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Some laboratory investigations on Yurimin P-99 as an experimental molluscicide

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Yurimin P-99 (3, 5-dibromo-4-hydroxy-4'-nitroazobenzene), which gives promise of proving effective against *Limnodrilus spp.* (Annelida: Oligochaeta), has been developed in Japan. One might anticipate that this compound would be useful against schistosome-bearing snails, since it has been shown to exert a toxic effect on *Fruticicola* (*Acusta*) *despecta sieboldiana* (Gastropoda: Bradybaenidae). Iijima *et al.* (1964) conducted a field study of the use of Yurimin P-99 (5% w/w granule form) in an endemic area of Yamanashi prefecture and concluded that in the field use of this compound, a dose of 5g/m^2 would be amply sufficient to obtain the same molluscicidal effect as a dose of 5g/m^2 sodium pentachlorophenate (NaPCP). In our immersion method with a 48-hour exposure period at a constant temperature of 25°C, the LC_{50} and its 95% confidence limits showed 5.4 (4.3-6.9) ppm in Yurimin P-99, while those of NaPCP indicated 0.51 (0.26-0.62) ppm. Such a marked discrepancy between the results of laboratory and field tests with Yurimin P-99 stimulated the authors to examine the molluscicidal property of this compound in the laboratory.

Materials and Methods

Oncomelania nosophora snails were obtained from field collections in Yamanashi prefecture. Their viability was assured by selecting only those snails which crawled out of water-filled Petri dishes.

The purified base and 5% w/w granular form of Yurimin P-99 were received through the courtesy of Dr. M. Konishi, Non-Medical Product Section, Chugai Pharmaceutical Company Limited, Tokyo. The granular form was used in the form of suspensions in distilled water as it is hardly soluble in water.

To bring the snails in contact with the compound, our immersion method (Komiya *et al.*, 1962) was adopted. After a certain hour's exposure to the chemicals in an incubator at 25°C, the snails were rinsed with running tap water and transfered to aerated tap water. Then deaths were recorded after a further 48 hours. The snails were judged as being dead when they showed no movement after their transfer into water. Those which showed no movement were crushed afterwards to confirm their death. For the determination of the LC_{50} either the method of Reed and Muench (1938) or Litchfield and Wilcoxon (1949) was adopted.

Results

1. Efficacy of Yurimin P-99 against *Oncomelania* snails in the immersion method

In order to know the relation between the efficacy of Yurimin P-99 and various periods of exposure, the mortality in the immersion method was examined at intervals of 6, 12, 24 and 48 hours after placing the snails into the chemicals. The LC_{50} and respective 95% confidence limits were calculated (Table 1). In tests with 6- and 12- hour exposures it was impossible to compute the LC_{50} because the applied concentration of the compound was a little too low. This information indicates that Yu-

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rimin P-99 does not kill rapidly as does a benzimidazole derivative, NC 3447 (Komiya *et al.*, 1965).

Table 1 LC_{50} and respective 95 percent confidence limits for various periods of exposure in the immersion method

Periods of exposure (hours)	LC_{50}(95% confidence limits)
6	not computable
12	not computable
24	6.3(4.3-9.1)ppm
48	5.2(4.2-6.5)ppm

As it was anticipated that Yurimin P-99 might act on the snails as a stomach poison, the immersion method was slightly modified; the bottom of the containers was covered with a piece of filter-paper possibly participating in the eating activity of snails. Controls without filter-paper were run simultaneously. From the mortality data after 48-hour exposure the LC_{50} were estimated as follows: 4.2 ppm with filter-paper and 3.9 ppm without filter-paper. No marked difference was thus found to exist between the dishes with and without filter-paper.

2. Influence of hard water upon the efficacy of Yurimin P-99

Experiments were performed to know whether Yurimin P-99 is rendered less effective by the hardness of water. The hard water used in this test, according to the WHO scheme, was composed of 600 ppm calcium chloride and 139 ppm magnesium chloride. Parallel tests with distilled water were run. The results of tests with a 48-hour exposure period show that the hardness of water plays but a small part in the reduction ot molluscicidal activity of Yurimin P-99 (Table 2).

