Residue Analyses on 2-Amino-4-phenylthiazole, a Piscine Anesthetic, in Fishes-IV. GC/MS Analysis on Rainbow Trout

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When a piscine anesthetic, 2-amino-4-phenylthiazole, was absorbed by rainbow trout, *Salmo gairdneri irideus*, from an anesthetic solution, a whole body concentration of 6.7 and 13 ppm was attained at 3 hours and 6 hours, respectively. After anesthetization for 6 hours, fish were transferred into 500 l of fresh water flowing at a rate of 2 l per minute. The anesthetic previously absorbed by the fish was excreted into the environmental water by this process. Residues of the anesthetic in the fish were measured by gas chromatography-mass spectrometry (GC/MS). The total concentration of anesthetic in the fish declined at a rate comparable to that predicted by a computed theoretical biexponential curve obtained by a previously described mathematical procedure. The biological half-life of the anesthetic under these experimental conditions was approximately 40 minutes in the primary step and 22 hours in the secondary step. The whole body residues which remained at 24 and 48 hours were 0.1 ppm and 0.05 ppm, respectively.

A previous pharmacokinetic study on ³H-2-amino-4-phenylthiazole, a piscine anesthetic, in medaka (killifish), *Oryzias latipes*, revealed that both the anesthetic and its *N*-glucuronyl conjugate may be excreted mainly through the gills.^{1,2)} Excretion of the anesthetic followed a biexponential curve and that total recovery of radioactivity in environmental water was 102% of the anesthetic initially absorbed by the fish. While 89% of the total 2-amino-4-phenylthiazole absorbed was present in the medaka body as the *N*-glucuronyl conjugate at the start of the excretion experiment, residues of that conjugate in carp or rainbow trout were only 12 or 8%, respectively.^{1,3)} As the use of radioactive tracers in open field experiment is prohibited in this country, there has been considerable effort to develop a nonradioactive analytical method of a high sensitivity and accuracy that could be used to obtain pharmacokinetic data in large scale test. The usual analytical methods such as spectrophotometry, fluorometry, high pressure liquid chromatography or FID gas chromatography were not suitable to obtain a sufficient sensitivity and accuracy, because of high concentrations of fat and accompanying substances in the fish which interfered with the quantitative microanalysis of this anesthetic. Recently, the technique of gas chromatography coupled with mass spectrometry (GC/MS) has become available and we have

applied this new analytical method to the analysis of residues of 2-amino-4-phenylthiazole. In this paper, we report the use of GC/MS to measure the elimination with respect to time of this anesthetic from rainbow trout.

Materials and Methods

Biological Procedure

One year-old rainbow trout (99.5±17.9 g, 17.3±1.0 cm) were used in this study. Tap water for these experiments was vigorously aerated for 72 hours prior to transferring of fish. Thirty-six rainbow trout were maintained in 500 l water for 7 days at 14°C for acclimatization and were used for the experiment after 2 days starvation. Fish were anesthetized by exposing them for 6 hours at 14°C to 500 l of aerated solution containing 6 g of 2-amino-4-phenylthiazole hydrobromide monohydrate. Under these conditions, rainbow trout were well sedated at a grade of I-2 according to modified MCFARLAND's criterion for anesthetization.⁴⁾ After a given exposure time, the fish were transferred into 500 l of fresh water flowing at a rate of 2 l per minute. Groups of three rainbow trout were removed from the acclimation water for an analytical sample just before the anesthetization, at 3 and 6 hours during the anesthetization, and at 0.25, 0.5, 1, 2, 4, 6, 18, 24

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and 48 hours after initiation of the recovery process. Each group of fish was immediately frozen at -20°C .

