魚類麻酔剤2-アミノ-4-フェニールチアゾールの残留分析3

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Residue Analyses on 2-Amino-4-phenylthiazole, a Piscine Anesthetic, in Fishes—III Metabolism in Rainbow Trout and Carp

Akira SUZUKI*, Masaru SHIMURA*, Takahiko KIKUCHI*, and Yasuharu SEKIZAWA*

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The major biotransformation product of 2-amino-4-phenylthiazole in rainbow trout (*Salmo gairdneri irideus*) was isolated from water following exposure of fish to the anesthetic. The isolated crystalline metabolite was shown by means of ultraviolet, infrared and optical rotatory dispersion spectroscopy and gas chromatography to be identical to 2-amino-4-phenylthiazole-2-*N*-β-mono-D-glucopyranosiduronic acid, the major biotransformation product previously found in medaka (killifish, *Oryzias latipes*). The major biotransformation product in carp (*Cyprinus carpio*) was also identified as 2-amino-4-phenylthiazole-2-*N*-β-mono-D-glucopyranosiduronic acid by molecular sieve, thin layer and gas chromatography. Conversion of 2-amino-4-phenylthiazole to the *N*-glucuronyl conjugate was 8 and 12%, respectively, in rainbow trout and carp as shown by thin layer chromatography of extracts from fish treated with ^{3}H-labeled anesthetic. In addition, a minor metabolite of the anesthetic in rainbow trout was isolated as a yellowish-white crystalline powder and identified as 2-acetamido-4-(4′-hydroxyphenyl)-thiazole by means of ultraviolet and infrared spectroscopy, NMR and mass spectrometry. Chromatography suggested that this same metabolite was also formed in carp but in concentrations too low for isolation and definitive identification.

The major biotransformation product of the anesthetic 2-amino-4-phenylthiazole in medaka was previously isolated in crystalline form and its chemical structure was identified as 2-amino-4-phenylthiazole-2-*N*-β-mono-D-glucopyranosiduronic acid.[1] Pharmacokinetic studies with ^{3}H-2-amino-4-phenylthiazole indicated that the anesthetic is extensively metabolized by medaka with about 89% conversion to the *N*-glucuronide occurring under conditions of limited water exchange.[2] Excretion of both the anesthetic and its biotransformation product followed similar biexponential curves.

Despite development of an isolation technique for the *N*-glucuronyl conjugate of 2-amino-4-phenylthiazole from medaka, this procedure was not effective for the isolation of the *N*-glucuronyl conjugate from other species of freshwater fishes including rainbow trout and carp.[2] Thus, from the negative results in these earlier studies, the biotransformation product of the anesthetic in these fishes seemed likely to be quite labile and formed only in small amounts.

To clarify these uncertainties, a radiochromatographic experiment was performed using ^{3}H-2-amino-4-phenylthiazole and the isolation procedure previously described for

* Research Laboratories, Meiji Seika Kaisha, Ltd., Morooka-cho, Kohoku-ku, Yokohama (鈴木 明・志村 勝・菊池孝彦・関沢泰治: 明治製菓中央研究所).

medaka. In this paper, details will be reported on the radiochromatographic analysis, isolation and identification of the biotransformation product of the anesthetic in rainbow trout and carp.

Materials and Methods

Preparation of radioactive material 2-Amino-4-phenylthiazole was tritiated by the WILZBACH method[8]. After elimination of exchangeable tritium, radioactive impurities were removed by repeated recrystallization of the tritiated anesthetic from aqueous methanol containing a small amount of sodium hydroxide followed by chromatography on a silica gel column (about 30 g of silica gel, 25×300 mm) eluted with chloroform-ethyl acetate (10: 1 v/v). Thin layer chromatography (TLC) of the resulting ^{3}H-2-amino-4-phenylthiazole (specific activity 11.9 μCi/mg) gave a single radioactive peak (Rf=0.33) when the plates (Silica gel F-254, 0.25 mm, E. Merck, Dalmstadt, West Germany) were developed in chloroform-ethyl acetate (4: 1 v/v). The radioactivity was detected with a Packard Model 7201 radiochromatogram scanner.

