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Short communication

Persistence of gentian violet and leucogentian violet in channel catfish (*ictalurus punctatus*) muscle after water-borne exposure[1]

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Abstract

Gentian violet is a triphenylmethane dye that is an antifungal/antiparastic agent. GV is similar to malachite green that has been used in the aquaculture industry for treatment or prevention of external fungal and parasitic infections in fish and fish eggs although it (MG) is not approved for this use. For these reasons, GV's potential for misuse by the aquaculture industry is high. The uptake and depletion of gentian violet (GV) were determined in channel catfish (*Ictalurus punctatus*) after water-borne exposure (100 ng ml^{-1}, 1 h) under simulated aquaculture farming conditions. Leucogentian violet (LGV) was rapidly formed, concentrated in the muscle tissue, and very slowly eliminated from muscle tissue. An isocratic (60% acetonitrile–40% water; 0.05 *M* ammonium acetate buffer, pH 4.5) HPLC system consisting of a 5 μm LC–CN 250×4.6 mm I.D. column, a 20×2.0 mm I.D. PbO_2 oxidative post-column, and a UV–VIS detector set at 588 nm were used to determine uptake and depletion of tissue residues of GV and LGV with time. GV was rapidly depleted and converted to its major metabolite, LGV, which was detected out to 79 days. Therefore, LGV is the appropriate target analyte for monitoring exposure of channel catfish to GV. © 1999 Published by Elsevier Science B.V.

Keywords: Gentian violet; Leucogentian violet

1. Introduction

Gentian violet (GV) is a triphenylmethane dye that is used in inks, used as a dye for wood, silk, paper, and used as a biological stain, microbicide and as an anthelmintic [1]. GV has also been used as an inhibitor of mold and fungal growth in poultry feeds. GV has been determined to be mutagenic to *Bacillus subtilis, Escherichia coli, and Salmonella typhimurium* [2] and cytotoxic to mammalian cells [3]. GV is structurally similar to other triphenylmethane dyes such as rosaniline, which has been linked to increased risk of human bladder cancer [4]. The leuco form of rosaniline induces renal, hepatic, and lung tumors in mice [5]. In a number of species, including humans, it has been demonstrated that intestinal microflora systems convert GV to the leuco form [6].

Malachite green (MG), very structurally similar to GV, is reported to be used in the aquaculture industry for treatment or prevention of external fungal and

[1] The opinions expressed in this manuscript are solely those of the authors and not necessarily those of the U.S. Food and Drug Administration.

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parasitic infections in fish and fish eggs [7–9]. MG is typically administered as an aqueous bath treatment, alone or in combination with formalin. MG is readily absorbed by fish during water-borne exposure [10–14]. Leucomalachite green (LMG), the reduced form of MG, is recognized as the major metabolite in animal tissues [11,15–19]. From preliminary investigations with catfish exposure to GV, we have determined that GV is also readily absorbed and that leucogentian violet (LGV) is the major metabolite which was expected since GV and MG are structurally related. Structures of GV, MG, LGV, and LMG are shown in Fig. 1.

GV is not currently approved by the Food and Drug Administration (FDA) for use in the aquaculture industry; however, the potential for misuse by the industry is high because of its antifungal and antiparasitic properties. Information concerning the characteristics of GV and its metabolite, LGV, in edible fish fillet tissue is significant because of the potential of their residues to cause untoward affects on human health. The time required for clearance of these chemicals from the muscle tissue is also of concern.

The objective of this study was to determine the persistence of GV and LGV in aquacultured channel catfish after water-borne exposure to GV. Our recently developed analytical chemical procedure [20] was used to determine the uptake and depletion of GV and LGV in tissue residues with time under simulated farming conditions.

