STUDIES ON ASPIRIN DERIVATIVES WITH VERY LITTLE SIDE EFFECTS. III.¹⁾ ABSORPTION, DISTRIBUTION, EXCRETION AND METABOLISM OF TRITIUM-LABELED ASPIRIN-ISOPROPYLAN-TIPYRINE (AIA) IN RATS

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The absorption, distribution, excretion, metabolism and protein binding of orally administered tritium-labeled aspirin-isopropylantipyrene (AIA) were demonstrated in rats. ³H-AIA having 0.1 μ Ci/mg of specific activity and 93.2% of radiochemical purity was prepared by the Wilzbach's method. When ³H-AIA was administered orally to rats, about 20% of the given ³H was absorbed from gastro-intestinal tracts in 30 min and about 50% in 3 h, 72% of the dose was excreted in the feces and urine during 5 d, and 10% was excreted in the bile in 24 h. The highest accumulation of ³H in most organs was found in one to three hr after oral administration and ³H was concentrated in the liver. The major metabolites excreted in urine within 24 h after administration were salicylic acid-isopropylantipyrene (SIA) sulfate (57.7%) and SIA glucuronide (30.5%). The amount of free SIA excreted in urine was 1.2%. The carboxylamide bond of AIA was never cleaved *in vivo* to give salicylic acid and 3-aminomethylisopropylantipyrene. About 58% of ³H in blood 1 h after the administration was bound with serum protein.

Keywords—aspirin derivative; aspirin-isopropylantipyrene; tritium-labeled compound; absorption; distribution, excretion; metabolism; serum protein binding

Previously, we synthesized a new aspirin derivative, *N*-3'-propyphenazonyl-2-acetoxymethylbenzamide (aspirin-isopropylantipyrene, AIA) which has very little side effects unlike aspirin, and shows analgesic, antipyretic, anti-inflammatory and potent platelet anti-aggregant activities like aspirin.^{1,2)} In this paper, for an understanding of the biological activities and the toxicities *in vivo*, the absorption, distribution, excretion and metabolism of its tritium-labeled compound were demonstrated in rats.

MATERIALS AND METHODS

Materials and Animals—Gentisic acid and reagents for liquid scintillation mixture were obtained from Nakarai Chemicals, Ltd., tritium gas from Nippon Isotope Kyokai, salicylic acid and silica gel from Wako Pure Chemicals, Ltd., isopropylantipyrene (IA) from Toho Shinyaku Ltd. and Sephadex G-50 from Seikagaku Kogyo,

Ltd. β -Glucuronidase was a product of Worthington Biochem. Corp. Aspirin was a product of Yoshitomi Seiyaku, Ltd. Arylsulfatase was a product of Sigma Chem. Co. AIA, salicylic acid-IA (SIA), 3-aminomethyl-IA (IANH₂) and salicyluric acid were synthesized in our laboratory as described previously.²⁾ ³H-AIA was prepared by the Wilzbach's method.³⁾ Briefly, AIA was contacted with tritium gas, and the products were dissolved in a mixture of distilled water and CH₃OH and lyophilized 3 times to remove unstable ³H. ³H-AIA was recrystallized from ethyl acetate. The specific activity was 0.1 μ Ci/mg and the radiochemical purity which was assayed with thin layer chromatography (TLC) on silica gel B-5 using CHCl₃-CH₃OH (9:1) and ether, was 93.2%. When ³H-AIA was hydrolyzed by refluxing in 12 N HCl for 3 h⁴⁾ and the hydrolysate was analyzed for the radioactivity on TLC using ether, 58.9% of the radioactivity in ³H-AIA molecule existed in

the part of IANH_2 , 32.2% in the part of salicylic acid and 8.9% in the part of acetyl group. The animals used were Wistar male rats weighing 200–250 g.

^3H Analysis in Rats administered with ^3H -AIA — All rats received orally ^3H -AIA suspended in 5% acacia in a dose of 200 mg/kg. The counting samples for radioactivity were placed in counting vials and 10 ml of scintillation mixture was added. The scintillation mixture consisted of naphthalene 150 g, 2, 5-diphenyloxazole 10 g and *p*-bis [2, 5-phenyloxazoly] benzene 0.3 g per 1.0 l of dioxane. The radioactivity was counted in a liquid scintillation spectrometer (Bechman, Model LS-150). The apparent counts were corrected by each recovery rate (75–100%) of ^3H into extracts from tissues, blood or excrements added with ^3H -AIA *in vitro* and by an internal standard method.

