three female rabbits (average weight 2.5 kg.), 400 mg./kg. of taurinophenetidine in water suspension was administered orally.

Five organs (heart, lung, liver, kidney, and brain) were used for the determination of taurinophenetidine and its metabolites. The sample of each organ after bleeding was taken in equal weight from five mice, three rats, or three rabbits. The sample was homogenized with 5 *N* HCl, and the homogenate was kept at 37 ± 2° for 1 hr. This homogenate was neutralized with 5 *N* sodium carbonate solution, and the supernatant from centrifugation was shaken with methylene dichloride for 20 min. The methylene dichloride solution was evaporated to dryness, and the residue was dissolved in 0.1 ml. of methylene dichloride. Fifty microliters of methylene dichloride solution was spotted on TLC (1), and the metabolites of taurinophenetidine were determined by the method for the assay of metabolites of taurinophenetidine in urine (1). The experimental conditions are shown in Table I.

Distribution of Nicotinoyltaurinophenetidine in Animal Organs—*Mice*—To five male mice (dd strain, average weight 13 g.), 2500 mg./kg. of nicotinoyltaurinophenetidine in 0.2% tragacanth solution was administered orally.

Rats—To three female rats (Wistar strain, average weight 120 g.), 2000 mg./kg. of nicotinoyltaurinophenetidine in 0.2% tragacanth solution was given orally.

Table II—Binding of Taurinophenetidine or Nicotinoyltaurinophenetidine with Serum Protein following Oral Administration to Rabbits[a]

Compound	Dose, mg./kg.	Hours after Dose	Assortment	Cp (*MV* ± *SE*) Buffer (Inside), ml. 0	0.5	1.0
TP	200	1.0	T	29 ± 4	39 ± 1	31 ± 4
			F	24 ± 3	21 ± 5	19 ± 9
			R	18 ± 2	37 ± 3	35 ± 4
		2.0	T	26 ± 7	35 ± 1	20 ± 2
			F	19 ± 2	19 ± 7	15 ± 8
			R	24 ± 2	37 ± 4	34 ± 3
	280	1.0	T	29 ± 1	32 ± 0.4	39 ± 9
			F	31 ± 7	31 ± 4	30 ± 8
			R	5 ± 5	8 ± 8	22 ± 4
		2.0	T	29 ± 4	30 ± 5	28 ± 8
			F	25 ± 5	27 ± 1	28 ± 6
			R	19 ± 2	10 ± 1	2 ± 2
	360	1.0	T	35 ± 1	48 ± 3	49 ± 3
			F	16 ± 8	26 ± 2	27 ± 2
			R	57 ± 7	49 ± 6	60 ± 2
		2.0	T	30 ± 2	36 ± 2	27 ± 5
			F	16 ± 6	24 ± 1	10 ± 2
			R	37 ± 2	35 ± 4	54 ± 9
NTP HCl	240	1.0	T			114 ± 2
			F			91 ± 2
			R			19 ± 2
		2.0	T			94 ± 3
			F			69 ± 1
			R			24 ± 1
	320	1.0	T			123 ± 4
			F			68 ± 1
			R			43 ± 9
		2.0	T			95 ± 3
			F			59 ± 1
			R			37 ± 6
	400	1.0	T	159 ± 1	162 ± 2	158 ± 9
			F	87 ± 1	92 ± 1	91 ± 1
			R	45 ± 6	43 ± 8	42 ± 9
		2.0	T	138 ± 3	144 ± 3	126 ± 2
			F	81 ± 1	81 ± 1	81 ± 1
			R	40 ± 5	44 ± 2	36 ± 1

[a] Cp = concentration of the compound (mcg./ml.) after equilibrium dialysis (outside of bag); T = total compound (mcg./ml.); F = free compound (mcg./ml.); R = binding ratios (%) of the compound with serum protein; *SE* = standard error; TP = taurinophenetidine; and NTP HCl = nicotinoyltaurinophenetidine hydrochloride. Experimental conditions were: rabbit serum/ml. inside the bag, and 8 ml. of the phosphate buffer solution (pH 7.4) outside the bag.

The experimental conditions were the same as described for the distribution of taurinophenetidine in animal organs. After extraction from the homogenate of each organ, the methylene dichloride solution was submitted to TLC; nicotinoyltaurinophenetidine and its metabolites were determined by the method for assay of metabolites of nicotinoyltaurinophenetidine in urine (1).