Biological materials and methods Zero year old rainbow trout (1.5–2.0 g, 6–7 cm) and carp (0.8–1.2 g, 2–3 cm) were used in this study. Both species were raised in tanks in which water temperatures were 20±1°C and 24±1°C, respectively. Fish were used for the experiment after 4 days starvation.

To determine biotransformation product, 4 rainbow trouts were maintained in 500 ml of water containing 2.05 mg of tritiated 2-amino-4-phenylthiazole for 48 hours at 20±1°C with aeration. In a similar manner, 10 carps were held in 800 ml of water containing 5.12 mg of the tritiated compound for 48 hours at 24±1°C with aeration. At the end of the exposure period, each fish was rapidly dried over calcium chloride in a desiccater under vacuum. The dried fish were pooled and extracted by grinding with 10 m*l* of methanol 5 times to attain complete extraction. The resultant extract and environmental water were both evaporated under diminished pressure to a volume less than 0.5 m*l*. The concentrated solutions then were applied as a band to 5×20 cm TLC plates. The plates were developed up to 10 cm in length with a solvent system consisting of chloroformethyl acetate (4: 1 v/v) or *n*-buthanol-acetic acid-water (4: 1: 1 v/v). A small amount of nonradioactive 2-amino-4-phenylthiazole and chemically synthesized 2-amino-4-phenylthiazole-2-*N*-β-mono-D-glucopyranosiduronic acid as previously described were used for a marker which were applied to the TLC plates separately from the sample band. After drying, radioactivity on the TLC plates was measured by the Packard radiochromatogram scanner. Positions of the radioactive materials coincided with those of the authentic anesthetic and *N*-glucuronyl conjugate. Conversion ratios for the biotransformation product of the anesthetic were calculated from the peak areas in each radiochromatogram scan.

Isolation of biotransformation products HP-20 resin, purchased from Mitsubishi Kasei Chemicals Co., was used for the adsorption chromatography. Sephadex G-25 was used for molecular sieve chromatography.

Instrumentation for structural determination Hitachi EPS-3T and 215 recording spectrophotometers were used for determination of ultraviolet and infrared absorption spectra, respectively. NMR spectra were measured with a Varian XL-100 nuclear magnetic resonance spectrometer. A Hitachi 073 gas chromatography equipped with FID ditector was used for the gas chromatographic analyses. Optical rotation or dispersion were measured by a Nihon Bunko ORD/UB-5. For determination of the molecular weight and the ultimate analysis of the biotransformation products, a JMS-01SG high performance mass spectrometer, from Japan Electron Optics Laboratory Co., Ltd. was used.

Results

TLC analysis of the biotransformation products derived from ^{3}H-2-amino-4-phenylthiazole TLC of the concentrate from environmental water of rainbow trout and carp gave two UV absorbing spots corresponding to standards of 2-amino-4-phenylthiazole and the *N*-glucuronyl conjugate, respectively (Fig. 1). The two radioactive peaks corresponded to UV absorbing reference standards of the anesthetic and its *N*-glucuronyl conjugate, respectively (Fig. 2). The percent conversion of ^{3}H-2-amino-4-phenylthiazole to its *N*-glucuronide was calculated from the radiochromatogram scans following TLC of either the environmental water or methanol extracts of the fish body in solvent system