Gastro-intestinal Absorption and Blood Level — Rats were given ^3H -AIA 10 min after the ligation of total bile duct. After the prescribed time intervals, blood and total gastro-intestinal tracts with contents were removed. The gastro-intestinal tracts were excised and homogenized in 2 volumes of CH_3OH using a waring blender. An additional CH_3OH was added to the homogenate to a total volume of 50 ml and the mixture was centrifuged at 3000 rpm for 15 min. One ml of the supernatant was subjected to counting. The radioactivity in tissues of gastro-intestinal tracts was regarded as unabsorbed ^3H because it is very difficult to clearly separate ^3H in tissues from ^3H in their contents. Two ml of blood was homogenized with 3 ml of CH_3OH in a glass homogenizer. After centrifugation, 1 ml of the supernatant was subjected to counting.

Excretion in Urine and Feces — Each rat was kept in a metabolic cage permitting a separate collection of urine and feces periodically. The counting sample of feces was prepared as described in gastro-intestinal tract, and the urine sample was prepared as that for blood.

Excretion in Bile — Rat was inserted with cannula through the common bile duct under ether-anesthesia. After sample administration, bile was

collected at every 2 h and its counting sample was prepared from 0.2 ml of bile as that for blood.

Tissue Distribution — After sample administration, lobule of liver, brain, heart, spleen, adrenal and lung were excised and homogenized with 2 volumes of CH_3OH in a glass homogenizer, respectively. After dilution of the homogenate to 10 ml (except to 3 ml in adrenal), the mixture was centrifuged, and 1 ml of the supernatant was counted.

Analyses of Metabolites in Urine — Urine collected within 24 h after sample administration was lyophilized and the residue was extracted with CH_3OH containing 5 mg of AIA as a carrier. The recovery of ^3H from urine was 93.1%. The CH_3OH extract was analyzed by using TLC with silica gel B-5. The radioactive spots on the plate were extracted with CH_3OH , followed by counting. Enzymatic hydrolysis was carried out by incubating sample solution (7.5 ml) with 1 ml of β -glucuronidase solution 10000 Fishman unit/ml at 37°C for 48 h or with sulfatase solution (2100 unit of sulfatase containing β -glucuronidase activity/ml) at 37°C for 96 h. The hydrolysates were lyophilized and the residue was extracted with CH_3OH , followed by analysis on TLC.⁵⁾ Sample and enzyme were dissolved in 0.1M acetate buffer, pH 4.5.

Serum Protein Binding — One hr after sample administration, blood was collected and centrifuged. Two ml of serum was diluted with an equal volume of 0.15M phosphate buffer, pH 7.4, and applied on gel filtration with Sephadex G-50 column (1.3 $\times$ 34 cm) equilibrated with the buffer.⁶⁾

RESULTS

Gastro-intestinal Absorption and Blood Level

As shown in Fig. 1, about 20% of the given ^3H was absorbed from gastro-intestinal tracts 30 min after oral administration of sample, and the absorption increased gradually up to 3 h and reached to about 50% at 6 h. In blood, the radioactivity showed the maximum uptake during comparatively fast periods of 30 min to 1 h after sample administration, then gradually

decreased to one third the concentration of the maximum by 6 h.

Excretion in Urine and Feces

Table I showed urinary and fecal excretion of ^3H . At the first day, 51% of dose was excreted in urine and feces. During the periods of 5 d after

FIG. 1. *Gastro-intestinal Absorption and Blood Level of ^3H after Oral Administration of ^3H -AIA in Rats*
Each point represents mean $\pm$ s.e. of 3 rats.

FIG. 2. *Cumulative Excretion of ^3H in Bile after Oral Administration of ^3H -AIA in Rats*
Each point represents mean $\pm$ s.e. of 3 rats.

TABLE I. *Excretion of ^3H in Urine and Feces after Oral Administration of ^3H -AIA in Rats*

	Excretion of ^3H (% of dose) ^{a)}				
	Day after administration				
	1	2	3	4, 5	Total
Urine	7.3 $\pm$ 0.6	0.96 $\pm$ 0.13	0.33 $\pm$ 0.04	0.21 $\pm$ 0.10	8.8 $\pm$ 1.7
Feces	43.6 $\pm$ 2.7	13.0 $\pm$ 0.4	5.7 $\pm$ 0.8	1.4 $\pm$ 0.01	63.7 $\pm$ 2.6
Total	50.9 $\pm$ 3.5	14.0 $\pm$ 0.3	6.0 $\pm$ 3.8	1.6 $\pm$ 0.10	72.5 $\pm$ 2.1

a) Each value represents mean $\pm$ s.e. (n=3).