Collection of Rabbit and Rat Biles—Female rabbits, varying in weight from 2.5 to 3.0 kg., were anesthetized with urethan, 1 g./kg. s.c., following a fasting period of approximately 24 hr.

Blood pressure was recorded by a mercury manometer connected to a cannula inserted in the carotid artery, and respiration was recorded on a kymograph.

The experimental conditions were designed by the method described in a previous paper (5). Taurinophenetidine hydrochloride or nicotinoyltaurinophenetidine hydrochloride, 300 mg./kg. dissolved in 20 ml. of water, was administered orally. Each group consisted of three rabbits. Water (20 ml.) was given to each control rabbit. Male rats (Wistar strain, body weight 190–210 g.) were treated by the same method as for the rabbits. Each group consisted of five rats. Taurinophenetidine hydrochloride or nicotinoyltaurinophenetidine hydrochloride (300 mg./kg.) dissolved in 0.5 ml. of water was given orally. Control rats received 0.5 ml. of water each.

Determination of Taurinophenetidine or Nicotinoyltaurinophenetidine and Its Metabolites in Rabbit and Rat Bile—Rabbit bile from each group was combined and saturated with sodium chloride. The bile was shaken with 150 ml. of methylene dichloride for 2 hr. and the supernatant, after centrifugation at 12,000 r.p.m., was evaporated. The residue (A) obtained was mixed with 2 ml. of 5 *N* HCl and incubated at 37 ± 2° for 1 hr. This solution was neutralized with 2 ml. of 5 *N* sodium carbonate solution and shaken with about 7 ml. of methylene dichloride for 20 min. Residue B, obtained after evaporation of the methylene dichloride layer, was dissolved in 1 ml. of methylene dichloride, and 30 μl. of this solution was submitted to TLC. The component from each spot was determined by the method for assay of metabolites of taurinophenetidine in feces. Other parts of Residue A were submitted to the same TLC as Residue B. Determination of taurinophenetidine and its metabolites in rat bile was carried out by the same method as for rabbit bile, using 50 ml. of methylene dichloride instead of 150 ml. of the solvent for extraction.

Rabbit or rat bile collected after ingestion of nicotinoyltaurinophenetidine hydrochloride was treated in the same way as in the case of administration of taurinophenetidine and submitted to TLC. The component from each spot was determined by the method described for the assay of metabolites of nicotinoyltaurinophenetidine in urine.

Acute Toxicity of Taurinophenetidine, Taurinophenetidine Hydrochloride, Nicotinoyltaurinophenetidine, and Nicotinoyltaurinophenetidine Hydrochloride to Mice—The experiments were designed by the same method described in a previous report (6). Taurinophenetidine and nicotinoyltaurinophenetidine were administered as a suspension in 0.2% tragacanth solution, and the hydrochlorides of both compounds were given to mice as a solution in water. The volume of the test sample was adjusted to keep constant at 0.4 ml./10 g. body weight.

Tail-Pinching Test—The reaction time of mice pinched on the tail with an artery clip was recorded every 15 min. for 1.5 hr. after administration of the compounds.

Effect of Taurinophenetidine, Nicotinoyltaurinophenetidine, and Related Compounds on Squirming and Capillary Permeability—Male dd strain mice, 15 g., were given the test compounds orally. The compounds were screened through 200 mesh and suspended in

Table III—Distribution of Taurinophenetidine (Micrograms per Gram of Each Organ) after Oral Administration to Animals[a]

Organ	Animal	Taurino-phenet-idine	*p*-Amino-phenol	*p*-Phenet-idine	*p*-Acet-amido-phenol
Heart	Mouse	131			
	Rat	42			
	Rabbit	58			
Lung	Mouse	300			
	Rat	55			
	Rabbit	71			
Liver	Mouse	204			
	Rat	5		4	
	Rabbit	6			
Kidney	Mouse	273			Trace
	Rat	74	10		
	Rabbit	256	9	44	
Brain	Mouse	56			
	Rat	1			
	Rabbit	26			

[a] The data were obtained at the time of peak blood levels, 1.5 hr. for mice and rats and 2.0 hr. for rabbits, after oral administration of the compound, 2500 mg./kg. to mice, 2000 mg./kg. to rats, and 400 mg./kg. to rabbits.

0.2% tragacanth solution. The experiments were carried out using a method described in a previous paper (6).

Tail-Withdrawal Reflex in Mice—The same method for analgesimetry described in the paper of Ben-Bassat *et al.* (7) was used.