3. Influence of pH of water upon the efficacy of Yurimin P-99

Hydrogen-ion determinations were made by a Beckman Model G pH meter, and

Table 2 Effect of hard water on the toxicity of Yurimin P-99

Concentration in ppm	Hard water*			Distilled water		
	Alive	Dead	%Mortality	Alive	Dead	%Mortality
Water control	30	0	0			
3.125	28	2	6.7	30	0	0
6.25	12	18	60.0	6	24	80.0
Water control	30	0	0			
2.5	21	9	30.0	21	9	30.0
5	16	14	46.7	2	28	93.3
10	1	29	96.7	0	30	100

* WHO standard hard water (600 ppm calcium chloride plus 139 ppm magnesium chloride)

Table 3 Percent mortality of *O. nosophora* after 24-hour exposure to Yurimin P-99 in distilled water with certain pH levels

Concentration in ppm	pH range				
	4.5-5.2	5.6-6.0	6.4-6.5	7.3-7.8	8.1-8.7
Water control	0	0	0	0	0
2.5	10	30	30	70	50
10	70	90	90	100	90
40	100	90	90	90	90
Water control	0	0	0	0	0
2.5	0	50	40	70	70
5	30	70	70	90	90
10	80	80	70	90	100

desired pH levels were obtained using sodium hydroxide and hydrochloric reagents. The water was first adjusted to the desired pH, then Yurimin P-99 was added. Oncomelania snails were introduced and the pH was again recorded. The results of two replications of tests (Table 3) indicate that acid waters (pH 4.5–5.2) decrease the toxicity of Yurimin P-99, whereas alkaline waters (pH 7.2–8.7) increase the toxicity of the compound.

4. Influence of sunlight upon the efficacy of Yurimin P-99

A series of twofold dilutions of Yurimin P-99 in water was made up. Petri dishes each containing 100 ml of the dilutions, in a layer 0.5 cm thick, were exposed to direct sunlight for four hours. The irradiated samples, and corresponding unirradiated controls, were tested by the immersion method for their molluscicidal effect on Oncomelania snails. Parallel tests with NaPCP were run. From the percentages of snails killed in 48 hours by irradiated and control dilutions, the LC_{50}s and respective 95% confidence limits were calculated (Table 4). The lethal effects of irradiated and untreated control dilutions of Yurimin P-99 were not significantly different. Contrasting with that, the toxicity of NaPCP was highly effected by sunlight, and after 4 hours a decline in efficacy down to 1/6 was found biologically.

5. Photochemical degradation of Yurimin P-99 solution in ethanol

A concentration of 7 ppm Yurimin P-99 pure base, in ethanol solution, was divided into two samples of 30 ml each, which was contained in 100 ml Petri dishes in a layer 0.3 cm thick. Sample 1 was covered with a 0.08 mm thickness of polyethylene sheet which transmitted approximately 50% of light at a wavelength of 250mμ; and sample 2 was covered with a Wako Haze UV filter which cut off all wavelengths below 360mμ. A Toshiba GL-15 germicidal lamp(15 watt) which has a peak at 254 mμ was used at a distance of 20cm above the surface of the liquid. Each sample was irradiated for 18 hours, and then assayed colorimetrically. For the determination of the compound in ethanol the following was used:

A standard solution was prepared by dissolving 100 mg of the pure base of Yurimin P-99 in 100 ml of ethanol, and diluting 10 ml of this with ethanol to give a volume of 100 ml. Three, 5, 7 and 10 ml each of the standard solution were placed in 100-ml flasks to which 1 ml of 0.1N ethanol-KOH was added; and ethanol was added to give a volume of 100 ml. A suitable volume of each was removed to a 5-ml cuvette with a light path of 1 cm for determination of optical density at 500 mμ in Hitachi EPO-B spectrophotometer. Calibration curves were drawn up (Fig. 1), from which the

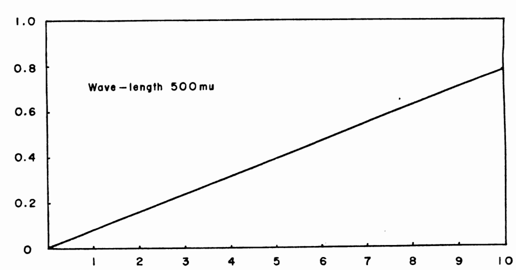

Fig. 1 Relation between concentration of Yurimin P-99 base in ethanol and density of light transmitted.

