Determination of leucogentian violet and gentian violet in catfish tissue by high-performance liquid chromatography with visible detection

Larry G. Rushing*, Sharon F. Webb[1], Harold C. Thompson Jr.

Department of Health and Human Services, Public Health Service, Food and Drug Administration, National Center for Toxicological Research, 3900 NCTR Drive, Jefferson, AR 72079-9502, USA

Abstract

A sensitive analytical procedure for the determination of residues of leucogentian violet (LGV) and gentian violet (GV) in catfish tissue is presented. Frozen ($-20°C$) catfish fillets were cut into chunks and then blended in a Waring blendor. A 10-g amount of catfish muscle tissue was homogenized and extracted with acetonitrile–buffer, partitioned against methylene chloride, and cleaned up on tandem neutral alumina and propylsulfonic acid cation-exchange solid-phase extraction cartridges. Samples of 100 μl (0.5 g equiv.) were chromatographed isocratically in 15 min using an acetonitrile–buffer mobile phase on a cyano phase column in-line with a post-column PbO_2 oxidation reactor. The PbO_2 post-column reactor efficiently oxidized the LGV to the chromatic GV permitting visible detection at 588 nm for both LGV and GV. Linearity was demonstrated with standards over the range 0.5–50 ng per injection. Recoveries of LGV and GV from catfish tissues fortified at 20, 10, and 1 ng/g were 83.1 ± 1.2, 78.4 ± 4.0, 84 ± 8 and 92.7 ± 1.8, 95.0 ± 2.2, 93 ± 2 (mean $\pm$ S.D., $n = 4$), respectively.

1. Introduction

Gentian violet (GV), a triphenylmethane dye, is on the Food and Drug Administration's (FDA's) priority list for fish drugs that need analytical methods development. Although GV is not approved by FDA for use in the aquaculture industry it has the potential for misuse

because of anti-parasitic, anti-fungal properties and structural similarity to malachite green (MG). MG has been used by the aquaculture industry since the early 1930s to combat ecto-parasites and control fungus on fish eggs, fingerlings, and adult fish. MG also is not approved by the FDA for use in the aquaculture industry; however, circumstantial evidence suggests that MG continues to be used [1]. GV is structurally related to other triphenylmethane dyes such as rosaniline which has been linked to increased risk of human bladder cancer. The leuco form of rosaniline induces renal, hepatic and lung tumors in mice [2]. In a number of species including

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* Corresponding author.
[1] Present address: Auburn University, 2031 S. College 35, Auburn, AL 36830, USA.

man, it has been shown that the intestinal microflora systems [3] are capable of converting GV to the leuco form (LGV). The FDA, therefore, has need of a sensitive analytical method for the determination of residues of GV and its metabolite LGV in catfish to monitor illicit use and for potential use in enforcement proceedings. The structures for LGV and GV are shown in Fig. 1.

Several methods have been reported in the scientific literature for LGV and/or GV in a variety of matrices. Most of these methods employed liquid chromatography with UV–visible detection or electrochemical detection [4–8]. In 1991 Allen and Meinertz [9] reported a HPLC method for separating the leuco and chromatic forms of two triphenylmethane dyes [leucomalachite green (LMG)/MG and LGV/GV]. The leuco form was oxidized to the chromatic form with an in-line post-column cartridge packed with 10% PbO₂–Celite 545 with subsequent detection of both forms by visible spectrophotometry. Their method eliminated the need to split the sample or assay the LGV by difference following determination of the GV before and after complete oxidation. No methods were found in the literature for analysis of LGV and GV in catfish tissue. However, Roybal et al. [10] reported a method for the analysis of the structurally similar dye and its metabolite, MG and LMG in catfish tissue. Roybal's method employing HPLC with PbO₂ post-column oxidation (LMG → MG) and visible detection was modified for our application for analyses of LGV and GV in edible catfish tissue.

2. Experimental

2.1. Chemicals

LGV was obtained from Aldrich (Milwaukee, WI, USA) and GV was obtained from Hilton-Davis (Cincinnati, OH, USA). Both were used as received. Lead dioxide (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. Fish samples

Preparation

Several pounds of catfish fillets were purchased at a local market and stored at −20°C, then cut into chunks and blended in a Waring blendor. These were stored in a zip-lock plastic bag until required for processing and analysis.