Anti-Inflammatory Activity—Inflammation was produced by injecting into the plantar surface of the hind paw of a male rat (Wistar strain, average weight 200 g.) carrageenin (0.1 ml. of 2% suspension), formaldehyde solution (0.2 ml. of 0.74% solution), or croton oil (0.1 ml. of 1% in sesame oil). Normal saline solution was given to the other hind paw as a control.

The test compound was administered in an aqueous solution, whose volume was kept constant at 0.1 ml./100 g. body weight for intraperitoneal dosage. One-half of the total dose of the test compound was given immediately after injection of the phlogistic agent, and the remaining half was given approximately 6 hr. later. Intraperitoneal injections were given in either caudal quadrant of the abdominal wall. Control animals received injections of the vehicle.

Each group consisted of five rats. The effect of the test compound on the edema was determined volumetrically 24 hr. after injection of the phlogistic agent. The method reported by Benitz and Hall (8) was used to design the experimental conditions.

Antianaphylactic Activity—Female guinea pigs (Hartley strain, average weight 400 g.) were used. The experiment was carried out by the procedure reported by Labelle and Tislow (9). Guinea pigs were challenged exactly 1 hr. after drug administration by rapid injection of 1 ml. of horse serum into the saphenous vein.

Antihistamine Activity—Female guinea pigs (Hartley strain, average weight 400 g.) were used in the histamine tests. The test was performed under the same condition as described in a previous paper (6).

Antipyretic Activity—To male rabbits weighing 2.2–2.8 kg., 15 mcg./kg. of nonanaphylactogenic polysaccharides obtained from

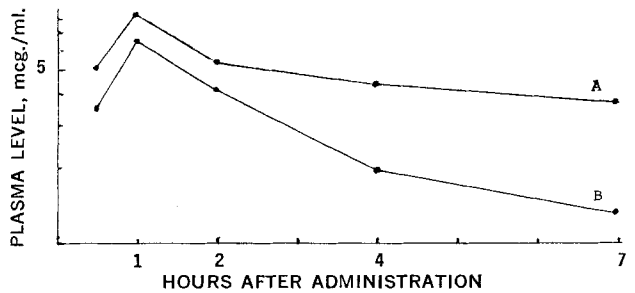

Figure 1—*Mean plasma level of free and total acetophenetidin after oral administration to four rabbits of 250-mg./kg. dose. Key: A, total acetophenetidin; and B, free acetophenetidin.*

Table IV—Distribution of Nicotinoyltaurinophenetidine and Its Metabolites (Micrograms per Gram of Each Organ) after Oral Administration of Nicotinoyltaurinophenetidine to Animals[a]

Organ	Animal	Weight of Organ, g. ($MV \pm SE$)[b]	Nicotinoyl-taurino-phenet-idine, mcg./g.	Taurino-phenet-idine, mcg./g.
Heart	Mouse	0.1 ± 0.0	131	0
	Rat	2.1 ± 0.2	45	34
Lung	Mouse	0.17 ± 0.0	89	26
	Rat	4.2 ± 1.2	66	39
Liver	Mouse	1.0 ± 0.0	139	6
	Rat	15.2 ± 0.3	49	9
Kidney	Mouse	0.2 ± 0.0	103	16
	Rat	3.6 ± 0.1	58	39
Brain	Mouse	0.3 ± 0.0	14	14
	Rat	3.4 ± 0.1	25	0

[a] The data were obtained at the time of peak blood levels, 1.5 hr. after oral administration of the compound, 2000-mg./kg. dose. [b] $MV \pm SE$ = mean value ± standard error.

Pseudomonas fluorescens[1] was administered intravenously; solution of the test compound was given subcutaneously 1.75 hr. after the administration of the pyrogen solution. Body temperature was recorded every 15 min. for 6 hr. by a thermistor-thermometer.[2] Each group consisted of three rabbits.

RESULTS AND DISCUSSION

Plasma of mice and rats, as well as of rabbits (1), after oral ingestion of taurinophenetidine or nicotinoyltaurinophenetidine, contained no detectable metabolites of the chemical in TLC.