TABLE II. *Distribution of ^3H in Tissues after Oral Administration of ^3H -AIA in Rats*

Tissue	Distrubution (dpm/mg) ^{a)}		
	1 h	3 h	24 h
Liver	18.8 $\pm$ 1.3	12.8 $\pm$ 1.5	3.3 $\pm$ 1.0
Heart	2.3 $\pm$ 0.4	2.4 $\pm$ 0.4	1.2 $\pm$ 0.2
Brain	0.75 $\pm$ 0.07	0.86 $\pm$ 0.07	0.85 $\pm$ 0.15
Spleen	9.2 $\pm$ 1.9	9.0 $\pm$ 1.7	3.5 $\pm$ 0.9
Adrenal	5.7 $\pm$ 0.6	5.8 $\pm$ 1.8	2.4 $\pm$ 0.4
Lung	3.9 $\pm$ 0.6	5.8 $\pm$ 0.5	2.4 $\pm$ 0.5

a) Each value represents mean $\pm$ s.e. (n=3).

the administration, 8.8% of the dose was excreted in the urine and 63.7% in the feces.

Excretion in Bile

As shown in Fig. 2, ^3H excretion in the bile increased linearly with time up to 12 h after the administration. At 24 h, 10% of the dose was

TABLE III. *R_f Values of Authentic Compounds and Metabolites in Urine after Oral Administration of ^3H -AIA in Rats*

	<i>R_f</i> value		
	Solvent system ^{a)}		
	I	II	III
AIA	0.63	0.80	0.13
SIA	0.63	0.80	0.26
Aspirin	0.43	0.82	0.70
Salicylic acid	0.24	0.73	0.63
Gentisic acid	0.31	0.80	0.62
Salicyluric	0.30	0.70	0.34
IA	0.65	0.66	0.56
IANH ₂	0.37	0.37	0.06
Fr. A	0	0.60	0
Fr. B	0.63	—	0.26, 0
Fr. A hydrolyzed with β -glucuronidase	0.63, 0	0.80, 0.60	0.26, 0
Fr. A hydrolyzed with sulfatase	0.63, 0	0.80, 0.60	0.26, 0

a) System I: CHCl_3 - CH_3OH (9:1), System II: *n*-butanol- CH_3COOH - H_2O (4:1:1), System III: ether.

FIG. 3. Thin Layer Chromatogram of CH_3OH Extract of 24 h Urine after Oral Administration of ^3H -AIA in Rats

Solvent I: CHCl_3 - CH_3OH (9:1), Solvent II: *n*-butanol- CH_3COOH - H_2O (4:1:1), Solvent III: ether, Development: I_2 vapor.

excreted in the bile.

Tissue Distribution

As shown in Table II, the liver showed the highest degree of accumulation of ^3H , 1, 3, and 24 h after oral administration of the sample. Spleen, adrenal, lung, heart and brain showed accumula-

tion of ^3H in the decreasing order. A high degree of accumulation was shown at 1 to 3 h and it was lowered at 24 h except in the brain.

Analyses of Metabolites in Urine

The CH_3OH extract of 24 h urine after oral administration of ^3H -AIA was developed on TLC

FIG. 4. Thin Layer Chromatogram of Fr. A hydrolyzed with β -Glucuronidase
Solvent I: $\text{CHCl}_3\text{-CH}_3\text{OH}$ (9:1), Solvent II: $n\text{-butanol-CH}_3\text{COOH-H}_2\text{O}$ (4:1:1), Solvent III: ether,
Development: I_2 vapor.

FIG. 5. Thin Layer Chromatogram of Fr. A hydrolyzed with Sulfatase
Solvent I: $\text{CHCl}_3\text{-CH}_3\text{OH}$ (9:1), Solvent II: $n\text{-butanol-CH}_3\text{COOH-H}_2\text{O}$ (4:1:1), Solvent III: ether,
Development: I_2 vapor.

