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Journal of Chromatography B, 688 (1997) 325–330

JOURNAL OF CHROMATOGRAPHY B

Simultaneous determination of malachite green, gentian violet and their leuco metabolites in catfish or trout tissue by high-performance liquid chromatography with visible detection

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Received 27 February 1996; revised 26 June 1996; accepted 27 June 1996

Abstract

A sensitive analytical procedure for the determination of residues of leucomalachite green (LMG)–malachite green (MG) and leucogentian violet (LGV)–gentian violet (GV) in catfish or trout tissue is presented. Frozen (−20°C) fish fillets were cut into small pieces and blended in a Waring blender. A 20-g amount of homogenized fish tissue was 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 (0.8 g equiv.) were chromatographed isocratically in 10 min using an acetonitrile–buffer mobile phase on a short-chain deactivated (SCD) reversed-phase column (250×4.6 mm I.D.) in-line with a post-column PbO_2 oxidation reactor. The PbO_2 post-column reactor efficiently oxidized LMG to the chromatic MG, and LGV to the chromatic GV permitting visible detection at 588 nm for all four compounds. Linearity was demonstrated with standards over the range of 0.5–50 ng per injection. Recoveries of LMG, MG, LGV and GV from catfish tissues fortified at 10 ng/g were 75.4±3.0, 61.3±4.1, 72.6±3.7 and 87.9±2.5, respectively, while trout tissues fortified at 10 ng/g yielded recoveries of 82.6±2.3, 48.6±1.8, 72.1±2.1 and 83.8±4.6 (mean±S.D., n=4), respectively.

Keywords: Malachite green; Gentian violet; Leucomalachite green; Leucogentian violet

1. Introduction

Malachite green (MG) and gentian violet (GV), triphenylmethane dyes, are on the Food and Drug Administration's (FDA's) priority list for fish drugs that need analytical methods development. MG and GV are not approved by the FDA for use in the aquaculture industry. However, they have potential for misuse because of their anti-parasitic and anti-microbial properties. 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. Currently, circumstantial evidence suggests that MG continues to be used [1]. MG and GV are 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 man, it has been shown that the intestinal microflora systems [3] are capable of converting GV to the leuco form (LGV). The FDA,

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0378-4347/97/$17.00 Published by Elsevier Science B.V.
PII S0378-4347(96)00298-8

therefore, has need of a sensitive analytical method for the determination of residues of MG and GV, and their metabolites LMG and LGV in catfish and trout to monitor illicit use and for potential use in enforcement proceedings. The structures of these dyes and their leuco forms are shown in Fig. 1.

Several methods have been reported in the scientific literature for the determination of LMG–MG and for LGV–GV in a variety of matrices. Most of these methods employed liquid chromatography with UV-Vis detection or electrochemical detection [4–8]. In 1991 Allen and Meinertz [9] reported a high-performance liquid chromatography (HPLC) method for separating the leuco and chromatic forms of two triphenylmethane dyes LMG–MG and LGV–GV. The leuco form was oxidized to the chromatic form with an in-line post-column cartridge packed with 10% PbO_2–Celite 545 with subsequent detection of both forms by visible spectrophotometry. Their method eliminated the need to split the sample to assay the leuco forms by difference before and after complete oxidation. No methods were found in the literature for the simultaneous analysis of all four compounds in catfish or trout tissues. However, Roybal et al. [10] reported a method for the analysis of MG and its metabolite (LMG) in catfish tissue. Roybal's method employing HPLC with PbO_2 post-column oxidation (LMG→MG) and visible detection was modified for our application for concurrent analyses of LMG, LGV, MG and GV in edible catfish and trout samples.