Table 4 Comparison of molluscicidal activities of irradiated* and untreated control dilutions of Yurimin P-99 and NaPCP

Molluscicide	LC_{50} (95% confidence limits)	
	Irradiated	Untreated
Yurimin P-99	5.6(4.4-7.1)ppm	5.4(4.3-6.9)ppm
NaPCP	3.0(2.4-3.7)ppm	0.51(0.26-0.62)ppm

* Exposed to sunlight for 4 hours

Table 5 Photochemical degradation of Yurimin P-99 ethanol solution exposed to a germicidal lamp and sunlight

Filter	Germicidal lamp			Sunlight		
	Hours exposed	Concentration(ppm)	c^*/co	Hours exposed	Concentration(ppm)	c^*/co
Polyethylene sheet**	0	7.0	1.00	0	7.0	1.00
	18	3.7	0.53	5	6.8	0.97
Kenko Haze UV(excludes light of wavelength less	0	7.0	1.00	0	7.0	1.00
than 360mμ)	18	7.1	1.01	5	7.0	1.00

* *c*/co is the fraction of Yurimin P-99 remaining as determined by colorimetric analysis.

** The transmission rates (%) are 51.0, 67.4, 72.0, 75.6 and 79.2 at wavelengths of light of 230, 250, 270, 290 and 310mμ, respectively.

content of Yurimin P-99 in ethanol after irradiation was evaluated.

The results (Table 5) showed that the specimens under the UV filters were not affected by the light, indicating that the wavelengths above 360 mμ were not contributory. The loss of Yurimin P-99 in the specimens under the polyethylene sheet therefore resulted from absorption of light of wavelength less than 360 mμ.

To determine the effects of sunlight on dilute ethanol solutions of the compound, preparations identical to those mentioned above were exposed to approximately 5 hours of direct sunlight. No remarkable photochemical degradation of Yurimin P-99 ethanol solution was observed in the specimens under the polyethylene sheet (Table 5).

Discussion

A review of our immersion method of testing molluscicides in the laboratory reveals that in general satisfactory correlation has been observed between the performance of the molluscicides obtained in the laboratory and in the field. In the field use of Yurimin P-99 in a dose of 5g/m² was more efficient than the dose of 5g/m² NaPCP (Iijima *et al.*, 1964); yet, Yurimin P-99 was less efficient than NaPCP in the immersion method in the laboratory. Evidence from the present experiments designed to evaluate the influence of sunlight revealed that Yurimin P-99 was not detoxified after a 4-hour exposure, whereas NaPCP was highly detoxified after the exposure. The experiments with a germicidal lamp which has a peak at 254 mμ indicated that the concentration of Yurimin P-99 in ethanol was reduced from the original 7 ppm to 3.7 ppm after a 18-hour exposure. The information presented here plus that already reported by Kutsumi (1963) concerning the decomposition of NaPCP by ultraviolet irradiation with the germicidal lamp identical to that used in the present experiment, show that Yurimin P-99 is much more likely to be protected against photochemical action. Light thus appeared to be an important factor participating in the above-mentioned discrepancy between results of laboratory and field test with this compound.

The properties of a good molluscicide are that it should be cheap, safety to handle and easy in its method of application. It should, of course, be lethal to snails and stable under different conditions. In spite of its having several undesirable qualities, NaPCP is still considered to be one of the most promising molluscicides because of its effectiveness, availability and the fact that it has been thoroughly tested. From the above laboratory experiments it would appear that Yurimin P-99 has an advantage over NaPCP for protection against photochemical action. In Japan where the majority of the important snail habitats are sun-baked small irrigation ditches, and where vegetation has to be cleared from the habitats before the use of molluscicides, the use of Yurimin P-99 for the most parts would be appropriate. In addition the chemical is relatively cheap (US$ 0.45/1b) and very easy to handle in the form of an aqueous suspension.

