モリネートによるコイの貧血症に対するメナジオン亜硫酸水素ジメチルピリミジノールの予防効果

誌名	日本水産學會誌
ISSN	00215392
著者	川津, 浩嗣 池田, 輝和
巻/号	54巻10号
掲載ページ	p. 1731-1736
発行年月	1988年10月

Nippon Suisan Gakkaishi 54(10), 1731–1736 (1988)

Anti-anemic Effect of Menadione Dimethylpyrimidinol Bisulfite against Molinate-induced Anemia in Common Carp*1

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(Received April 27, 1988)

Anti-anemic effect of menadione dimethylpyrimidinol bisulfite (MPB) against molinate-induced anemia was investigated in a bioassay test. Common carp fingerlings were arranged in 12 groups of 20 animals each. Five groups of fish were exposed to molinate at a concentration of 0.10 ppm, 6 groups of fish at 0.32 ppm, and one group of fish served as controls. Concentrations of MPB were set at 0.0, 0.4, 1.2, 3.6 and 10.8 ppb for the test solutions with 0.10 ppm of molinate, and at 0.0, 0.4, 1.2, 3.6, 10.8 and 32.4 ppb for those with 0.32 ppm of molinate, respectively. After 21-day exposure to molinate solutions with different concentrations of MPB, hemoglobin level was determined on all surviving fish. The mortality and hemoglobin level data indicated that MPB was highly effective in preventing anemia. The effective concentrations of MPB were estimated to to be 3.6 ppb in water containing 0.10 ppm of molinate and 32.4 ppb in water containing 0.32 ppm of molinate, respectively.

The herbicide Molinate induces anemia in common carp, due to excessive blood loss from extensive lesions of the gill capillaries.[1] It has been reported that oral administration of menadione is highly effective in preventing this anemia.[2] If the water-soluble types of menadione derivative in the ambient water penetrate through the gills into the body, effective precautionary measures against molinate-induced anemia will be achieved by exposing fish to these compunds. From the viewpoint of direct uptake of menadione from the water, we conducted bioassay tests to prevent anemia using menadione dimethylpyrimidinol bisulfite (MPB).

The present paper describes the anti-anemic effect of MPB and its effective concentrations in water to prevent anemia.

Materials and Methods

Bioassay Test

Common carp fingerlings *Cyprinus carpio*, weighing an average of 15.9 g, were arranged in 12 groups of 20 animals each. They were kept in glass tanks (45×30×30 cm) containing 30 *l* of test solution. Twenty liters of test solution in each tank was renewed every day. Five groups of fish were exposed to molinate at a concentration of 0.10 ppm, 6 groups of fish at 0.32 ppm, and one group of fish served as controls were exposed to 50 ppm acetone alone (Table 1). Concentrations of MPB were set at 0.0, 0.4, 1.2, 3.6 and 10.8 ppb in the test solutions with 0.10 ppm of molinate, and at 0.0, 0.4, 1.2, 3.6, 10.8 and 32.4 ppb in those with 0.32 ppm of molinate. For preparing stock solutions, molinate was dissolved in acetone at concentrations of 640 and

Table 1. Composition of test solution

Lot No.	Composition of test solution		
	Molinate (ppm)	MPB (ppb)	Acetone (ppm)
1	0.10	—	50
2	0.10	0.4	50
3	0.10	1.2	50
4	0.10	3.6	50
5	0.10	10.8	50
6	—	—	50
7	0.32	—	50
8	0.32	0.4	50
9	0.32	1.2	50
10	0.32	3.6	50
11	0.32	10.8	50
12	0.32	32.4	50

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200 mg/100 m*l*, and MPB was dissolved in distilled water at a concentration of 40 mg/*l*. For preparing test solutions, 0.5 m*l* of stock solution of molinate was diluted with 10 *l* of water, and different volumes of MPB stock solution were added to make solutions of different concentrations of MPB. Water temperature was maintained at 25±1°C. Fish were daily fed on a commerial pellet food. During the experiment, test solutions in all tanks were examined every day for the occurrence of bleeding from the gills using a commercially available occult blood test paper,* "Hemastix-III'" (Miles-Sankyo Co., Ltd., Tokyo). After 21-day exposure, blood samples were taken from all surviving fish for determination of the hemoglobin level. Hemoglobin level was measured by the cyanmethemoglobin method.[3]

Method of Analyzing Molinate in Water

Molinate in water was analyzed by Gas Liquid Chromatography (GLC)[4] according to the following procedures.