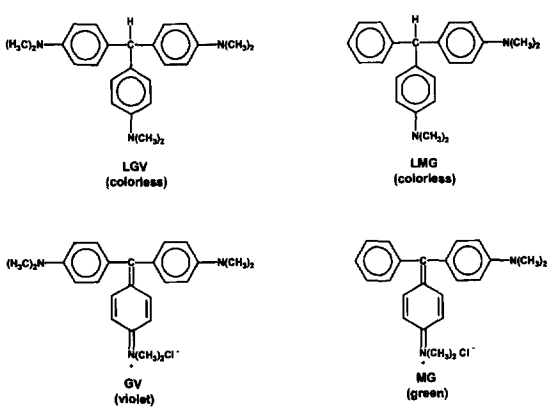

Fig. 1. Chemical structures of leucomalachite green (LMG), leucogentian violet (LGV), malachite green (MG) and gentian violet (GV).

2. Application

An analytical method is presented that is capable of assaying concurrently LMG, LGV, MG and GV at levels as low as 5 ng/g in trout and 10 ng/g in catfish. This method, therefore, may be applicable for determining residues of MG and GV, and their metabolites LMG and LGV in catfish and trout in order to monitor for their illicit use and for potential use in enforcement proceedings.

3. Experimental

3.1. Chemicals

LMG, LGV and malachite green oxalate were obtained from Aldrich (Milwaukee, WI, USA) and were used as received (Aldrich listed no purity data). GV was obtained from Hilton-Davis (Cincinnati, OH, USA) and had previously been assayed to contain 94.8% GV, 4.3% methyl violet (MV) and 0.5% LGV. Lead dioxide (PbO_2) and hydroxylamine hydrochloride were from Mallinckrodt (Chesterfield, MO, USA) and were AR grade. Basic alumina, Brockman activity I, and di(ethylene glycol) were purchased from Fisher (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).

3.2. Fish samples

3.2.1. Preparation

Several pounds of catfish and trout fillets were purchased at a local market and stored at −20°C. Individual fish were then cut into small pieces and blended in a Waring blender. These were stored in individual zip-lock plastic bags until required for processing and analysis.

3.2.2. Extraction

Quadruplicate 20.0-g fish samples were weighed into 250-ml Falcon polypropylene tubes obtained from Becton Dickinson (Lincoln Park, NJ, USA). A 3-ml volume of aqueous 0.25 g/ml hydroxylamine

hydrochloride, 5 ml of aqueous 0.05 *M* *p*-TSA, and 20 ml of aqueous 0.1 *M* 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) was 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.

3.2.3. 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 di(ethylene glycol) were added to each. The separatory funnels were then shaken vigorously by hand for 1 min and left to stand 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) rotary evaporator at 65°C to approximately 2–5 ml. At this point the samples can be reserved overnight in the dark.

3.2.4. Solid-phase extraction

J.T. Baker (Phillipsburg, NJ, USA) 6-ml (1000 mg) neutral alumina cartridges and Varian (Harbor City, CA, USA) 2.8-ml (500 mg) Bond Elut PRS cartridges were pre-washed with 5 ml acetonitrile. The alumina cartridge was then placed on top 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 were also applied to the cartridge assemblies. Finally, a 5-ml amount of acetonitrile was rinsed through each cartridge. All wash fractions and the alumina cartridges were then discarded. A 2-ml volume of deionized water was rinsed through each PRS cartridge followed by a 1 ml volume of acetonitrile–0.1 *M* ammonium acetate buffer (50:50) adjusted to pH 4.5 with glacial acetic acid. These fractions were also discarded. The LMG, MG, LGV and GV residues were eluted from the PRS cartridges with 2 ml of the above acetonitrile–buffer and collected in graduated 2.5-ml centrifuge tubes containing 0.5 ml of 2.5 mg/ml hydroxylamine hydrochloride in water. Samples, standards and controls alike were therefore contained in 2.5 ml of a mixture of 40% acetonitrile–buffer. All fish extract samples were 8 g equiv./ml (i.e., 0.8 g equiv./100 μl injection).