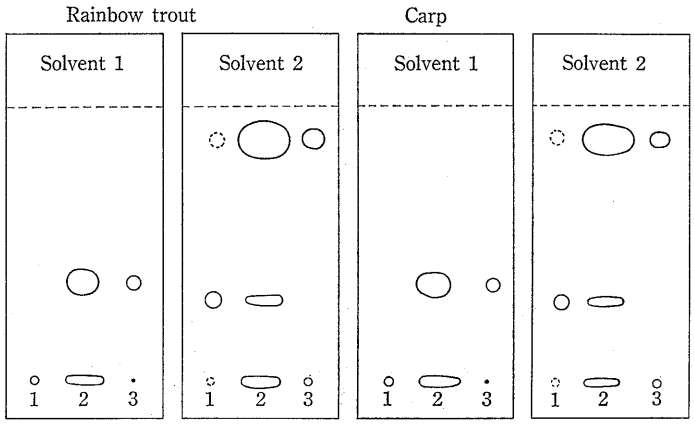


Fig. 1. TLC of reference compounds and the concentrated environmental water of rainbow trout and carp after treatment with ^{3}H-2-amino-4-phenylthiazole. Spots were located by the quenching of fluorescence by irradiating the plates with 253.5 nm UV light. Solvent 1: chloroform-ethyl acetate (4: 1); Solvent 2: *n*-buthanol-acetic acid-water (4: 1: 1); Sample 1: *N*-glucuronyl conjugate; Sample 2: concentrated environmental water; and Sample 3: 2-amino-4-phenylthiazole.

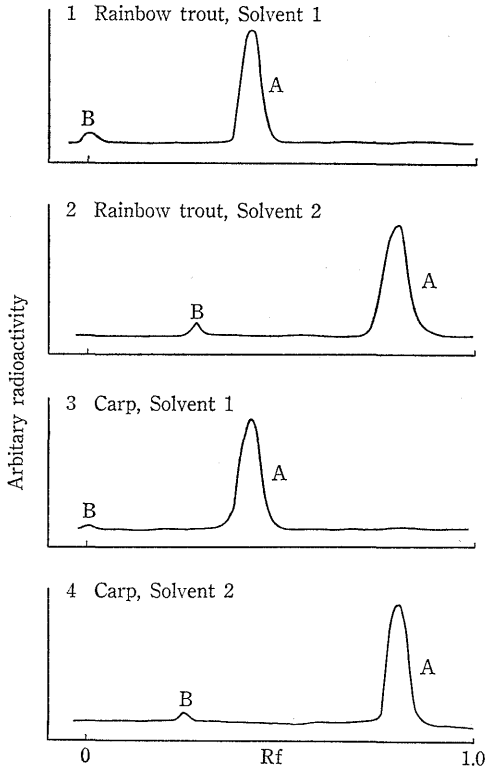


Fig. 2. Radiochromatogram scans of the concentrated environmental water from rainbow trout and carp treated with ^{3}H-2-amino-4-phenylthiazole. Peak A corresponds to unchanged ^{3}H-2-amino-4-phenylthiazole and peak B to the *N*-glucuronyl conjugate of ^{3}H-2-amino-4-phenylthiazole. Solvent 1: chloroform-ethyl acetate (4: 1); and Solvent 2: *n*-buthanol-acetic acid-water (4: 1: 1).

2 and the results are summarized in Table 1.

Isolation of the biotransformation products The procedure employed for isolation of the biotransformation products of 2-amino-4-phenylthiazole in rainbow trout or carp is illustrated in Fig. 3. Absorption chromatography on HP-20 resin was quite effective in concentrating the biotransformation products. Molecular sieve chromatography on Sephadex G-25 alone was not suitable for separating metabolites in these two cases because of the presence of large amounts of the accompanying material excreted by these two species of fishes.

Using this isolation procedure, we recovered in crystalline form from the Sephadex G-25 column about a few hundred milligrams of unchanged 2-amino-4-phenylthiazole (fraction No. 70 to 85), 6 mg of the major *N*-glucuronide metabolite (fraction No. 24 to 30) and 3 mg of a minor biotransformation product (fraction No. 91 to 110) after exposure of rainbow trout to 1.2 g of the anesthetic (Fig. 4). Following a similar procedure, we obtained from the environmental water of carp by Sephadex G-25 chromatography, about a few hundred milligrams of 2-amino-4-phenylthiazole

Table 1. Percent conversion of ^{3}H-2-amino-4-phenylthiazol to its *N*-glucuronide in fish and in the environmental water of rainbow trout and carp.