Reagents used were of analytical grade (or equiv.). Dichloromethane and acetone were distilled before use. Molinate standard was obtained by distillation of technical molinate (Stauffer Chemical Co., CAL.). The purity of this standard was 99.8%.

GLC was performed with a system incorporating a Shimadzu Model GC-5A equipped with a flame photometric detector and a Shimadzu Model R-12 recorder. Operating conditions were as follows. Column: glass-column (3 mm I.D.× 1 m) packed with 10% DC-200/Gaschrom Q 60–80 mesh. Temperatures (°C): column 180, injection 200, detector 200. Gas flows (m*l*/min): N_2 carrier gas 100, air 40, H_2 40. Sensitivity: attenuation 10×(32–128). Flame photometric detector 394 nm.

Molinate standard was dissolved in n-hexane at concentrations of 0.2, 1.0, and 5.0 μg/m*l*. Five microliters of these solutions were injected respectively. Peak height of molinate was determined and the calibration curve was plotted on logarithmic graph paper.

To a 500 m*l* separating funnel was transferred 250 m*l* of water which was then acidfied with 5 drops of hydrochloric acid. After adding 20 m*l* of diclorometha ne, the funnel was shaken for 5 min. The dichloromethane layer was drained into a 100 m*l* Erlenmeyer flask. The extracion was repeated with 20 m*l* of dichloromethane.

The extract was dried with sodium sulfate anhydrous as follows: ca. 3 g of sodium sulfate anhydrous was added to the extract and shaken for 1 min. The mixture was settled, then dried extract was decanted into an eggplant type flask. Precipitate was washed 4 or 5 times with ca. 5 m*l* of dichloromethane by decanting. Washings were added to the dried extract.

An aliquot of the dried extract was concentrated to ca. 1 m*l* with a vacuum rotary evaporator at 40°C. Then the concentrate was evaporated to dryness under nitrogen gas at room temperature. The residue was dissolved in a known volume of acetone.

Five microliters of the prepared solution was injected. The concentration of molinate was calculated from the peak height found from the calibration curve.

Method of analyzing MPB in Water

MPB in water was analyzed by High Performance Liquid Chromatography (HPLC) according to Abe *et al.*[5] with several modifications.

Reagents used were of analytical grade. Menadione and sodium borohydride were obtained from Wako Pure Chemicals (Osaka). The derivatization reagent was prepared by dissolving sodium borohydride in absolute ethanol at a concentration of 0.2 g/*l* and degassing by ultravibration for 5 min. HPLC mobile phase methanol and ethanol were flushed with a stream of nitrogen gas for 20 min (50 m*l*/min) before use, respectively.

HPLC was performed with a system incorporating a Shimadzu Model LC-5A constant-flow pump, a Rheodyne Type 7125 HPLC injection valve equipped with a 20 μ*l* sample loop and a Shimadzu Model RF-530 fluorescence spectrophotometer. Operating conditions were as follows. Column: Zorbax ODS (250×4.6 mm I.D.). Mobile phase:methanol-1% acetic acid solution (9: 1). Flow rate: 1.5 m*l*/min. Injection volume: 20 μ*l*. Integrator: Shimadzu Model C-R2AX, Fluorescence spectrophotometer: ex. 320 nm, em. 430 nm.

Menadione standard solutions were prepared by dissolving in methanol at concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 μg/m*l*. One milliliter of the standard solution and 1 m*l* of the derivatization reagent were mixed, and 20 μ*l* of this mixture was injected. Corrected areas of menadione were determined in each standard solution from

* Occult blood: concealed hemorrhage.

a note integrator reading and plotted calibration curve.

Two hundred milliliters aliquot of water was transferred to a 500 m*l* brown separating funnel. After adding 40 m*l* of chloroform and 15 m*l* of 12.5% (W/V) sodium carbonate solution, the funnel was shaken for 5 min.