3.3. Liquid chromatography

The liquid chromatograph consisted of a Waters (Milford, MA, USA) Model 510 pump and a Rheodyne (Cotati, CA, USA) Model 7125 injector with a 200-μl loop. Separation system A consisted of a 20×2.0 mm I.D. pellicular CN guard column, a Supelco (Bellefonte, PA, USA) 5 μm LC–CN 250×4.6 mm I.D. column and a 20×2.0 mm I.D. PbO_2 oxidative post-column. The mobile phase was 60:40 acetonitrile–buffer. The buffer was prepared by adding 3.85 g ammonium acetate 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 a pressure of 103 bar. Separation system B consisted of a 20×2.0 mm I.D. pellicular C_{18} guard column, a SynChrom (Lafayette, IN, USA) 5 μm SynChropak 150×4.6 mm I.D. SCD-100 column, and a 20×2.0 mm I.D. PbO_2 oxidative post-column. The mobile phase was 55:45 acetonitrile–buffer. The buffer was prepared by adding 0.4 g ammonium acetate and 1 ml of triethylamine (TEA) to approximately 400 ml of water. The pH was then

adjusted to 3.6 with glacial acetic acid. This was diluted with water to 450 ml and added to 550 ml of acetonitrile. The flow-rate was 2 ml/min at a pressure of 138 bar. The oxidative post-column on both systems was hand packed with PbO_2 with no Celite 545 added. Detection was with a Hewlett Packard (Atlanta, GA, USA) Model 1050 UV-Vis detector set at 588 nm. 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. Quantitation was performed using external standards and was based on peak areas. The controls, having no peak areas to measure, were quantified by using the baseline peak-to-peak noise.

3.4. Recovery experiments

Quadruplicate 20.0-g fish samples were weighed into 250-ml Falcon polypropylene tubes and fortified with 0, 100, 200 or 400 ng LMG, MG, LGV and 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.

4. Results and discussion

An isocratic HPLC system similar to that used by Roybal et al. [10] to assay LMG–MG in catfish and repeated by Rushing et al. [11] to assay LGV–GV in catfish was attempted for the four component admixture LMG, LGV, MG and GV. The isocratic chromatography of a 5 ng/100 μl injection of an admixture standard of LMG, LGV, MG and GV on a cyano column resulted in coelution of LMG and LGV at a retention time (t_R) of 5.8 min. The t_R of MG and GV were 10.2 and 11.9 min, respectively. The very small peak at 10.7 min was an impurity (MV) in the GV standard. This separation was deemed inadequate due to its inability to separate LMG from LGV. Complete separation of this admixture standard on a short-chain deactivated (SCD) reverse phase column in under 10 min is shown in Fig. 2. This column separated all four components. The LMG–LGV pair

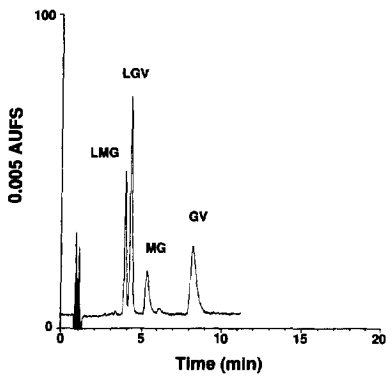

Fig. 2. HPLC chromatogram of a 4-ng admixture standard of LMG, LGV, MG and GV on a SCD column, 100 μl injection, detection at 588 nm.

were separated with almost baseline resolution. The t_R of LMG and LGV were 4.0 and 4.3 min and the t_R of MG and GV were 5.3 and 8.2 min, respectively. The very small peak at 6.1 min was MV.