Species	Fraction	2-amino-4-phenylthiazole (peak A)*	*N*-glucuronyl conjugate (peak B)*	Conversion percentages (B)/(A)+(B)×100 (%)
rainbow trout	fish	230	20	8.0
carp	—	96	13.5	12
rainbow trout	water	108	36	25
carp	—	640	15	2.3

* Peak area (mm^2) in a given recording chart.

(fraction No. 70 to 85) and a few milligrams of the crystalline *N*-glucuronide conjugate (fraction No. 24 to 30). In the case of carp, the presence of a minor metabolite of the anesthetic was also indicated by a shoulder on the Sephadex G-25 elution curve at

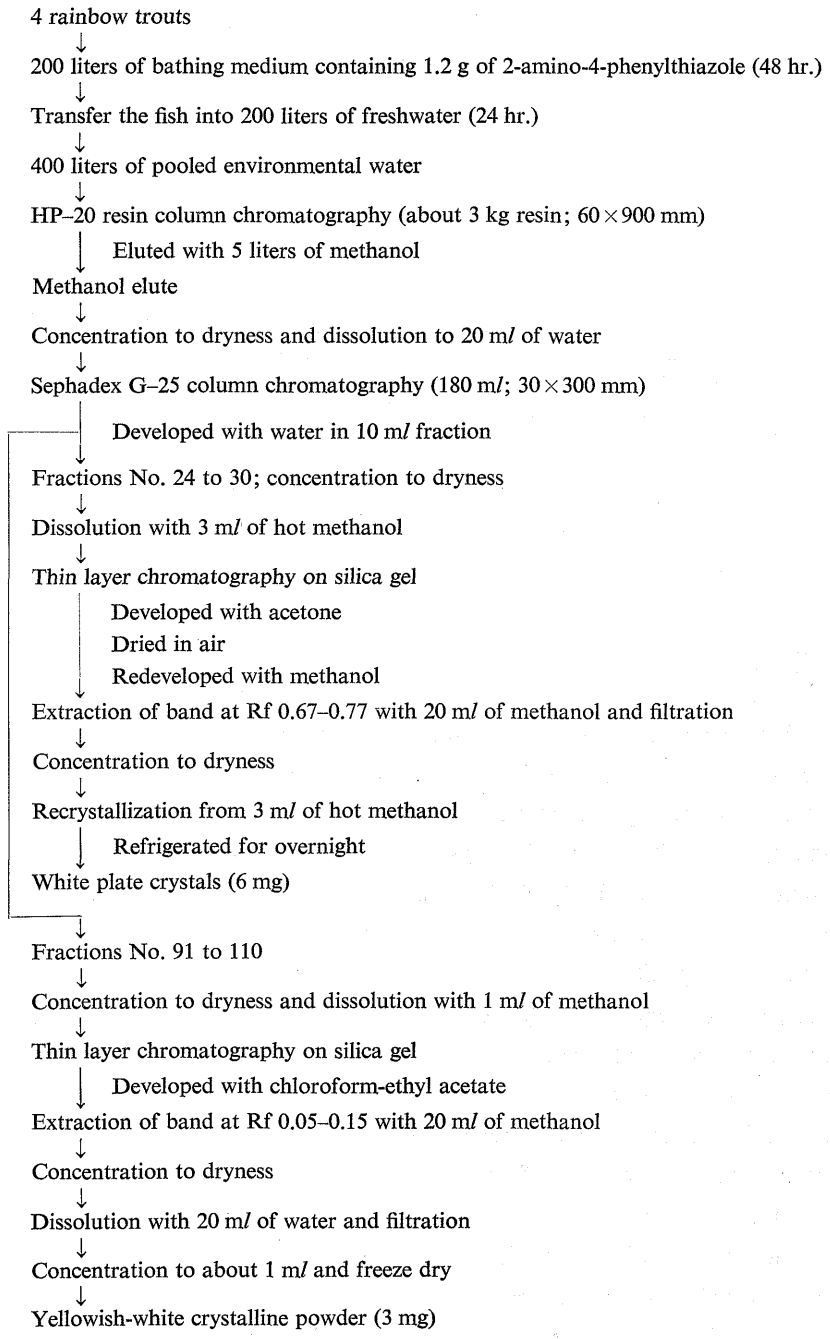


Fig. 3. Procedure for the isolation of the biotransformation products of 2-amino-4-phenylthiazole in rainbow trout.