With regard to the phytotoxicity of this chemical, no visible damage to waterfield rice plants has been observed during field trials in a paddy field with a dose of 5g/m^2 (Yamanashi Prefectural Experimental Farm Report in 1964). Concerning the effect on fresh-water fishes, Ihara Chemical Laboratory (1963) reported that the LC_{50} for a 24-hour exposure was 0.825 ppm in goldfishes, 0.16 ppm in carp and 0.2-2.0 ppm in killifishes. It can be said that the lethal concentration of Yurimin P-99 is a little higher for fishes than for snails and that the absolute fish toxic dose does not present any difference comparing NaPCP and Yurimin P-99. The acute oral LD_{50} in mice was 167.9 mg/kg with the active ingredient and 1.659 g/kg with a 5% w/w granule form (Ihara Chemical Lab., 1963). By far more important than the acute toxicity is the chronic toxicity. An exact knowledge of the toxicity of molluscicides in vegetables and animals other than snails is of paramount importance, as contamination of crops, fishes, farm animals and human being with water containing molluscicides cannot always be avoided. More laboratory and field tests should be carried out to further appraise the rate of damage of this chemical for human being it can be widely accepted as a standard molluscicide for use on a larger scale.

Summary

Laboratory experiments designed to evaluate the efficacy of Yurimin P-99 as a molluscicide are described. Evidence from the experiments with the WHO standard hard water indicated that the hardness of water plays but a small part in the reduction of molluscicidal activity of Yurimin P-99. The toxicity of Yurimin P-99 is also affected by pH and it appears that Yurimin P-99 was a more effective molluscicide in alkaline waters (pH 7.2-8.7), when compared with the same or greater concentration in acid waters (pH 4.5-5.2). It was proved that Yurimin P-99 has an advantage over NaPCP for protection against photochemical action. The results are encouraging and it is felt that there would be a certain scope for its use against Oncomelania snails.

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実験的殺貝剤 Yurimin P-99 の実験室内検討

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わが国で開発された新殺貝剤 Yurimin P-99 (3,5-dibromo-4-hydroxy-4'-nitroazoben) は，飯島ら (1964, 1965) の野外試験によればそのミヤイリガイ殺貝効果において従来広く使用されているNaPCP よりすぐれている．しかしながらわれわれの実験室内における直接浸漬法では，Yurmin P-99，5% w/w 顆粒の LC_{50} (25 °C，48時間作用) は 4.8 (3.9～5.9) ppm を示し，その効果はNaPCP のそれよりはるかに劣っている．このような野外と実験室内の試験結果の間に見られる discrepancy に関与する要因を追求する目的で，本薬剤におよよぼす水の pH，硬度および日光等の影響をしらべた．WHO の standard hard water (600 ppm calcium chloride+139ppm magnesium chloride) 中では LC_{50} は 4.5 (3.4～5.9) ppm を示し，対照の蒸留水中での LC_{50} 3.0 (2.4～3.7) ppm に比しわずかに効力の低下が見られた．一方，本薬剤の殺貝効果は acid water (pH 4.5～5.2) 中で若干低下し，alkaline water (pH 7.3～7.8; 8.1～8.7) において上昇する傾向が認められた．次に Yurimin P-99 と対照薬剤として NaPCP の各倍々稀釈液 100ml をそれぞれシャーレに容れ，液の深さを0.5 cm として太陽光に4時間暴露した後にミヤイリガイを投入し，直接浸漬法によって LC_{50} 値を算出した．Yurimin P-99 では暴露液による LC_{50} は 5.6(4.4～7.1) ppm，対照の室内散光下に置かれたものの LC_{50} は 5.4 (4.3～6.9) ppm を示し．両者間に有意差は認められなかった．しかし NaPCP においては暴露液の LC_{50} が 3.0 (2.4～3.7) ppm であるのに対し，散光下のそれは 0.51 (0.26～0.62) ppm を示し，あきらかに有意差が見られた．一方，Yurimin P-99 結晶の 7ppm ethanol 液を東芝 GL-15 殺菌灯下に18時間暴露した後に日立 EPO-B 光電比色計で定量した．250mμ の光の50%を透過する polythylen sheet で蔽われた試料では薬剤の約47%が破壊されたが，UV filter で蔽われた試料では変化が全く認められなかったことから，Yurimin P-99 は 360mμ 以下の紫外線により破壊されることが明らかにされた．しかしこの Yurimin P-99 の紫外線による破壊は，さきに久津見ら (1963) がほとんど同様の条件下で NaPCP において認めたそれに比しはるかに低い．上記を総合し，Yurimin P-99 は NaPCP よりも日光の影響を受けにくいという利点を有し，この特性が本薬剤の野外と実験室内の効果のくいちがいの要因の一つとなっていると考えられる．わが国のミヤイリガイ棲息地の大部分が，ほとんど日蔭のない小溝であることから本薬剤はかなり有望な殺貝剤と考えられる．