LMG and LGV are chromatographed on the column as the leuco form (reduced form). After separation on the analytical column, they are oxidized by the PbO_2 post-column reactor from the colorless leuco form to their respective chromatic form. LMG was, therefore, detected as MG, and LGV was detected as GV. The LMG and LGV were distinguished from MG and GV by their earlier t_R. 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 ng/g level analytes were totally obscured by the control fish tissue background interferences (267 nm chromatogram not shown). Also, the PbO_2 oxidative post-column was packed with 100% PbO_2 rather than the 10% PbO_2–Celite 545 as previously described [10]. No increase in the back pressure of the LC system was noted when the 20×2.0 mm I.D. post-column was hand packed with 100% PbO_2. Detection was achieved using the visible maximum for GV at 588 nm (the maximum for MG at 618 nm was above the upper range [600 nm] of the detector). Linearity was demonstrated with standards over the range of 0.5–50 ng per injection. The regression coefficients for a linear fit are presented in Table 1. The correlation coefficient

Table 1
Regression coefficients for linearity line ($y=A_0+A_1x$)

Compound	A_0	A_1	r^2	S.E.	n
LMG	−19±3	511.1±0.1	1.000000	5.1	4
LGV	−51±22	921.1±0.9	0.999998	36.4	4
MG	−30±13	336.8±0.5	0.999995	21.5	4
GV	−66±36	810.6±1.4	0.999994	60.1	4

A_0=y intercept; A_1=slope of line; r^2=correlation coefficient of regression line; S.E.=standard error of regression line; n=single data point for each of n concentration levels; y=area response; x=ng/inj (100 μl); range over which linearity studied=0.5–50 ng/injection.

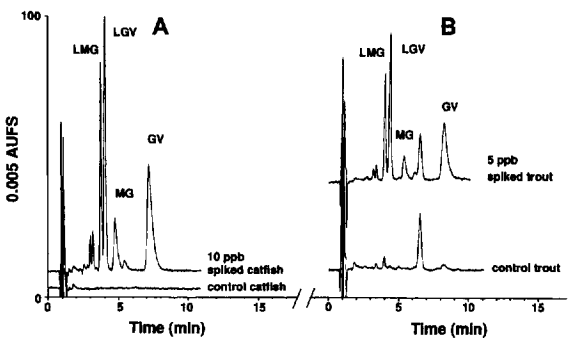

Fig. 3. (A) Overlay chromatograms of 10 ng/g (ppb) LMG, LGV, MG and GV fortified catfish and control catfish, 0.8 g equiv./100 μl injection, SCD column, @ 588 nm. (B) Overlay chromatograms of 5 ng/g (ppb) LMG, LGV, MG and GV fortified trout and control trout, 0.8 g equiv./100 μl injection, SCD column, @ 588 nm.

(r^2) for LMG and LGV demonstrated that the oxidation of the PbO_2 post-column was efficient and linear.

The isocratic separation of LMG–MG by Roybal et al. [10] included the addition of the ion pairing agent, *p*-TSA. In the present study, the addition of this agent had no effect on the t_R or on peak shape and was therefore omitted from the mobile phase. However, experiments were not conducted on the effects of this pairing agent on the recoveries of LMG, LGV, MG and GV from catfish or trout matrices and as a result the *p*-TSA was retained in the extraction process as described by Roybal et al. [10].

Fig. 3A is a composite of chromatograms of a 10 ng/g LMG, LGV, MG and GV fortified catfish sample and its corresponding catfish control sample. No interfering peaks were seen in the control. Fig. 3B is a composite of chromatograms of a 5 ng/g LMG, LGV, MG and GV fortified trout sample and its corresponding trout control sample. The 6-min peak in the control trout chromatogram did not interfere with quantification of any of the analytes of interest. Fig. 3 demonstrates that there should be no difficulty in assaying these four analytes at the 5 ng/g level.