The chloroform layer was drained into a brown eggplant type flask through phase separator filter paper ("1PS" by Whatman Ltd., Maidstone, England) and saved. The extraction was repeated with 40 m*l* of chloroform. The lower layer was added to the above-saved extract.

An aliquot of the chloroform layer was concentrated to 1–2 m*l* with a rotary vacuum evaporator at 40°C. Then the concentrate was evaporated to dryness under nitrogen gas at room temperature. The residue was dissolved in 2 m*l* of methanol by ultravibration for 1 min.

One milliliter of this solution and 1 m*l* of the derivatization reagent were mixed, and 20 μl of the mixture was injected. The corrected area of menadione was determined from a note integrator reading, and mendione μg/m*l* was obtained from standard curve. The concentration of menadione dimethylpyrimidinol bisulfite in water was calculated from menadione μg/m*l*.

Results

Mortality

Remarkable differences in mortality were observed among the groups of fish exposed to molinate at a concentration of 0.32 ppm, *i.e.*,

Table 2. Cumulative mortality of fish exposed to different concentrations of molinate and MPB

Lot No.	Test solution		Cumulative mortality during 21-day exposure																	Mortality (%)	Number of test fish
	Mol. (ppm)	MPB (ppb)	1–5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		
1	0.10	—																		0.0	20
2	0.10	0.4						1	1	1	1	1	1	1	1	1	1	1	1	5.0	20
3	0.10	1.2															1	1	1	5.0	20
4	0.10	3.6																		0.0	20
5	0.10	10.8																		0.0	20
6	—	—																		0.0	20
7	0.32	—			2	2	3	4	4	5	5	6	6	6	6	6	8	8	8	40.0	20
8	0.32	0.4								1	1	1	2	3	3	3	4	4	4	20.0	20
9	0.32	1.2									1	1	1	1	1	1	1	1	2	10.0	20
10	0.32	3.6																		0.0	20
11	0.32	10.8																		0.0	20
12	0.32	32.4																		0.0	20

Table 3. Occult blood test for detecting bleeding into ambient water. Test was performed every day just before renewing test solution

Lot No.	Test solution		Reaction of occult blood test during 21-day exposure*																			
	Mol. (ppm)	MPB (ppb)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	0.10	—	−	−	−	−	−	−	±	+	++	++	++	+	±	±	−	+	±	±	±	−	+
2	0.10	0.4	−	−	−	−	−	±	+	+	++	++	+	+	±	+	±	±	±	±	−	−	−
3	0.10	1.2	−	−	−	−	−	−	±	+	+	+	+	+	+	+	±	+	±	±	−	−	−
4	0.10	3.6	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−	−
5	0.10	10.8	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
6	—	—	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
7	0.32	—	−	−	−	−	+	++	++	++	++	++	++	++	++	++	+	±	±	±	±	−	±
8	0.32	0.4	−	−	−	−	±	±	±	±	++	++	++	++	++	++	++	+	+	±	±	±	−
9	0.32	1.2	−	−	−	−	−	−	−	+	+	+	+	++	++	++	++	+	+	+	±	±	−
10	0.32	3.6	−	−	−	−	±	±	+	++	++	++	++	+	+	+	±	−	±	±	±	−	−
11	0.32	10.8	−	−	−	−	−	±	±	±	±	+	+	±	±	±	±	±	±	±	±	±	−
12	0.32	32.4	−	−	−	−	−	−	−	±	−	±	±	±	−	−	−	−	±	−	−	−	−

* −: negative; ±: slightly positive; +: moderately positive; ++: strongly positive.