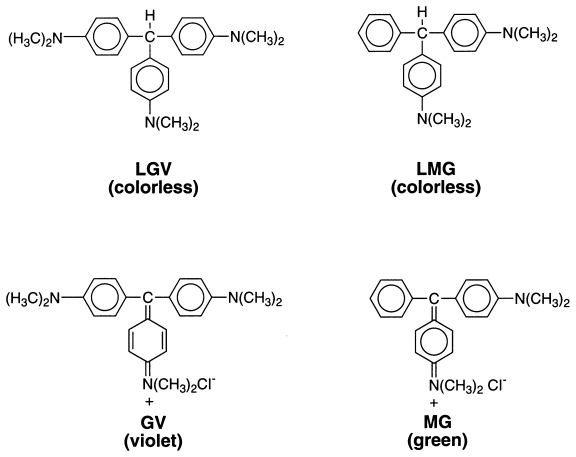

Fig. 1. Structures of gentian violet (GV), malachite green (MG), leucogentian violet (LGV), and leucomalachite green (LMG).

2. Experimental

2.1. Chemicals

LGV was obtained from Aldrich (Milwaukee, WI, USA) and GV was obtained from Hilton-Davis (Cincinnati, OH, USA). Lead oxide [PbO_2] and hydroxylamine hydrochloride were from Mallinckrodt (Chesterfield, MO, USA) and were AR grade. Basic alumina (Brockman activity I) and diethylene glycol were purchased from Fisher Scientific (Springfield, NJ, USA). The *p*-toluene sulfonic acid (*p*-TSA) and ammonium acetate were purchased from Fluka (Ronkonkoma, NY, USA). The glacial acetic acid and the HPLC grade acetonitrile were obtained from J.T. Baker (Phillipsburg, NJ, USA).

2.2. Catfish exposure and sampling:

Catfish exposure, holding in the 0.2 ha pond, sampling, and processing (sacrificing, filleting, packaging, and freezing) were performed at the University of Arkansas at Pine Bluff's Experimental Aquaculture Facilities. There ninety-five channel catfish in the 0.45–0.91 kg range were placed in a stainless steel (SS) tank containing 1560 L of water. The temperature of the tank water was 26.7°C and the pH was 8. GV (154.4 mg, dissolved in 100 ml DI water at pH 3.2) was added to the tank water. Aeration was provided to keep the water oxygenated. Fish were exposed to GV at a nominal concentration of 100 ng ml^{-1} for 1 h, then transferred in mass to a hauling truck using a seine. The hauling truck was driven approximately 200 meters to an experimental catfish pond (approximately 0.2 hectare×1.25 m depth) and the fish were immediately transferred to a vinyl-coated wire catfish cage in the pond. The pH of the pond water was 8.5. Over the 79 days of the study (July 17–October 3), pond temperatures ranged from 25°C to 35°C.

Five catfish were withdrawn from the SS tank prior to exposure to serve as controls. Five fish were withdrawn from the wire cage in the catfish pond at the following time intervals after exposure: 1, 2, 4,

and 7 h., then at 1, 2, 5, 8, 15, 22, 33, 51, and 79 days. Each fish was skinned and filleted. Fillets from each fish were immediately placed in a Zip-lock bag and frozen at −60°C.

2.3. Sample preparation

The fillets from each Zip-lock bag were cut into chunks while still frozen and macerated in a Robot Coupe RSI2Y1 (Jackson, MS, USA) blender. A 10-g aliquot was removed and the remaining homogenate placed in the Zip-lock bag and returned to −60°C storage.

2.4. Extraction

A ten (10.0) g sample was weighed into 250 ml Falcon polypropylene tubes (Becton Dickinson Labware, Lincoln Park, NJ, USA) for each of the five fish taken at each sampling interval. Samples were then extracted, liquid–liquid partitioned, and cleaned up on solid-phase extraction cartridges as described in Rushing et al. [20]. Briefly, acetonitrile/buffer pH 4.5 was added to the samples, homogenized at 20,000 rpm for 1 min, 20 g basic alumina added, centrifuged, and the supernates decanted into separatory funnels. Deionized water, methylene chloride, and diethylene glycol were added to the superanate in the separatory funnels. After shaking, the methylene chloride was concentrated and applied to stacked alumina/PRS cation-exchange solid-phase extraction cartridges. Acetonitrile/buffer was then used to elute the LGV and GV.