Blood level of acetophenetidine, which has a chemical structure similar to taurinophenetidine, was determined in rabbits for the sake of comparison, and the results are shown in Fig. 1. Blood level of taurinophenetidine is clearly higher than that of acetophenetidin at

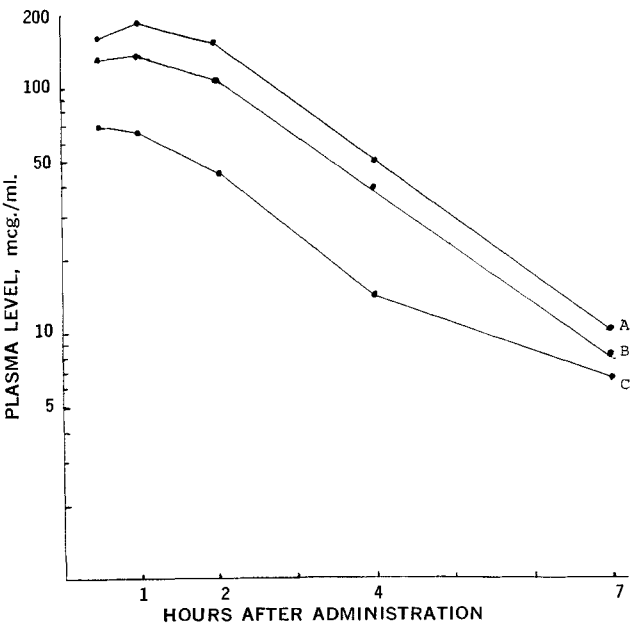

Figure 2—*Mean plasma level of nicotinoyltaurinophenetidine after oral administration of nicotinoyltaurinophenetidine hydrochloride to four rabbits of different doses. Each group consisted of four rabbits. Key: A, 300 mg./kg.; B, 225 mg./kg.; and C, 150 mg./kg.*

[1] T.T.G. No. 1, Fujisawa Yakuhin Kogyo Co. Ltd., Osaka, Japan.
[2] PSM-402, Eiwa Denshi Keiki Seisakusho Co. Ltd., Kyoto, Japan.

Table V—Acute Toxicity of Taurinophenetidine and Its Related Compounds in Mice[a]

Route[b]	Compound[c]	LD_{50}, mg./kg.[d]
p.o.	TP	4500 (3965–5108)
i.p.	TP	1100 (924–1309)
p.o.	TP HCl	1550 (1192–2015)
i.p.	TP HCl	570 (416–781)
s.c.	TP HCl	590 (552–630)
p.o.	NTP	6800 (6326–7310)
i.p.	NTP	1650 (1510–1803)
p.o.	NTP HCl	4650 (3577–6045)
i.p.	NTP HCl	470 (376–588)
p.o.	AP	3100 (2903–3311)

[a] Each group consisted of five male and five female mice (dd strain average weight 17 g.). The LD_{50} was calculated by the Litchfield–Wilcoxon method (12). [b] i.p. = intraperitoneal injection; p.o. = oral administration; and s.c. = subcutaneous injection. [c] TP = taurinophenetidine; TP HCl = taurinophenetidine hydrochloride; NTP = nicotinoyltaurinophenetidine; NTP HCl = nicotinoyltaurinophenetidine hydrochloride; and AP = acetophenetidin. [d] ( ) = 95% confidence limits.

the same dose, and this fact shows that the former is absorbed more easily than the latter.

Blood level of nicotinoyltaurinophenetidine is higher than that of taurinophenetidine at the same dose, as shown in Fig. 2. Therefore, nicotinoyltaurinophenetidine is more desirable than taurinophenetidine for use in pharmacological experiments as far as absorption is concerned.

The binding of taurinophenetidine or nicotinoyltaurinophenetidine with rabbit serum proteins *in vivo* at different concentrations of blood level is shown in Table II. It was confirmed that the binding ratio of taurinophenetidine or nicotinoyltaurinophenetidine with serum proteins varies according to experimental conditions. As a result, the averaged binding ratios of taurinophenetidine and nicotinoyltaurinophenetidine were roughly 40–50% at high doses, such as 360 mg./kg. of taurinophenetidine or 400 mg./kg. of nicotinoyltaurinophenetidine, but the binding ratios of both compounds with serum proteins were questionable at low doses.

Metabolites of taurinophenetidine in mouse urine were examined. The same metabolites were detected in mice as in rabbits (1), namely, unchanged taurinophenetidine, *p*-acetamidophenol, *p*-aminophenol, and *p*-phenetidine, having R_f values of 0.25, 0.35, 0.53, and 0.83, respectively.

Metabolites of nicotinoyltaurinophenetidine in rabbit urine were studied in the freeze-dried residue of the urine within 48 hr. after oral administration. Only two metabolites, unchanged nicotinoyltaurinophenetidine and taurinophenetidine, having R_f values of 0.31 and 0.20, respectively, were detected. *p*-Phenetidine, *p*-aminophenol, and *p*-acetamidophenol, the metabolites of taurinophenetidine, were not detected.