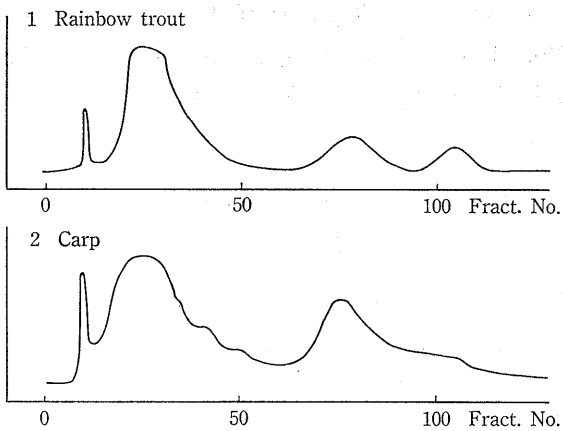
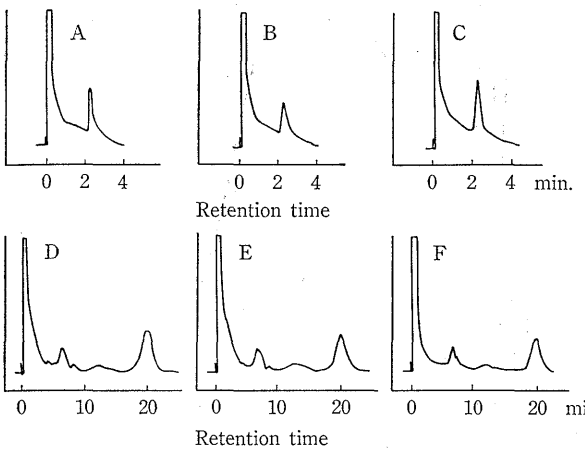


Fig. 4. Molecular sieve chromatography of the excreted anesthetic and its biotransformation products in rainbow trout and carp. The absorption of the elute was measured by a UV monitor, UVICONE. Aqueous concentrate (20 m*l*) of the methanol eluted from the HP–20 resin was applied to a Sephadex G–25 column, the column eluted with water, and 10 m*l* fractions collected.

fractions 91 to 110 (Fig. 4).

The *N*-glucuronyl conjugate was confirmed by the TLC analysis, naphthoresorcinol reaction for sugars and gas chromatography of the acid hydrolysate of the fractions. 2-Amino-4-phenylthiazole was identified by TLC analysis and gas chromatography. As shown in Fig. 4, the minor biotransformation product of the anesthetic in rainbow trout (fraction No. 91 to 110) gave one symmetrical peak. This peak was not found in the molecular sieve chromatography of medaka[1,2] and, moreover, the Rf value of these fractions under several conditions of TLC analysis was different from those of 2-amino-4-phenylthiazole and its *N*-glucuronyl conjugate.

Identification of *N*-glucuronyl conjugate: the major biotransformation product in rainbow trout and carp Sephadex G-25 (fraction No. 24 to 30) were pooled, concentrated under vacuum and crystallized with hot methanol to give white plates melting at 170 to 172°C under decomposition. The major biotransformation product of the anesthetic was optically active: $[\alpha]_D^{20}=-60°$ (c=0.87, water). The ultraviolet and infrared absorption spectra of this metabolite coincided with those of the *N*-glucuronyl conjugate derived from medaka described in the earlier report.[1] As shown in Fig. 5, the retention time of the aglycon moiety after acid hydrolysis of the biotransformed and synthetic *N*-glucuronyl conjugates coincided with that of authentic 2-amino-4-phenylthiazole. The retention time of the methanolized and trimethylsilylated hexuronic acid moieties from the two *N*-glucuronide samples also coincided with that of authentic D-glucuronic acid. The details for this procedure were previously reported.[1] Gas chromatography was conducted on a Hitachi 073 gas chromatograph with a FID detector. The aglycon moiety was separated at 180°C on a 3 mm×50 cm glass column containing Chromsorb WAW



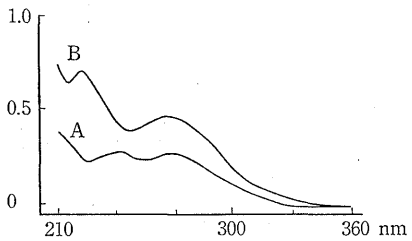


Fig. 5. Fig. 6.