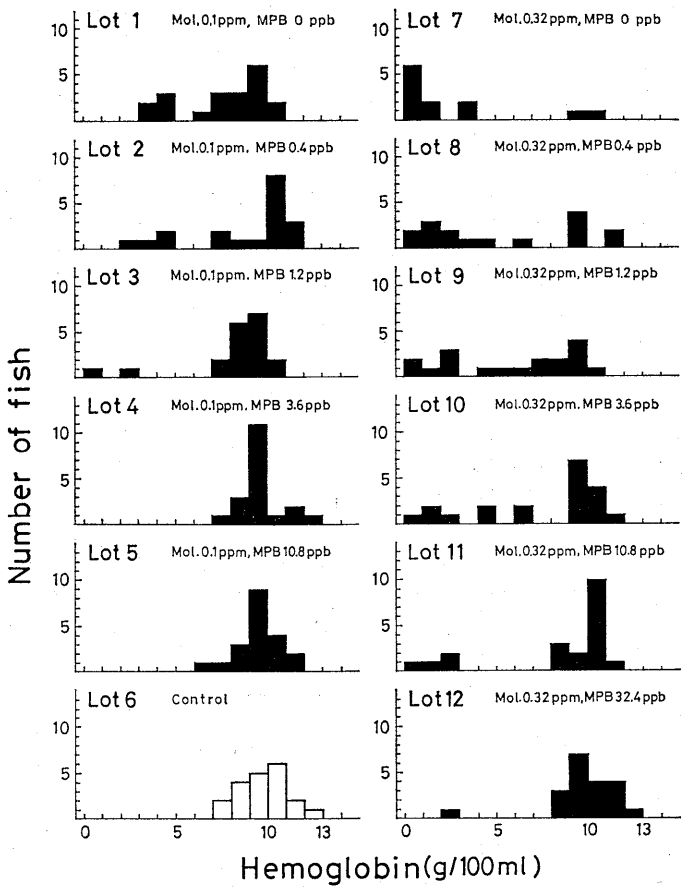

Fig. 1. Frequency distributions of hemoglobin level in fish after 21-day exposure to different concentrations of molinate and MPB.

mortality reached 40% in the test solution without addition of MPB, 20% with 0.4 ppb and 10% with 1.2 ppb of MPB, respectively (Table 2). No mortality was recorded at more than 3.6 ppb of MPB. Mortality was very small in the groups of fish exposed to 0.10 ppm molinate.

Bleeding

In the test solutions with 0.10 ppm of molinate, a positive reaction in the occult blood test was recorded at 0 to 1.2 ppb of MPB and a negative one at more than 3.6 ppb (Table 3). In test solutions with 0.32 ppm of molinate, a positive reaction was recorded at 0 to 10.8 ppb of MPB and a negative one at 32.4 ppb.

Hemoglobin Levels

Frequency distributions of hemoglobin levels determined for all survived fish after 21-day exposure are shown in Fig. 1. In the control group, the hemoglobin level ranged from 7.0 to 13.0 g/100 m*l*. In the test solutions with 0.10 ppm of molinate, anemic fish were observed at 0 to 1.2 ppb of MPB and no anemic fish at more than 3.6 ppb. On the other hand, anemic fish were observed in all groups exposed to molinate at a concentration of 0.32 ppm, whereas the number of anemic fish decreased with increasing of MPB concentration. Only one individual revealed a low hemoglibin level at a concentration of 32.4 ppb of MPB.

Changes of Molinate and MPB Concentrations in the Test Solutions

In order to confirm the actual exposing condition, molinate and MPB concentration in the test solutions were monitored on the 16th day. Each 500 m*l* of test solution was collected from all tanks 0, 1, 2, 6 and 24 h after renewing the test solution.

In the test soltuions being set up at 0.10 ppm of molinate, actual molinate concentrations

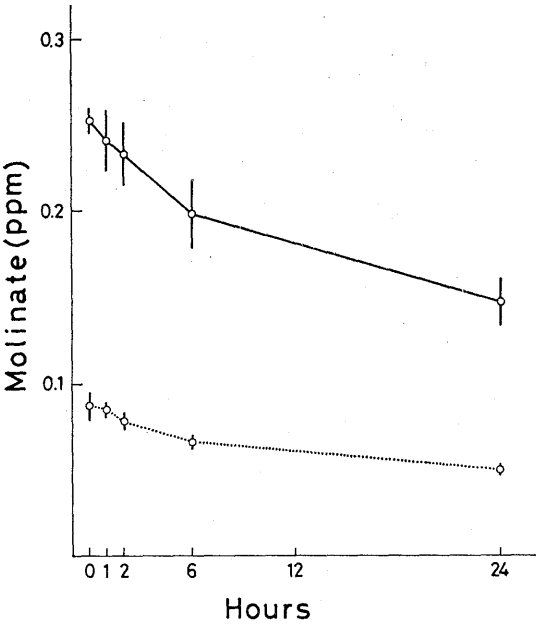

Fig. 2. Changes of molinate concentration in test solutions after renewal. ○------○: Lots 1 to 5; ○——○: Lots 7 to 12. Open circles with vertical lines show mean±standard deviation.