The results of quantitative determination of the metabolites in urine suggested that 9% of taurinophenetidine and 91% of unchanged nicotinoyltaurinophenetidine of the total metabolites were excreted; the ratio of the total metabolites to the dose ingested could not be calculated because of incomplete urine collection. However, 112 mg. of taurinophenetidine and 1200 mg. of nicotinoyltaurinophenetidine were recovered from urine of rabbits within 48 hr. after oral administration of a total of 4 g. of nicotinoyltaurinophenetidine.

Taurinophenetidine in feces of each rat was only 841 ± 132 mcg. when 2000 mg./kg. of the compound was given to a rat weighing about 200 g. The total weight of feces from each rat for 4 days was 39 ± 8 g. No metabolites except unchanged taurinophenetidine were detected in rat feces through TLC.

Table VI—Tail-Pinching Test after Oral Administration to Mice[a]

Compound	ED_{50}, mg./kg.[b]
Taurinophenetidine	580 (523–644)
Taurinophenetidine hydrochloride	365 (294–453)
Nicotinoyltaurinophenetidine	425 (322–561)
Acetophenetidin	750 (684–823)

[a] Each group consisted of five male and five female mice (dd strain, average weight 15 g.). The experiment was performed by the method of Haffner (10), and the results were obtained 30 min. after administration. The ED_{50} was calculated by the Litchfield–Wilcoxon method (12). [b] ( ) = 95% confidence limits.

Table VII—Effect of Test Compounds on Squirming and Capillary Permeability after Oral Administration[a]

Compound	Inhibition of Squirming, ED_{50}, mg./kg.[b]	Inhibition of Permeability, ED_{50}, mg./kg.[b]
Sodium acetylsalicylate	155 (80–301)	
Acetophenetidin	430 (261–710)	825 (453–1502)
Taurinophenetidine	285 (143–560)	
p-Acetamidophenol	970 (485–1940)	
p-Phenetidine hydrochloride	710 (458–1101)	950 (519–1739)
p-Aminophenol	490 (314–764)	
Taurinophenetidine hydrochloride	198 (99–396)	
Nicotinoyltaurinophenetidine	130 (74–229)	
Nicotinoyltaurinophenetidine hydrochloride	128 (82–200)	

[a] Each group consisted of 12 male mice (dd strain, average weight 15 g.). [b] ( ) = 95% confidence limits.

The amounts of taurinophenetidine distributed in the heart, lung, liver, kidneys, and brain of mice were fairly larger than those in rats and rabbits, as shown in Table III. Distribution of taurinophenetidine (micrograms per gram of each organ) showed almost similar values in rats and rabbits, except for the compound in the kidney. A considerable amount of taurinophenetidine was distributed in the kidneys of rabbit, but no metabolites were found in most animal organs except in rat liver and kidneys and in rabbit kidneys. It was also ascertained that taurinophenetidine and its metabolites do not remain in any of the organs of mice, rats, and rabbits 24 hr. after oral administration of the compound.

As shown in Table IV, nicotinoyltaurinophenetidine and taurinophenetidine are distributed in animal organs but none of the metabolites such as *p*-aminophenol, *p*-acetamidophenol, and *p*-phenetidine is found when nicotinoyltaurinophenetidine is administered orally to mice and rats. Nicotinoyltaurinophenetidine and its metabolites do not remain in any of the organs of mice and rats 24 hr. after oral ingestion of the compound, as in the case of taurinophenetidine.

The hydrochloride of taurinophenetidine or nicotinoyltaurinophenetidine at the dose of 300 mg./kg. had no effect on arterial blood pressure, respiration, bile flow, or total bile volume excreted for 7.5 hr. compared with untreated control rabbits. The evaporated residue from methylene dichloride used for extraction of rabbit or rat bile contained no metabolites when the hydrochloride of taurinophenetidine or nicotinoyltaurinophenetidine was administered orally. Even when the evaporated residue was treated with hydrochloric acid since the binding of a drug with bile components can possibly be prohibited by the action of hydrochloric acid, no metabolites were found after oral administration of the hydrochloride of taurinophenetidine or nicotinoyltaurinophenetidine to rabbits or rats. Taurinophenetidine or nicotinoyltaurinophenetidine in rabbit and rat bile, excreted for 7.5 hr. following oral administration of a 300-mg./kg., dose, was almost zero in either rabbits or rats in the former (taurinophenetidine), or 395 and 2325 mcg./kg. in the latter (nicotinoyltaurinophenetidine) at the mean value obtained from three rabbits and five rats.