2.5. Liquid chromatography

The LC system consisted of a 20×2.0 mm I.D. pellicular CN guard column, a 5 μm Supelco LC–CN 250×4.6 mm I.D. analytical column and a 20× 2.0 mm I.D. PbO_2 oxidative post-column. The detector was a Hewlett-Packard Model 1050 UV–VIS detector set at 588 nm. The mobile phase was 60% acetonitrile 40% aqueous buffer. Ammonium acetate (3.85 g) was added to approximately 380 ml H_2O that was adjusted to pH 4.5 with glacial acetic acid. This was diluted to 400 ml with H_2O then mixed with 600 ml acetonitrile. The final solution was 0.05 *M*. The flow-rate was 1 ml min^{-1} at 10.34 MPa. All injections were 100 μl. A single determination was made for each of the five fish sampled at each time interval ($n=5$). Chromatographic data was collected on HP Vector QS/16S Chemstation with HP3365 series II Chemstation software version A.03.21.

3. Results and discussion

Typical treatment schemes of MG for dermal contact of external fungus and parasitic infections in fish cover a broad range from 100 ng ml^{-1} for a few seconds of dip [21] to an indefinite treatment of 0.1 ng ml^{-1} in ponds [22]. For the current study, we chose to investigate the 100 ng ml^{-1} level concentration of GV. This dose was selected after performing a pilot study using two aqueous (pH ~7) dose levels (10 and 100 ng ml^{-1}) for exposure of catfish at each level for 1 h. After exposure of the catfish in the current study, the SS tank water was poured through large filters containing activated carbon. The filter contents, including the carbon, were incinerated at 1250°C.

LGV and GV were separated isocratically by HPLC on a cyano column (Fig. 2). The LGV was chromatographed on the column as the leuco form but after separation on the analytical column it was oxidized by the PbO_2 post-column reactor to the chromatic form. Both compounds were detected as GV; however, LGV was distinguished from GV by its earlier retention time. The 10× expansion of Fig.

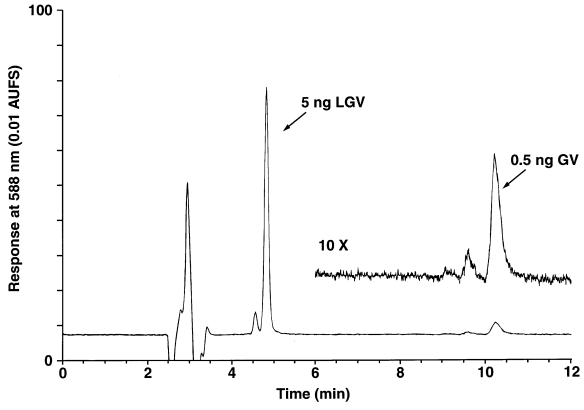

Fig. 2. Isocratic separation of LGV and GV on a cyano column.

2 illustrates the sensitivity and noise levels obtained while assaying a 0.5 ng standard of GV. Since almost all of the GV was rapidly converted to the LGV, the chromatograms in Fig. 3 were attenuated to display this major metabolite. Fig. 3 illustrates composite overlays of HPLC chromatograms of the control reagents blank; LGV (5 ng) and GV (0.5 ng) standard; 0.5 g equiv. of control catfish sample; 1 h post-dosing sample [11.7 ng g^{-1} LGV and 0.5 ng g^{-1} GV]; 7 h post-dosing sample [15.5 ng g^{-1} LGV and <MDL=0.2 ng g^{-1} GV]; 15-day post-dosing sample [5.7 ng g^{-1} LGV and <MDL GV]; and 79-day post-dosing sample [3.1 ng g^{-1} LGV and <MDL GV]. The additional peak in Fig. 3 at approximately 8 min was an extraneous peak but not one that interfered with the quantification of LGV or GV. Also it was not a late eluter from a previous injection. This peak was not only in the fish samples but also in the reagent blank samples and thus did not originate from the catfish. Rather it has to have originated from the reagents added or the manipulate steps applied to the samples. Extraction of individual reagents, alumina, and PRS cartridges used in this procedure failed to produce any peak at this retention time. Additionally, sample set on days 1, 15, and 22 indicated little or no extraneous peak at 8 min. Therefore it remains an unknown peak but not an interfering peak. Table 1 lists the means and standard