Extraction

Quadruplicate ten (10.0) g fish samples were weighed into 250-ml Falcon polypropylene tubes obtained from Becton Dickinson Labware (Lincoln Park, NJ, USA). Three ml of aqueous 0.25 g/ml hydroxylamine hydrochloride, 5 ml of aqueous 0.05 M p-TSA and 10 ml of aqueous 0.1 M
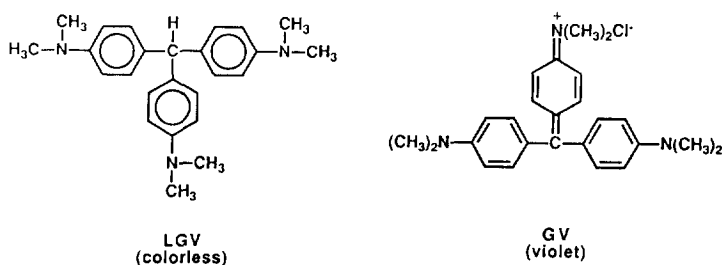

Fig. 1. Chemical structures of leucogentian violet (LGV) and gentian violet (GV).

ammonium acetate (adjusted to pH 4.5 with glacial acetic acid) were added to each sample. These were homogenized for 1 min at 20 000 rpm using a Tekmar (Cincinnati, OH, USA) Ultra-Turrax T25 tissuemizer. Acetonitrile (90 ml) was added to each and the samples were homogenized for an additional 10 s. The Falcon tubes were capped and shaken vigorously by hand for 1 min. Basic alumina (20 g) were added and the tubes were again shaken vigorously for 1 min. The four tubes were centrifuged (centrifuge speed was not critical) and the supernatants were decanted into 250-ml separatory funnels. Acetonitrile (30 ml) was added to the Falcon tubes and the samples were extracted, centrifuged and decanted again into the separatory funnels.

Liquid–liquid partition

To the combined supernatants in the separatory funnels, 100 ml of deionized distilled water, 50 ml of methylene chloride and 2 ml of diethylene glycol were added to each. The separatory funnels were then shaken vigorously by hand for 1 min. Separation of the layers occurred after standing for 45 min. The bottom layer of each was collected in a 500-ml round-bottom flask containing several boiling chips. An additional 50 ml of methylene chloride was added to the separatory funnels which were again shaken for 1 min. The layers generally separated in less than 5 min and were added to their respective 500-ml round-bottom flask. These samples were then concentrated on a Büchi (Flawil, Switzerland) roto-evaporator at 65°C to approximately 2–5 ml. The samples can be reserved overnight in the dark at this point.

Solid-phase extraction

J.T. Baker 6-ml (1000 mg) neutral alumina cartridges and Varian (Harbor City, CA, USA) 2.8-ml (500 mg) Bond Elut PRS cartridges were prewashed with 5 ml acetonitrile. The alumina cartridge was then placed atop of the PRS cartridge using an adapter. This assembly was then attached to an Alltech (Deerfield, IL, USA) solid-phase extraction vacuum manifold. The sample flow control valves were replaced with 15

gauge needles to reduce dead volume. A 2-ml amount of methylene chloride was added to each 500-ml sample flask which was then swirled to dissolve the residue. A 5-ml amount of acetonitrile was added to the flask prior to the addition of the sample extracts to the cartridge assemblies. The flasks were rinsed with an additional 2 × 5 ml of acetonitrile which was also applied to the cartridge assemblies. Finally 5 ml of acetonitrile was rinsed through each cartridge. All wash fractions and the alumina cartridges were then discarded. A 1-ml amount of acetonitrile–0.1 M ammonium acetate buffer (50:50) adjusted to pH 4.5 with glacial acetic was then washed through each PRS cartridge and also discarded. The LGV/GV residues were eluted from the PRS cartridges with 1.5 ml of the above acetonitrile–buffer and collected in graduated 2.5-ml centrifuge tubes containing 0.5 ml deionized distilled water. Samples, standards and controls alike were therefore contained in 2.0 ml of a mixture of 37.5% acetonitrile–buffer. All catfish extract samples were 5 g equiv/ml (i.e., 0.5 g equiv/100 μl injection).

Recovery experiments

Quadruplicate ten (10.0) g fish samples were weighed into 250-ml Falcon polypropylene tubes and fortified with 0, 10, 100, or 200 ng LGV/GV contained in 20 μl of methanol using a 25-μl liquid chromatographic syringe equipped with a Chaney adaptor. The samples were left in contact with the fortification solution for 30 min and then subjected to the entire analytical procedure to determine recovery efficiencies.

2.3. Liquid chromatography

The liquid chromatographic system consisted of a Waters (Milford, MA, USA) Model 510 pump, a Rheodyne (Cotati, CA, USA) Model 7125 injector with a 200 μl loop, a 20 × 2.0 mm I.D. pellicular CN guard column, a 5 μm Supelco (Bellefonte, PA, USA) LC-CN 250 × 4.6 mm I.D. column and a 20 × 2.0 mm I.D. PbO$_2$ oxidative post-column. This oxidative column was hand-packed with PbO$_2$ with no Celite 545 added.