Acute toxicity according to the number of dead mice 72 hr. after various doses of taurinophenetidine, nicotinoyltaurinophenetidine, or hydrochlorides of both compounds, and intraperitoneal, subcutaneous, and oral LD_{50}'s are shown in Table V. Although the animals receiving intraperitoneal doses below 700 mg./kg. of

Table VIII—ED_{50} for Pain Reaction Times in Mice 60 min. after Intraperitoneal Injection of Test Compounds[a]

Compound	ED_{50}, mg./kg.[b]
Acetophenetidin	435 (300–631)
Taurinophenetidine	310 (238–403)
Taurinophenetidine hydrochloride	127 (81–199)
Nicotinoyltaurinophenetidine	210 (124–357)
Nicotinoyltaurinophenetidine hydrochloride	168 (116–244)

[a] Each group consisted of 10 male mice (dd strain, average weight 15 g.). [b] ( ) = 95% confidence limits.

Table IX—Effect of Test Compounds on the Edema Induced by Subplantar Injection of Phlogistic Agents[a]

Phlogistic Agent	Treatment[b]	Dose, mg./kg.	Mean Volume, $MV \pm SE$[c]	Inhibition, %
Formaldehyde	Control		0.864 ± 0.136	
	NTP HCl	200	0.536 ± 0.087[d]	38
		300	0.582 ± 0.107[d]	33
		400	0.408 ± 0.133[d]	53
		500	0.446 ± 0.130[d]	48
	SA	500	0.735 ± 0.140	
	TP HCl	200	0.872 ± 0.085	
Croton oil	Control		0.868 ± 0.293	
	NTP HCl	200	0.724 ± 0.140	
	TP HCl	200	1.012 ± 0.417	
Carrageenin	Control		0.924 ± 0.131	
	NTP HCl	200	0.562 ± 0.082[d]	39
		300	0.492 ± 0.128[d]	47
		400	0.396 ± 0.153[d]	57
	SA	300	0.870 ± 0.375	
		400	0.864 ± 0.439	
		500	0.832 ± 0.349	
	TP HCl	200	0.904 ± 0.047	
		300	0.772 ± 0.391	
		400	0.710 ± 0.274	

[a] Each group consisted of five male rats (Wistar strain, average weight 200 g.). [b] NTP HCl = nicotinoyltaurinophenetidine hydrochloride; SA = sodium salicylate; and TP HCl = taurinophenetidine hydrochloride. [c] $MV \pm SE$ = mean value ± standard error. [d] Statistically significant effect at 95% confidence limits.

taurinophenetidine, 280 mg./kg. of taurinophenetidine hydrochloride, 1200 mg./kg. of nicotinoyltaurinophenetidine, or 280 mg./kg. of nicotinoyltaurinophenetidine hydrochloride exhibited no behavioral abnormality, intraperitoneal toxic doses of these four compounds produced a transient ataxic walk with extended hind limbs and a marked reduction in spontaneous movements. Within 10 min. after the intraperitoneal administration of 1800 mg./kg. of taurinophenetidine, 800 mg./kg. of its hydrochloride, 2200 mg./kg. of nicotinoyltaurinophenetidine, or 1000 mg./kg. of its hydrochloride, all of the animals were dead. They exhibited no lacrimation, piloerection, or convulsions before death, except that convulsion before death was observed in the cases of intraperitoneal administration of hydrochlorides of both compounds. No significant difference in the behavioral effect or LD_{50} was observed between the male and female

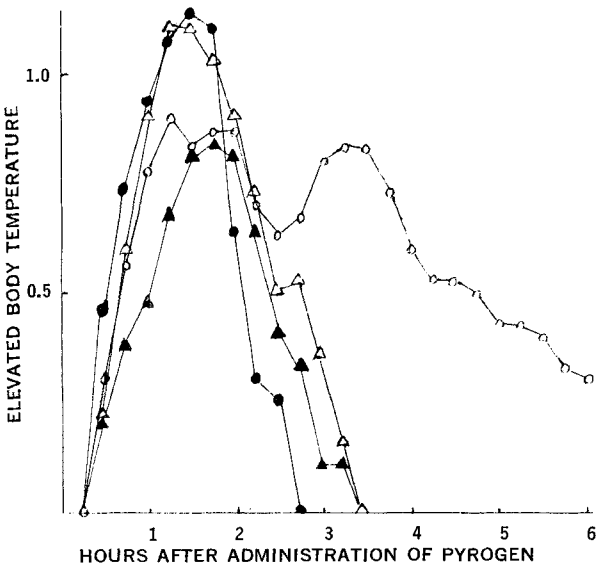

Figure 3—*Mean curve for the fall of elevated body temperature of male rabbits receiving pyrogen. Each group consisted of three rabbits. Key: ○, control group, mean basal body temperature (MBBT) 39.1°; ●, 125 mg./kg. of taurinophenetidine hydrochloride (TP HCl), MBBT 39.2°; ▲, 75 mg./kg. of TP HCl, MBBT 39.1°; and △, 50 mg./kg. of TP HCl, MBBT 39.2°.*