Fig. 5. Gas chromatography of 2-amino-4-phenylthiazole and its derivatives. A. Acid hydrolysate of rainbow trout biotransformation product; B. Acid hydrolysate of carp biotransformation product; C. Acid hydrolysate of authentic *N*-glucuronyl conjugate; D. Methanolized and trimathylsilylated biotransformation product of rainbow trout; E. Methanolized and trimethylsilylated biotransformation product of carp; and F. Methanolized and trimethylsilylated synthetic *N*-glucuronyl conjugate.

Fig. 6. Ultraviolet absorption spectra of the minor biotransformation product of 2-amino-4-phenylthiazole in rainbow trout and of authentic 2-amino-4-phenylthiazole hydrobromide. A. Minor biotransformation product in rainbow trout, 10 μg/m*l* in water; and B. Authentic sample of 2-amino-4-phenylthiazole hydrobromide, 10 μg/m*l* in water.

DMCS 60/80 mesh and 5% DC–QF–1. The hexuronic acid moiety was separated at 170°C on a 3 mm×1 m stainless steel column containing Anachrom ABS 100/110 mesh and 5% apiezon grease L.

Resolution of chemical structure of the minor biotransformation product in rainbow trout

Sephadex G-25 fractions No. 91 to 110 were pooled and freeze dried to yield a yellowish-white crystalline powder. The ultraviolet absorption spectrum of this metabolite showed two absorption maxima at 241 nm ($E^{1\%}_{1cm}$=200) and at 268 nm ($E^{1\%}_{1cm}$=190) in water (Fig. 6). The spectrum was similar to that of 2-amino-4-phenylthiazole hydrobromide except for a shift to the longer wave length and only about one-half of the extinction coefficient of the reference compound. The infrared absorption spectrum of this minor biotransformation product in nujol showed absorptions at 1645 and 1305 cm^{-1} (Fig. 7). NMR spectrum of the minor metabolite of 2-amino-4-phenylthiazole was determined in CD_3OD by a Varian XL-100. This minor biotransformation product gave two doublets at τ= 2.28 and τ=3.20, with a coupling constant of J=9 Hz each, and a singlet at τ=7.78. Although the presence of this minor metabolite was indicated in carp by Sephadex G-25 molecular sieve chromatography (Fig. 4), the paucity of the preparation derived from carp did not allow us to perform further analyses for definitive identification. The mass

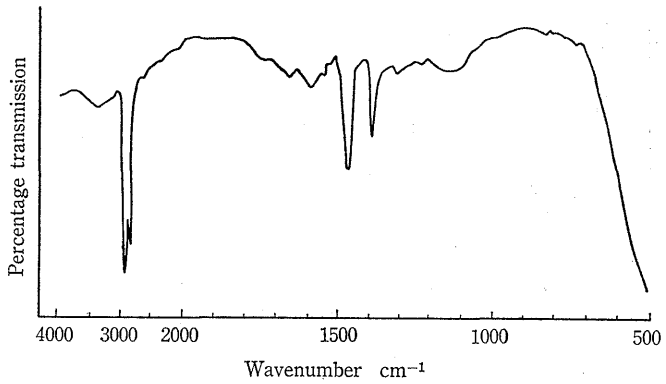


Fig. 7. Infrared absorption spectrum in nujol of the minor biotransformation product of 2-amino-4-phenylthiazole in rainbow trout.

Table 2. High resolution mass spectrum of the minor biotransformation product of 2-amino-4-phenylthiazole in rainbow trout and possible structural assignments.