Detection was with a Hewlett Packard (Atlanta, GA, USA) Model 1050 UV–visible detector set at 588 nm. The mobile phase was 60:40 acetonitrile–buffer. Ammonium acetate (3.85 g) was added to approximately 380 ml water which was then adjusted to pH 4.5 with glacial acetic acid. This was diluted to 400 ml with water and added to 600 ml of acetonitrile. The final solution was 0.05 M. The flow-rate was 1 ml/min at 10.34 MPa. All injections were 100 μl. The chromatographic data was collected on HP Vectra QS/16S Chemstation with HP 3365 series II Chemstation software version A.03.21.

3. Results and discussion

The isocratic separation of a 5 ng/100 μl injection of an admixture standard of LGV and GV on a cyano column is illustrated in Fig. 2. The retention times (t_R) of LGV and GV are 6.2 and 12.6 min, respectively. The very small peak at 11.3 min is an impurity (methyl violet) of the GV standard. The LGV is chromatographed on the column as the leuco form (reduced form). After separatio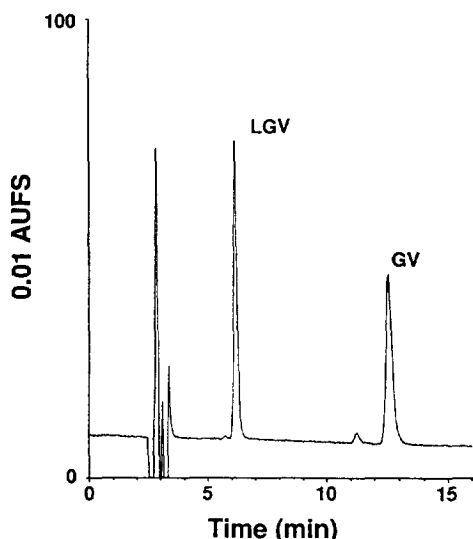n on the analytical column it is oxidized by the PbO$_2$ post-column reactor from the colorless leuco form to the chromatic form. Both compounds are therefore detected as GV; however, the LGV is distinguished from the GV by its earlier t_R. Detection is achieved using the visible maximum for GV at 588 nm. The isocratic separation of LMG/MG by Roybal et al. included the addition of the ion pairing agent, p-TSA [10]. In the present study, the addition of this agent had no effect on the t_R's of LGV and GV and was therefore omitted from the mobile phase. However, experiments were not conducted on the effects of this pairing agent on the recovery of LGV and GV from a catfish matrix and as a result the p-TSA was retained in the extraction process as described by Roybal et al.

The sensitivity of visible detection for LGV and GV is shown in Fig. 3. An admixture standard of LGV and GV at 100 pg each was injected onto the chromatographic system. Potentially even better sensitivity is possible with a visible detector having a tungsten visible light
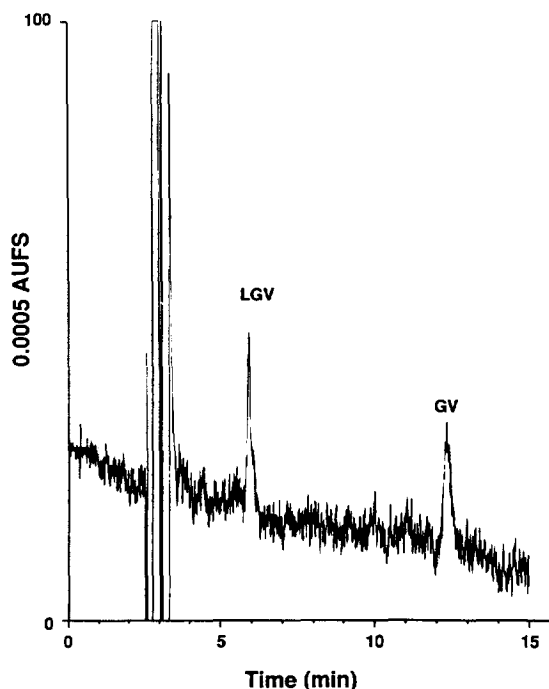

Fig. 2. HPLC chromatogram of 5 ng each of an admixture standard of LGV and GV on a cyano column; 100 μl injection.

Fig. 3. HPLC system sensitivity: 100 pg each of an admixture standard of LGV and GV; 100 μl injection.

source as opposed to the deuterium light source (reduced output in the visible range) in the detector that was available to conduct this study.