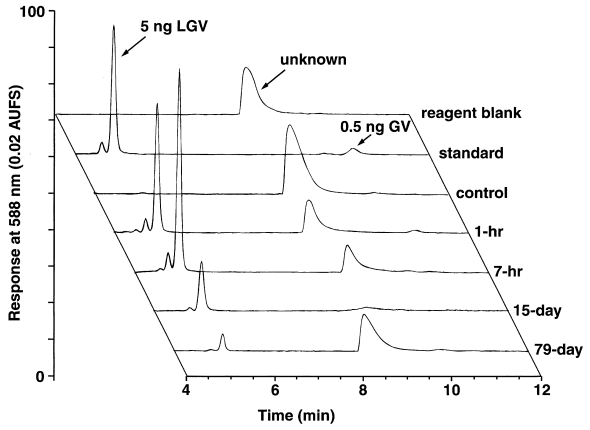

Fig. 3. Composite overlay of HPLC chromatograms of the control reagents blank; standard containing 5 ng LGV and 0.5 ng GV; 0.5 g equiv. of control catfish sample; 1-h post-dosing sample; 7-h post-dosing sample; 15-day post-dosing sample; and 79-day post-dosing sample. Samples are mean of $n=5$.

Table 1
Concentration of leucogentian violet (LGV) and gentian violet (GV) in muscle tissue of catfish exposed to 100 ng ml^{-1} GV in water for 1 h

Time Post-Dosing	LGV (ng g^{-1})	GV (ng g^{-1})
Controls[a]	0.0±0.1	<mdl[b]
1 h	11.7±1.8	0.5±0.1
2 h	16.8±2.2	0.8±0.3
4 h	15.9±4.3	<mdl
7 h	15.5±3.6	<mdl
1 day	15.1±3.1	<mdl
2 days	13.5±3.3	mdl
5 days	9.4±3.3	0.3±0.2
8 days	9.7±2.8	<mdl
15 days	5.7±2.2	<mdl
22 days	3.3±0.5	mdl
33 days	2.8±0.9	<mdl
51 days	1.5±0.6	mdl
79 days	3.1±0.5	<mdl

[a] Taken prior to dosing.
[b] mdl=0.2 ng g^{-1} for GV.
Mean and standard deviation of single determinations of five fish at each sampling interval.

deviations at each post-dosing time interval for LGV and GV in catfish muscle. LGV is the major metabolite of GV in catfish and is rapidly formed and deposited in muscle tissue. This rapid reduction of GV to LGV (~2 h) is responsible for the chromatic form (GV) not being detected in significant amounts in muscle tissue. The accumulation and depletion of LGV from catfish muscle with time is shown in Fig. 4. At 79 days post-dosing, the muscle tissue continues to have measurable residues of LGV (3.1 ng g^{-1}). Since GV is not a FDA approved chemical for use in the aquaculture industry, no measurable level of GV or its metabolite LGV are acceptable in catfish muscle or other aquacultured species. Although this study was terminated after 79 days post-dosing, it is apparent that measurable levels of LGV will persist for longer than 79 days. This study also confirms that LGV is an appropriate marker analyte for monitoring exposure of channel catfish to GV.