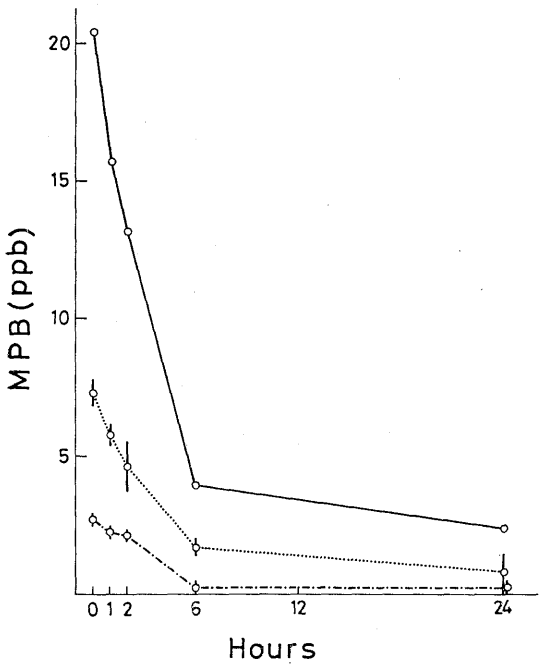

Fig. 3. Changes of MPB concentration in the test solutions after renewal. ○——○: Lot 12; ○------○: Lots 5 and 11; ○-·-·-○: Lots 4 and 10. Open circles with vertical lines show mean ±standard deviation.

(mean±SD) were 0.087±0.008 ppm just after renewing the test solution and 0.050±0.001 ppm after 24 h (Fig. 2). In the test solutions being set up at 0.32 ppm of molinate, actual molinate concentrations (mean±SD) were 0.253±0.008 ppm and 0.147±0.014 ppm just after renewing the test solution and after 24 h, respectively. Concentration of molinate in the test solution was reduced to 60% of its initial level during 24 h regardless of its initial level. Divergence between the directed and actual concentration of molinate in the test solution just after being renewed can be explained by mixing 20 *l* of newly prepared test solution and 10 *l* of the residual test soultion in the tank in which molinate concentration was reduced.

Actual concentrations of MPB in the test solution just after nenewal were 63–76% of the directed levels (Fig. 3). Divergence between the directed and actual concentration of MPB in the tests solution just after renewal can also be explained by the same reason described above. Concentration of MPB decreased rapidly during the first 6 h and slowly thereafter, *i.e.*, it was reduced to 8–23% of its initial level during 6 h in the test solutions at 3.6, 10.8 and 32.4 ppb. In the test solutions at 0.4 and 1.2 ppb of MPB, it was impossible to trace MPB concentration changes, because actual concentrations were near or below the detection limit.

Discussion

The data on mortality, occult blood test and hemoglobin determination indicate that MPB is highly effective in preventing molinate-induced anemia. Effective concentrations of MPB to prevent anemia were estimated to be 2.7 ppb in water containing 0.09–0.05 ppm of molinate and 20.5 ppb in water containing 0.25–0.15 ppm of molinate, respectively. The above figures are initial concentrations of MPB in the test solution just after renewal, because concentrations of both chemicals changed hourly and the reduction of MPB concentration was faster than that of molinate. If concentrations of chemicals could be held constant, the effective concentration of MPB would be lower than that obtained in this study. The effective concentration of MPB depends on the molinate level in the water.

These results sugggest that common carp is capable of uptaking MPB from the ambient water. The mechanism of uptake of this compound

across the body surface and its metabolism in the body remain for future study.

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