Observed	Relative Intensity	Calculated	Error	Empirical Formula							
				C12	C13	H	N	O	X	S	P
234.0443	55	234.0469	−2.5	19	0	6	0	0	0	0	0
		234.0429	+1.4	14	0	6	2	2	0	0	0
		234.0462*	−1.9	11	0	10	2	2	0	1	0
192.0350	64	192.0323	+2.6	12	0	4	2	1	0	0	0
		192.0357*	−0.6	9	0	8	2	1	0	1	0
150.0115	60	150.0105	+0.9	11	0	2	0	1	0	0	0
		150.0139*	−2.4	8	0	6	0	1	0	1	0
120.0442	45	120.0449*	−0.6	7	0	6	1	1	0	0	0
93.0336	40	93.0340*	−0.4	6	0	5	0	1	0	0	0

* Most probable empirable formula. These structures are most compatible with other data including mass spectra for 2-amino-4-phenylthiazole and its 2-acetamide derivatives (not given in this paper).

spectrum of the unknown minor metabolite, as shown in Fig. 8, is very similar to that of 2-amino-4-phenylthiazole. The parent peak occurred at 234 m/e which is 58 m/e larger than that of 2-amino-4-phenylthiazole. The results of high resolution mass spectrometry on the unknown metabolite are summarized in Table 2. On a basis of the high resolution mass spectrometry and other results the minor biotransformation product could be identified as 2-acetamido-4-(4′-hydroxyphenyl)-thiazole, $C_{11}H_{10}N_2O_2S$.

Discussion

Based on radiochromatography, column chromatography and subsequent instrumental analyses, the major biotransformation product of the anesthetic 2-amino-4-phenylthiazole in rainbow trout and carp was shown to be 2-amino-4-phenylthiazole-2-*N*-β-mono-D-glucopyranosiduronic acid, the major metabolite found previously in medaka[1,2]. Furthermore, a minor biotransformation product of the anesthetic in rainbow trout was

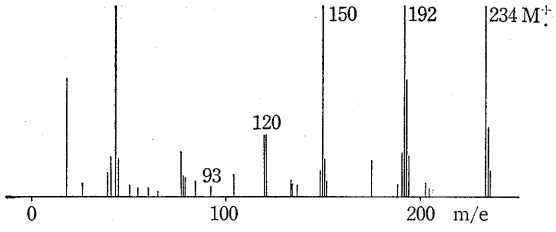


Fig. 8.

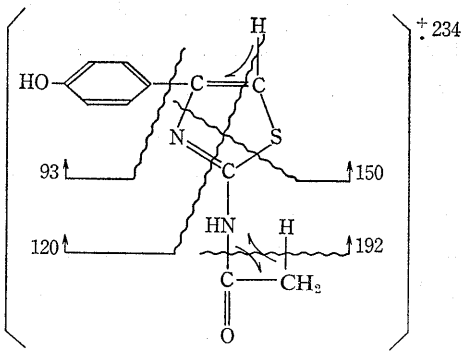


Fig. 9.

Fig. 8. Low resolution mass spectrum of the minor biotransformation product of 2-amino-4-phenylthiazole in rainbow trout.

Fig. 9. Fragmentation of the minor biotransformation product of 2-amino-4-phenylthiazole, $C_{11}H_{10}N_2O_2S$, in rainbow trout.