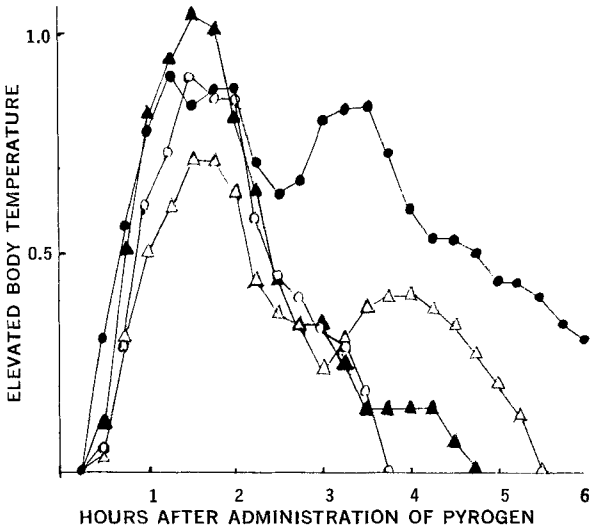

Figure 4—*Mean curve for the fall of elevated body temperature of male rabbits receiving pyrogen. Each group consisted of three rabbits. Key: ○, control group, mean basal body temperature (MBBT) 39.2°; ▲, 75 mg./kg. of nicotinoyltaurinophenetidine hydrochloride (NTP HCl), MBBT 39.3°; △, 50 mg./kg. of NTP HCl, MBBT 39.2°; and ○, 50 mg./kg. of* p*-acetamidophenol, MBBT 39.1°.*

mice. The LD_{50} of acetophenetidin was determined for the sake of comparison.

Results of the tail-pinching test are shown in Table VI. Taurinophenetidine and nicotinoyltaurinophenetidine show analgesic activity in the tail-pinching test and their potency is stronger than that of acetophenetidin.

The purpose of squirming and permeability tests was to obtain some information by measuring the concurrent changes in the peritoneal capillary permeability during squirming through the method of Whittle (11). Table VII records the values for the ED_{50}'s for inhibition of squirming and the ED_{50}'s for reduction of the permeability response. In most of the test compounds, reduction of the permeability response did not approach 50% over the range of doses which inhibited squirming. Sodium acetylsalicylate, acetophenetidin, and the metabolites of taurinophenetidine such as *p*-acetamidophenol, *p*-phenetidine, and *p*-aminophenol were used for comparative examination. None of the metabolites was more potent than taurinophenetidine at the same dose as the ED_{50} of taurinophenetidine for inhibition of squirming.

The results of tail-withdrawal reflex in mice are shown in Table VIII. Computation of ED_{50} was made by dichotomizing the reaction after 60 min. into two groups; "refractory," *i.e.*, pain reaction time of 2.0 sec. or more, and "nonrefractory" for the test compounds. From the above results, it was ascertained that analgesic action of nicotinoyltaurinophenetidine is more potent than that of taurinophenentidine which is more potent than that of acetophenetidin.

Table IX shows some results obtained for anti-inflammatory activity of taurinophenetidine hydrochloride, nicotinoyltaurinophenetidine hydrochloride, and sodium salicylate. It is interesting that the effect of sodium salicylate on the edema could not be found under the present experimental conditions, but nicotinoyltaurinophenetidine hydrochloride did show some anti-inflammatory actions on edema induced by formaldehyde solution and carrageenin. In contrast, taurinophenetidine hydrochloride shows no definite anti-inflammatory action on edema.

Effect of hydrochlorides of taurinophenetidine and nicotinoyltaurinophenetidine on antihistamine activity and antianaphylactic shock in guinea pigs was examined by the method of Labelle and Tislow (9). Neither of the compounds showed any activity at 300-, 500-, and 700-mg./kg. doses.