Chemical Procedure

The frozen samples of whole fish bodies were cut in cubic pieces of about 1 cm^3 and homogenized by Waring blender in the cold after addition of 1/10 part (v/w) of 0.05% cold sodium bicarbonate aqueous solution and 1/10 part (v/w) of 10 ppm 2-amino-5-methyl-4-phenylthiazole aqueous ethanolic solution as an internal standard. The partition coefficient of 2-amino-5-methyl-4-phenylthiazole in dichloromethane/water system or in acetonitrile/petroleum ether (1:1) system was close to that of 2-amino-4-phenylthiazole. Thus, the anesthetic and its internal standard behaved concomitantly along the extraction and clean-up process. Further, the difference of mass number coincided with one methyl group between anesthetic and its internal standard made GC/MS separation clear and convenient, and the relative peak areas against its internal standard were found to be quantitative as described later. Fifty grams of the homogenate

was placed in a Soxhlet's thimble, freeze-dried and the dried material was then extracted with dichloromethane for 24 hours. The extract was concentrated under vacuum to dryness, partitioned 2 times in acetonitrile and petroleum ether (1:1), and the acetonitrile layer separated and concentrated under vacuum to dryness. The resulting extract was dissolved in ethanol and then subjected to GC/MS analysis.

Analytical Procedure

A Du Pont Dimaspec Model 321 GC/MS instrument and 320 GC/MS data system were used for the analyses. Samples were injected into a gas chromatograph equipped with a glass column ($1.5\text{ m} \times 1.5\text{ I.D.}$) packed with 1% OV-1 on Gaschrom Q (80-100 mesh). Operational conditions for GC/MS included: column temperature (190°C); injection port temperature (220°C); ion source temperature (180°C); jet separator temperature (250°C); carrier gas (helium, 30 ml/min). Isobutane was used as reactant gas for chemical ionization. BSTFA* was used as silylating reagent for column conditioning. The anesthetic, 2-amino-

Fig. 1. The relationship between the concentrations of 2-amino-4-phenylthiazole and the internal standard, 2-amino-5-methyl-4-phenylthiazole, and the relative peak areas following GC/MS analysis.

* Abbreviation. BSTFA = *N,O*-Bis(trimethylsilyl)-trifluoroacetamide.

4-phenylthiazole and an internal standard, 2-amino-5-methyl-4-phenylthiazole, were monitored at *m/e* 177 (*M*+1) and 191 (*M*+1), respectively. The relationship between the relative concentrations of 2-amino-4-phenylthiazole and the internal standard, 2-amino-5-methyl-4-phenylthiazole, and the relative GC/MS peak area ratios under the defined experimental conditions is shown in Fig. 1. A linear relationship between the relative GC/MS peak area ratios and concentration ratios was given. As the amount of 2-amino-5-methyl-4-phenylthiazole added as an internal standard was known, the quantitation was done by the GC/MS peak area ratio found in a given experiment. The amount of anesthetic detectable by this analytical procedure was 12.5 pg per 1 μ l injection sample.

Results

The time course of elimination of 2-amino-4-phenylthiazole from rainbow trout is summarized in Table 1 and plotted in Fig. 2 along with a computed theoretical curve obtained by the mathematical procedure previously given.¹¹ Each data point is the average of triplicate analyses.

During anesthetization, the concentration of 2-

amino-4-phenylthiazole in rainbow trout attained 6.7 ppm at 3 hours and 13 ppm after 6 hours treatment. When the recovery process was started by transferring the fish into flowing fresh water, excretion of the anesthetic proceeded via a biexponential curve as shown in Fig. 2. Excretion was initially very rapid with a biological half-life of approximately 40 minutes. The second elimination proceeds was considerably slower with a half-life of about 22 hours. The residue of 2-amino-4-phenylthiazole remaining after 24 or 48 hours in the recovery process was 0.1 or 0.05 ppm, respectively.