Injections (100 μl) of LGV and GV standards in admixture over the range 0.5–50 ng were chromatographed to assess linearity. The linear fit for the LGV data was $y = (6.604 \cdot 10^{-4})x - 0.049$ where y was the concentration and x was the area response. The standard deviation of the slope and intercept were $2 \cdot 10^{-6}$ and 0.08. The linear fit for the GV data was $y = (6.505 \cdot 10^{-4})x + 0.099$. The standard deviation of the slope and intercept were $9 \cdot 10^{-6}$ and 0.41; $n = 4$ for both sets of data. The correlation coefficients for LGV and GV were 0.99999 and 0.9998, respectively. The correlation coefficient for LGV also demonstrated that the LGV to GV oxidation by the PbO_2 post-column was efficient and linear.

A composite overlay of a 10-ng LGV and GV standard (equiv. to 20 ppb fortification), a 20-, 10-, 1-ppb fortified LGV and GV in catfish and

the corresponding control catfish is shown in Fig. 4. Each injection represents 0.5 g/equiv. of fish injected onto the analytical column. The control chromatogram has very few peaks, none of which interfere with the quantification of either the LGV or GV peak. The PbO_2 oxidative post-column shifts the detection into the visible range which affords greater specificity as fewer interferences absorb light in this region. Without the PbO_2 column, detection would be at 267 nm (maximum for LGV). At this wavelength the 10-ppb LGV and GV peaks are totally obscured by the control background interferences (267 nm chromatogram not shown). An expanded view of the 1-ppb fortified catfish and the control catfish is shown in Fig. 5. The small interferences in the control chromatogram are equivalent to approximately 0.1 ppb for both the LGV and GV. Fig. 5 also demonstrates that this analytical method for the analyses of LGV and GV in catfish extends to the 1-ppb level.

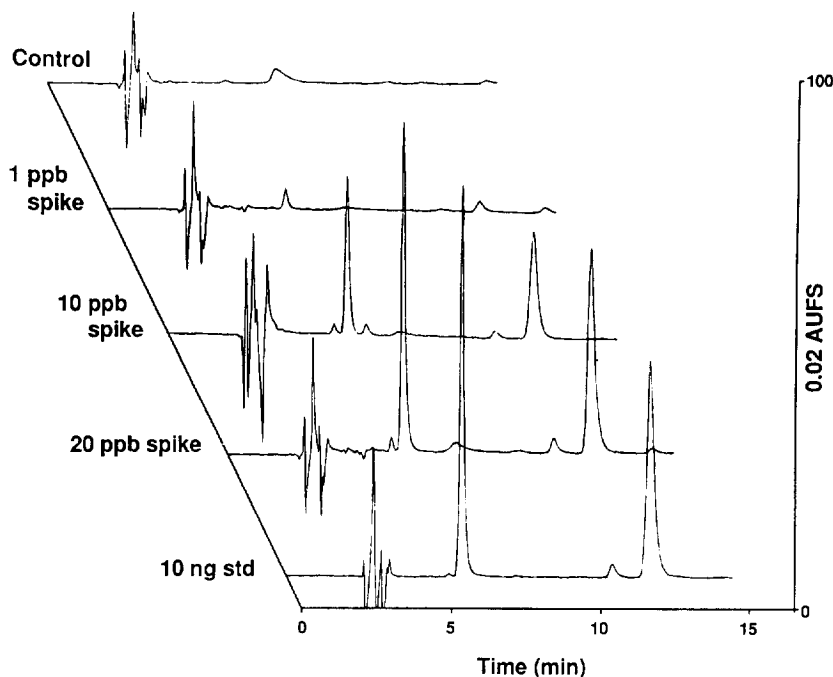Table 1 lists the recoveries for 20-, 10- and

Fig. 4. Overlaid HPLC chromatograms of 10 ng each admixture standard of LGV and GV (equivalent to 20 ppb in catfish); 20, 10, 1 ppb (ng/g) LGV and GV fortified catfish and control catfish.
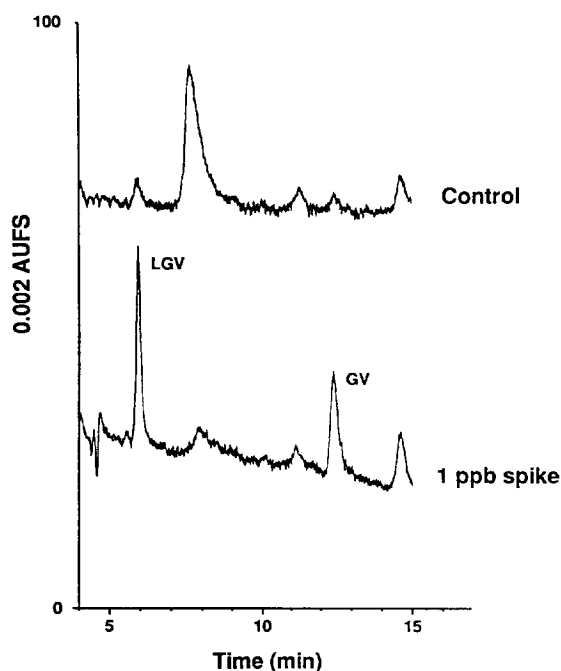
Fig. 5. Control catfish (0.5 g/100 μl injection) and 1 ppb fortified LGV and GV catfish.