and the R_f value of radioactive spot was compared with those of authentic compounds summarized in Table III. As shown in Fig. 3, when the CH_3OH extract of urine was developed on TLC using solvent I, the majority (95.4% of radioactivity of extract) was recovered in Fr.A at the origin and 5.6% in Fr.B (R_f value: 0.63) which coincided with the spot of AIA and SIA. On TLC using solvent II, Fr.B was separated into 2 spots with R_f value: 0.26 (1.8% of extract) which coincided with SIA and of the origin (1.5% of extract) which did not coincide with any authentic compounds. Fr.A gave a single spot with R_f : 0.60 and R_f : 0 in solvent II and III, respectively (Fig. 3), and it did not correspond to any authentic compounds (Table III). Unaltered AIA was not recognized on TLC analyses in Fig. 3. Since Fr.A seemed to be the conjugated products, it was hydrolyzed by β -glucuronidase and sulfatase. The hydrolysate of Fr.A by β -glucuronidase gave 2 spots which coincided with SIA (32.0% of radioactivity of Fr.A) and unaltered Fr.A in solvent I, II and III, as shown in Fig. 4. In order to elucidate 68% of the unaltered of Fr.A, Fr.A was

hydrolyzed by sulfatase containing β -glucuronidase activity, followed by analysis on TLC using solvent I, II, and III. As shown in Fig. 5, since 92.5% of Fr.A was recovered in the spot which coincided with SIA, 60.5% of Fr.A was identified as SIA-sulfate and 32.0% was identified as SIA-glucuronide. As mentioned above, the major metabolites in urine after oral administration of ^3H -AIA were SIA-sulfate (57.7% of extract) and SIA-glucuronide (30.5%), and a very little amount of SIA was detected.

Serum Protein Binding

Serum obtained 1 h after oral administration of ^3H -AIA, at which the radioactivity in blood showed maximum level as shown in Fig. 1, was fractionated with Sephadex G-50 column, as shown in Fig. 6. Two radioactive peaks were found. One coinciding with the protein peak detected with OD at 280 nm had 57.8% of the radioactivity in serum. The other peak in regard seemed probably to be ^3H -SIA or ^3H -AIA.

DISCUSSION

^3H -AIA was orally administered to rats in 200 mg/kg of biologically effective dose. The rate of absorption was calculated from the radioactivity of gastro-intestinal tracts containing their contents after oral administration of ^3H -AIA. Since a part of gastro-intestinal contents adheres to gastro-intestinal tissues and can not be separated from them, ^3H in gastro-intestinal tissues was regarded as unabsorbed ^3H . Absorption % of ^3H -AIA from gastro-intestinal tracts was about 51% at 6 h. A maximum blood concentration of ^3H -AIA was shown at 1 h. The excretion in urine was 7.3% of the dose and in feces 43.6% at 24 h, while 50–83% of aspirin (20–450 mg/kg, *p.o.*) was excreted in the urine in 24 h.^{7,8)} The total excretion in the urine and feces in 5 days was about 72% of the dose. The facts that 8.9% of radioactivity of ^3H -AIA used in this experiment existed in the acetyl group and 28% of ^3H was not reclaimed, may suggest that unstable and radioactive acetyl group and tritium which replace acetylated substances, H_2O and so on are still present in the body in addition to the basic structure of AIA.

FIG. 6. *Sephadex G-50 Chromatogram of Serum after Oral Administration of ^3H -AIA in Rats*
Column: 1.3×34 cm, Buffer: 0.15 M phosphate buffer (pH 7.4)

The basic structure of ^3H -AIA may be accumulated in fat tissues because AIA and SIA are highly insoluble in water. It is unlikely that the residual ^3H in the body originated from salicylic acid and IANH_2 , because their metabolites which should be rapidly excreted in urine⁷⁾ were not present in urine. Liver showed a higher accumulation than blood, and brain showed the lowest accumulation as shown in aspirin.⁹⁾ The major metabolites of ^3H -AIA in urine were conjugates of SIA with glucuronic acid and sulfate like salicylamide.¹⁰⁾ Aspirin, salicylic acid, IA, IANH_2 , gentisic acid and salicyluric acid were negligible in urine, that is, the carboxylamide bond of AIA was not cleaved *in vivo*, because if carboxylamide bond of AIA was cleaved, metabolites of salicylic acid or IANH_2 would exist in urine. These facts may explain that AIA showed weaker gastric ulcerogenic activity and toxicity than aspirin. Serum protein obtained 1 h after oral administration of ^3H -AIA had 57.8% of radioactivity. Considering the facts that acetyl group of ^3H -AIA possessed 8.9% of the radioactivity in ^3H -AIA molecule and that aspirin was transformed rapidly into salicylic acid by losing its acetyl group and yielding acetylated protein in serum,^{7,11)} it is probable that the binding % of AIA to serum protein was less than 57.8%, while that of aspirin was 70–90%.¹²⁾ Although it was reported that substitution on the carboxyl group of aspirin serves to lower the biological activity,⁹⁾ AIA with substituted carboxyl group of aspirin showed potent biological activities like aspirin.^{1,2)} This seemed to be mainly due to a lower binding of AIA to serum protein than aspirin.

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