also isolated in crystalline form. Resolution of the chemical structure of this latter metabolite was done by ultraviolet, infrared, NMR and mass spectrometry. The ultraviolet absorption spectrum of this minor metabolite was quite similar to that of the parent compound 2-amino-4-phenylthiazole, suggesting the presence of benzene and thiazole rings. The infrared absorption at 1645 cm^{-1} was indicative of a carboxyamide group, and NMR signals at $\tau=2.28$ and $\tau=3.20$ in doublet and $\tau=7.78$ in singlet revealed, respectively, the presence of a substituent group at the 4′ position of the phenyl ring and a methyl group, as in an acetamide group. The results of low and high resolution mass spectral analysis, summarized in Table 2 and Fig. 8 and 9, indicated that the molecular weight of this minor metabolite was 234 with a molecular formula of $C_{11}H_{10}N_2O_2S$. Fragments at 192, 150, 120 or 93 coincided, respectively to a molecular ion of 2-acetamido-4-(4′-hydroxyphenyl)-thiazole, having the molecular formula of $C_9H_8N_2OS$; a molecular ion having the formula of C_8H_6OS; a molecular ion having the formura of C_7H_6NO; and a molecular ion having the formula of C_6H_5O. Thus, the chemical structure of the minor biotransformation product of this anesthetic in rainbow trout was identified as 2-acetamido-4-(4′-hydroxyphenyl)-thiazole. Chromatography of a water concentrate from carp on Sephadex G-25 showed the presence of a similar biotransformation product. It was thus plausible that the structure of the minor biotransformation product in carp was identical with that found in rainbow trout.

Isolation and resolution of the chemical structure of the minor biotransformation product of 2-amino-4-phenylthiazole in rainbow trout indicated the formation of 2-acetamido-4-(4′-hydroxyphenyl)-thiazole in a minute amount. Formation of this metabolite revealed the probable participation of mixed-function oxygenase and *N*-acetyltransferase in the liver of this fish. Earlier findings described by Buhler and Rasmus-

son[4,5] for the ubiquitous distribution of mixed-function oxygenase that participated in the oxidative biotransformation of chemicals in the liver of fish strongly supported the above view. Because of the low energy of the tritiated compound and the low conversion ratio from the anesthetic, however, this minor biotransformation product was not detected on TLC of water samples from fish treated with ^{3}H-labeled 2-amino-4-phenylthiazole (Fig. 2). An analogy of this conversion in mammals suggests that the *N*-acetylation of 2-amino-4-phenylthiazole in fish might be also affected by a similar *N*-acetyltransferase with coenzyme A[6].

Because of the difficulty in isolation of the major metabolite of 2-amino-4-phenylthiazole from rainbow trout and carp despite application of an established procedure previously used in medaka, it had been tentatively concluded in our previous work[2] that these latter two species of fishes seemed to possess other pathway of drug biotransformation or to not have the ability to form the *N*-glucuronyl conjugate with this anesthetic. The situation was clarified in the present study by use of radiochromatography to demonstrate that the conversion ratios of the anesthetic to its *N*-glucuronyl conjugate in rainbow trout and carp were only 8% in rainbow trout and about 12% in carp, as compared to the 89% found in medaka. Moreover, it was difficult to conduct molecular sieve chromatography in the presence of the massive amounts of accompanying substances that were excreted during the rearing of these two species of fishes in a limited amount of environmental water. It was evident, therefore, that the low rate of conversion of the anesthetic to a *N*-glucuronyl conjugate and the excretion of the interfering substance made the earlier isolation procedure ineffective. Introduction of a step involving HP-20 resin chromatography in the isolation sequence overcame the above difficulty.

From the experimental evidence described and discussed above, it seems to be quite plausible that the chemical nature of biotransformation products formed by fish might be decided by the chemical properties of the drug or chemical rather than by the phylogenic characteristics of the fish. Furthermore, from the experience in this laboratory, it also appears likely that medaka are relatively resistant to anesthetization by 2-amino-4-phenylthiazole because they convert the anesthetic to the *N*-glucuronyl conjugate at a high rate. Other freshwater fishes, in turn including rainbow trout and carp are relatively sensitive to the anesthetic since they form the *N*-glucuronyl conjugate at a lower rate. Moreover, almost all marine fishes are susceptible to anesthetization suggesting that they exhibit a low conversion ratio of anesthetic to its *N*-glucuronide metabolite. It will be interesting to conduct a survey of the biotransformation product of 2-amino-4-phenylthiazole in marine fishes including yellowtail (hamachi), porgy (madai), flounder (hirame), plaice (karei) and common horse mackerel (maaji) in order to confirm this intriguing hypothesis.

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