1-ppb LGV and GV fortified catfish. Fortification was performed with admixture standards. No individual analyte recoveries were determined. The percent recoveries for LGV were typically in the low 80s while the percent recoveries of GV were typically in the low 90s. The control samples equated to 0.12 ppb LGV and 0.08 ppb GV. Samples fortified at 10 ppb or above typically had an imprecision of about 2%.

During the elution from the strong cation-exchange cartridge (PRS cartridge) which can be followed visually for GV, it was noted that a standard of GV with no fish matrix could be eluted in a tight band of about 200 μl. However, the GV band diffused in the presence of the catfish matrix and therefore the capture envelope was expanded to 1.5 ml.

Two preliminary range finding incurred residue samples of catfish treated for 1 h at two levels (100 ppb and 10 ppb) with gentian violet have been assayed. The 100-ppb GV treatment yielded residues of 118 ppb LGV and 0.8 ppb GV. The 10-ppb GV treatment yielded residues of 44 ppb LGV and 0.4 ppb GV. These findings indicate that the sensitivity and selectivity of the method for real-life applications were sufficient. The incurred residue samples all indicate that the metabolite (LGV) is the marker compound to follow. The results of the malachite green incurred residue study also support that it is the metabolite (LMG) that is the marker compound that should be followed.

In conclusion, an analytical method is presented that is capable of assaying concurrently LGV and GV in a catfish matrix at the 1-ppb level.

Acknowledgement

Table 1
Recoveries of leucogentian violet (LGV) and gentian violet (GV) from fortified catfish (mean ± S.D., $n = 4$)

LGV and GV added (ppb)	Recovered			
	LGV (ppb)	LGV (%)	GV (ppb)	GV (%)
20	16.6 ± 0.2	83.1 ± 1.2	18.5 ± 0.4	92.7 ± 1.8
10	7.84 ± 0.40	78.4 ± 4.0	9.50 ± 0.22	95.0 ± 2.2
10[a,b]	7.97 ± 0.20	79.7 ± 2.0	8.99 ± 0.10	89.9 ± 1.0
1	0.84 ± 0.08	84 ± 8	0.93 ± 0.02	93 ± 2
1[a]	1.00 ± 0.15	100 ± 15	0.94 ± 0.06	94 ± 6
0	0.12 ± 0.03	–	0.08 ± 0.04	–

[a] Fortified and assayed on 2nd day.
[b] $n = 3$.

References

[1] D.J. Alderman, J. Fish Dis., 8 (1985) 289.

[2] L.G. Rushing and M.C. Bowman, J. Chromatogr. Sci., 18 (1980) 224.

[3] J.J. McDonald and C.E. Cerniglia, Drug Metab. Dispos., 12 (1984) 330.

[4] E. Martinez and W. Shimoda, J. Assoc. Off. Anal. Chem., 72 (1989) 742.

[5] J.E. Roybal, R.K. Munns, J.A. Hurlbut and W. Shimoda, J. Assoc. Off. Anal. Chem., 73 (1990) 940.

[6] R.K. Munns, J.E. Roybal, J.A. Hurlbut and W. Shimoda, J. Assoc. Off. Anal. Chem., 73 (1990) 705.

[7] D. Heller, J. Assoc. Off. Anal. Chem. Int., 75 (1992) 650.

[8] J.E. Roybal, R.K. Munns, D.C. Holland, R.G. Burkepile and J.A. Hurlbut, J. Assoc. Off. Anal. Chem. Int., 75 (1992) 433.

[9] J.L. Allen and J.R. Meinertz, J. Chromatogr., 536 (1991) 217.

[10] J.E. Roybal, A.P. Pfenning, R.K. Munns, D.C. Holland, J.A. Hurlbut and A.R. Long, J. Assoc. Off. Anal. Chem. Int., 78 (1995) 453.