Table 2 lists the recoveries for 20, 10 and 5 ng/g LMG, LGV, MG and GV fortified trout and for 10 ng/g fortified catfish. Fortification was performed with admixture standards. No individual analyte recoveries were determined. The average percent recoveries for LMG, LGV, MG and GV from 20, 10 and 5 ng/g fortified trout were 81, 71, 53 and 87%,

Table 2
Recoveries of leucomalachite green (LMG), leucogentian violet (LGV), malachite green (MG) and gentian violet (GV) from fortified trout and catfish (mean±S.D., n=4)

Added (ng/g)	Recovered							
	LMG (ng/g)	LMG (%)	LGV (ng/g)	LGV (%)	MG (ng/g)	MG (%)	GV (ng/g)	GV (%)
Trout								
20[a]	15.3±0.4	76±2	13.5±0.2	68±1	10.0±0.3	50±1	17.3±0.1	86±1
10	8.3±0.2	83±2	7.2±0.2	72±2	4.9±0.2	49±2	8.4±0.5	84±5
5	4.1±0.1	83±3	3.7±0.1	73±2	3.0±0.1	60±2	4.5±0.2	90±3
0	0.2±0.1	–	0.1±0.0	–	0.2±0.0	–	0.6±0.3	–
Catfish								
10	7.5±0.3	75±3	7.3±0.4	73±4	6.1±0.4	61±4	8.8±0.3	88±3
0	0.1±0.1	–	0.0±0.0	–	0.2±0.0	–	0.2±0.0	–

[a] n=3

respectively. The control trout samples equated to 0.2, 0.1, 0.2 and 0.6 ng/g, respectively. The limit of detection (LOD) based on 3 times noise for LMG, LGV, MG and GV in trout were 0.6, 0.3, 0.6 and 1.8 ng/g and the limit of quantification (LOQ) based on five times the noise level were 1.0, 0.5, 1.0 and 3.0 ng/g, respectively. The results of LMG, LGV, MG and GV from 10 ng/g fortified catfish were similar with recoveries of 75, 73, 61 and 88%, respectively. The control catfish samples equated to 0.1, 0.0, 0.2 and 0.2 ng/g, respectively. The LOD for LMG, LGV, MG and GV in catfish were 0.3, 0.3, 0.6 and 0.6 ng/g and the LOQ were 0.5, 0.5, 1.0 and 1.0 ng/g, respectively.

Two preliminary range finding incurred residue samples of catfish treated with GV were assayed by this method. One fish was exposed to 100 ng/g GV in water for 1 h and another fish to 10 ng/g. The fish were then moved to clean aquaria for 24 h before being sacrificed. The filleted catfish were stored at −60°C until assayed. The 100 ng/g GV treatment yielded incurred residues of 118 ng/g LGV and 0.8 ng/g GV. The 10 ng/g GV treatment yielded incurred residues of 44 ng/g LGV and 0.4 ng/g GV (essentially the LOD). These observations indicate that the sensitivity and selectivity of the method for real-world applications were sufficient. The incurred residue samples also indicate that the leuco metabolite (LGV) is the marker compound to follow. Roybal's et al. [10] results of incurred residues of malachite green in catfish also support the leuco metabolite (LMG) as the marker compound. The LGV–GV incurred residue samples were subsequently assayed by LC–MS using a SynChropak SCD column with a serial number approximately 100 000 larger than the original HPLC–Vis assays. Modification of the mobile phase was required to obtain an equivalent separation. The acetonitrile content was increased from 55 to 60%, the TEA was removed (for LC–MS compatibility) and the amount of ammonium acetate reduced from 0.4 g/l to 0.2 g/l. The identity of the LGV and GV incurred residue peaks were confirmed by LC–MS (MS data to be published).

Acknowledgments

The authors wish to thank José E. Roybal of the Food and Drug Administration, Animal Drug Research Center, Denver Federal Center for his assistance and for providing a prepublication draft of his procedure for the determination of malachite green and leucomalachite green in catfish.

References

[1] D.J. Alderman, J. Fish Diseases, 8 (1985) 289.
[2] O.G. Prokofeva and M.A. Zabezhinskii, Vop. Onkol., 22 (1976) 66.
[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.
[11] L.G. Rushing, S.F. Webb and H.C. Thompson Jr., J. Chromatogr. B, 674 (1995) 125.