Taurinophenetidine hydrochloride decreased the elevated body temperature of all rabbits at doses above 75 mg./kg. The antipyretic action of the compound was higher than that of *p*-acetamidophenol even at low doses, such as 75 mg./kg., as shown in Figs. 3 and 4. On the other hand, nicotinoyltaurinophenetidine hydrochloride showed no antipyretic action at 50- and 75-mg./kg. doses. The question remains as to why there is a difference in the

antipyretic effect between taurinophenetidine hydrochloride and nicotinoyltaurinophenetidine hydrochloride; it is hoped that this problem will be solved in the near future.

In conclusion, taurinophenetidine and nicotinoyltaurinophenetidine are scarcely excreted in rat feces and in rat and rabbit bile. The amount of both compounds distributed in organs of mice and rats is very small. Taurinophenetidine has some analgesic and antipyretic activities, and nicotinoyltaurinophenetidine shows some analgesic and anti-inflammatory activities.

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Chromatographic Determination of Benzoic Acid Derivatives for Application to Metabolism Studies

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Abstract □ Thin-layer and gas chromatographic systems were developed in support of metabolism studies to detect 4-hydroxybenzoic, 2-hydroxyanisic, and 3-hydroxyanisic acids in the presence of benzoic acid. Chromatographic systems were also developed for the detection of 4-chlorobenzoic acid in the presence of benzoic and 4-hydroxybenzoic acids. Quantitative results were obtained by gas chromatography and by reverse TLC fluorimetry of the quenching of a fluorescent background.

Keyphrases □ Benzoic acid derivatives—determination as metabolites □ GLC—analysis □ TLC—analysis □ Fluorometry, quenching TLC—analysis

It was found necessary to develop procedures for the separation, identification, and assay of benzoic acid derivatives in support of studies in these laboratories of the importance of cleavage of the ethylenic bond in the metabolism of stilbenes (1). Sensitive methods applicable to rabbit urine extracts containing benzoic acid as a normal metabolite were required.

The initial stilbene under consideration was 4,4′-dimethoxystilbene. It was expected from the reported cleavage of *trans*-stilbene to benzoic acid (2, 3) that the cleavage product of 4,4′-dimethoxystilbene would be anisic acid. Moreover, since *O*-demethylation and aromatic hydroxylation are common metabolic reactions, it was felt that cleavage could also yield 4-hydroxybenzoic, 2-hydroxyanisic, and 3-hydroxyanisic acids. Therefore, it was necessary to develop analytical systems for the detection and estimation of these compounds as possible metabolites.

The major expected cleavage product of 4,4′-dichlorostilbene would be 4-chlorobenzoic acid; emphasis was placed on the analysis of this product in the presence of naturally occurring benzoic and 4-hydroxybenzoic acids.

TLC on a fluorescent background and gas chromatography of trimethylsilyl (TMS) esters met the requirements for the determination of the compounds of interest. The purpose of this paper is to describe the development of these procedures as general methods for the analyses of minor amounts of benzoic acid derivatives.

EXPERIMENTAL

Reagents—All common reagents and solvents utilized were of analytical reagent grade. The following reagents were used: silica gel G;[1] cellulose;[2] fluorescent indicator;[3] SE-52, OV-1, OV-17, and OV-25;[4] Chromosorb G, 100/120 mesh;[5] bis(trimethylsilyl)-acetamide (BSA);[4] benzoic acid;[6] 4-hydroxybenzoic acid;[7] 4-anisic acid;[8] and 2-hydroxy- and 3-hydroxyanisic acids prepared according to the literature (4–6).

TLC—Silica gel G TLC plates were prepared by coating five 20 × 20-cm. glass plates at a thickness of 250 μm. with a slurry composed of 21 g. of silica gel G, 2.1 g. of fluorescent indicator, and 50 ml. of water. Cellulose TLC plates were prepared by similarly coating five 20 × 20-cm. glass plates with a slurry composed of 10 g. of cellulose, 1.0 g. of fluorescent indicator, and 65 ml. of water. After air drying at room temperature, plates were activated at 105° for 30 min. prior to use. Spotting was performed with a 1.0-μl. pipet. Chromatograms were developed to a distance of 15 cm. by the ascending technique. Developing tanks were lined with filter paper and

[1] Warner-Chilcott Labs.
[2] Camag.
[3] Phosphor 2282, Research Specialties Co.
[4] Applied Science Labs.
[5] Johns-Manville Product Corp.
[6] J. T. Baker & Co., Phillipsburg, N. J.
[7] Eastman Organic Chemicals.
[8] Aldrich Chemical Co.