Discussion

The precise residual data related to commonly used piscine anesthetics were rare until relatively recent time. WALKER and SCHOETTGER⁶¹ first reported the residue of MS-222 (ethyl *m*-amino-benzoate methanesulfonate) in four salmonids following anesthesia. HUNN⁶¹ reported further the dynamics of MS-222 in the blood and brain of fresh water fishes during anesthesia. ALLEN, LUHNING and HARMAN⁷¹ reported the residue of MS-222 in northern pike, muskellunge and walleye. LUHNING reported the residues of MS-222, benzocaine and their metabolites in striped bass following anesthesia.⁸¹ And HUNN and ALLEN⁹¹ further reported the residue dynamics of quinaldine (2-methylquinoline) in rainbow trout.

As for a new piscine anesthetic, 2-amino-4-phenylthiazole, the presence of plenty of fat materials and interfering substances in fish body did not allow to apply the usual analytical methods mentioned and cited in this paper, because of insufficient sensitivity and accuracy which could not fulfill the requirement. As the governmental requirement as for data on residual amounts of drug and its metabolites left in a food commodity after treatment, an instrumental analytical procedure on this piscine anesthetic with sufficient sensitivity and accuracy other than radioactive assay method was established applying GC/MS.

It was confirmed by the time course survey in this study that the anesthetic 2-amino-4-phenylthiazole was excreted from rainbow trout also by a biexponential process. The biological half-life in the primary step was approximately 40 minutes and that in the secondary step was about 22 hours in a given experimental condition described in this paper. This type of excretion process in rainbow trout again indicates the presence of at least 2

Table 1. The relative GC/MS peak area ratios of 2-amino-4-phenylthiazole and the internal standard, 2-amino-5-methyl-4-phenylthiazole

Withdrawal time (h)	Relative GC/MS peak area ratio	Concentration of 2-amino-4-phenylthiazole in rainbow trout (ppm)	
		Experimental	Calculated [A]* ¹
[t]* ²			
-6	N.D.	0	—
-3	6.09	6.7	—
0	10.4	13.0	6.8
0.25	4.90	5.2	5.5
0.5	2.96	2.8	4.6
1	2.45	2.2	3.2
2	2.35	2.1	1.6
4	0.35	0.20	0.47
6	0.34	0.19	0.24
8	—	—	0.19
12	—	—	0.15
18	0.26	0.14	0.12
24	0.12	0.090	0.098
36	—	—	0.064
48	0.105	0.046	0.042

*¹ [A] is computed concentration of 2-amino-4-phenylthiazole in rainbow trout at a given withdrawal time.

*² [t] is each withdrawal time. The mathematical relationship between [A] and [t] was given by the formula shown in the inset of Fig. 2.

Fig. 2. Time course survey on the residue of the anesthetic, 2-amino-4-phenylthiazole, in rainbow trout. Experimental values (●) are the mean of the three determinations. Simulated curve: (—).

* Where, A is a concentration of the anesthetic, t is the withdrawal time in recovery process, e is the base of natural logarithm and P, Q, α and β are constants obtained by the least square mean method. See details in reference 1.

pharmacokinetic compartments in fish body. The above line of experimental evidence consistent with a previous small scale laboratory data using medaka and ^3H -2-amino-4-phenylthiazole¹¹. Comparing with the higher level of that conjugate in medaka, which showed a similar excretion kinetics to the anesthetic itself¹¹, the residue of that conjugate in rainbow trout is merely 8% of the total anesthetic absorbed into the fish body at the start of the excretion experiment.³¹ That lower level of the conjugate in rainbow trout was allowed to neglect in this study. The route of excretion of this anesthetic and its conjugate in rainbow trout was not surveyed in this study. It was assumed from the previous radiotracer experiment on carp¹⁰⁾ and Dr. HUNN's review related to the movement of

drugs across the gills of fish¹¹⁾ that the main excretion route of this anesthetic itself in rainbow trout might be also via gills.

An usefulness of the plot as in Fig. 2 is that the period of excretion treatment as for the anesthetic in a given conditions, which is required for fishes of food commodity, could be determined, when the tolerance level in food commodity in mammal is decided by the results from chronic toxicological tests and safety coefficient.

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