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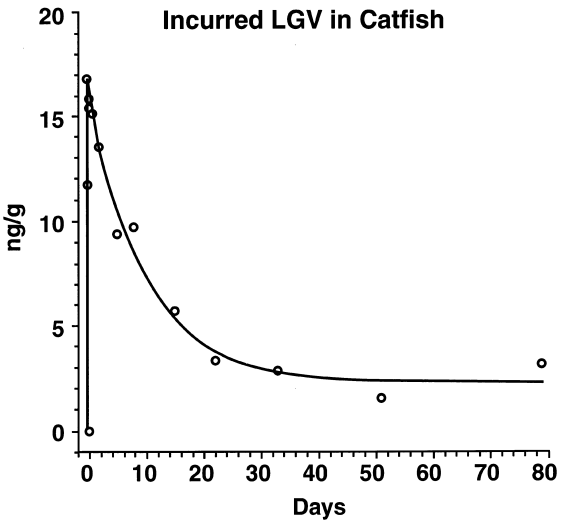

Fig. 4. Accumulation and depletion of LGV in fillet tissue of channel catfish after exposure to GV for 1 h at 100-ng ml^{-1}.

of Arkansas at Pine Bluff is appreciated for the maintenance and processing of catfish at UAPB. We also thank Richard Cullison of the FDA Center for Veterinary Medicine for conducting the 1-hour GV pilot study exposure of catfish and subsequently providing skinned fillets for the authors' analysis by HPLC.

References

[1] The Merck Index, Eleventh Edition, S. Budavari (Ed.), Merck & Co., Inc., Publisher, Rahway, NJ, 1989
[2] H. Fujita, A. Mizuo, K. Hiraga, Tokyo Toritsu Eisei Kenkyusho Kenkyu Nempo. 27 (1976) 153.
[3] K. Norrby, H. Mobacken, Acta Derm. Venereol. 52 (1972) 476.
[4] R.A.M. Case, J.T. Pearson, Brit. J. Indust. Med. 11 (1954) 213.
[5] O.G. Prokofeva, M.A. Zabezhinskii, Vop. Onkol. 22 (1976) 66.
[6] J.J. McDonald, C.E. Cerniglia, Drug Metab. Dispos. 12 (1984) 330.
[7] D.J. Alderman, J. Fish Dis. 8 (1985) 289.
[8] D. Manz, J. Togel, J. Nilz, F. Lutz, Tieraerztl. Umsch. 46 (1991) 543.
[9] K. Sagar, M.R. Smyth, J.G. Wilson, K. McLaughlin, J. Chromatogr. A 659 (1994) 329.
[10] M. Nakagawa, K. Murata, T. Shimokawa, T. Honda, S. Kojima, M. Uchiyama, Eisei Kagaku 30 (1984) 301.
[11] K. Bauer, H. Dangschat, H. O Knoeppler, J. Neudegger, Arch. Lebensmittelhyg. 39 (1988) 97.
[12] M. Kietzmann, H.-J. Hapke, M. Beeke, S. Stehle, Dtsch. Tieraerztl.Wochenschr. 97 (1990) 290.
[13] Y. Kasuga, M. Hishida, N. Tanahashi, M. Arai, J. Food Hyg. Soc. Jpn. 33 (1992) 539.
[14] D.J. Alderman, R.S. Clifton-Hadley, J. Fish Dis. 16 (1993) 297.
[15] G. Werth, A. Boiteux, Arzneim.-Forsch. 18 (1968) 39.
[16] W.E. Poe, R.P. Wilson, Prog. Fish-Cult. 45 (1983) 228.
[17] J.L. Allen, J.E. Gofus, J.R. Meinertz, J. AOAC Int. 77 (1994) 553.
[18] S.M. Plakas, K.R. El Said, G.R. Stehly, J.E. Roybal, J. AOAC Int. 78 (1995) 1388.
[19] J.E. Roybal, A.P. Pfenning, R.K. Munns, D.C. Holland, J.A. Hurlbut, A.R. Long, J. AOAC Int. 78 (1995) 453.
[20] L.G. Rushing, S.F. Webb, H.C. Thompson Jr., J. Chromatogr. B 674 (1995) 125.
[21] N.C. Nelson, National Technical Information Services, U.S. Department of Commerce, PB-235450 (1974)
[22] M.K. Stoskopf, Fish Medicine, W.B. Saunders, Philadelphia, 1993.