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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF GENTIAN VIOLET, ITS DEMETHYLATED METABOLITES, LEUCOGENTIAN VIOLET AND METHYLENE BLUE WITH ELECTROCHEMICAL DETECTION

JOSE E. ROYBAL*, ROBERT K. MUNNS, JEFFREY A. HURLBUT and WILBERT SHIMODA
Animal Drug Research Center, Food and Drug Administration, Denver, CO 80225-0087 (U.S.A.)
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SUMMARY

High-performance liquid chromatographic conditions are reported for the electrochemical detection (ED) of Gentian Violet, its demethylated metabolites, Leucogentian Violet and Methylene Blue. Gentian Violet, its demethylated metabolites and Leucogentian Violet were separated within 14 min on a cyano column eluted isocratically with methanol–buffer (60:40) as the mobile phase. ED responses for Gentian Violet, Leucogentian Violet and Methylene Blue were linear over the ranges 0.54–6.75, 0.50–25.2, and 5.7–285 ng, respectively. Under these conditions, the compounds were eluted in the following order: Leucogentian Violet, N″-2-tetramethylpararosaniline chloride, N′-1-tetramethylpararosaniline chloride, pentamethylpararosaniline chloride and Gentian Violet. Methylene Blue and Gentian Violet had essentially the same retention time under these parameters. The detection limit for Gentian Violet, its demethylated metabolites and Leucogentian Violet was determined to be 0.1 pmol. A detection limit of 3 pmol was established for Methylene Blue. Detector response, elution, separation, linearity and sensitivity of detection are discussed.

INTRODUCTION

Marketed in 1951, Gentian Violet (GV) (hexamethyl pararosaniline chloride, CAS 548-62-9), also known as Crystal Violet (Colour Index No. 42555) (Fig. 1), has been used as an agent in poultry feeds to inhibit mold and fungal growth. It has also been used to control fungal and intestinal parasites in humans and for other antimicrobial purposes in veterinary medicine.

A point of clarification should be made regarding terminology. Although "Gentian Violet" and "Crystal Violet" are used interchangeably, some distinctions should be mentioned. Crystal Violet is the pure hexamethylpararosaniline chloride, $C_{25}H_{30}ClN_3$. Methyl Violet refers to pentamethylpararosaniline chloride (PENTA). GV, commonly used as an antiseptic, is the commercial product, which is a mixture of Crystal Violet and Methyl Violet that may also contain some tetramethylpararosaniline chloride. The requirement of the *U.S. Pharmacopeia XXI* (USP XXI) for GV is

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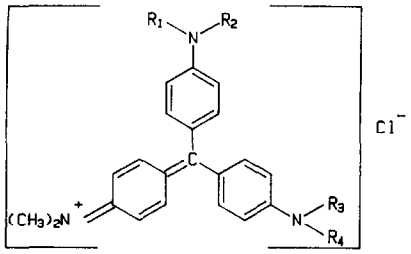
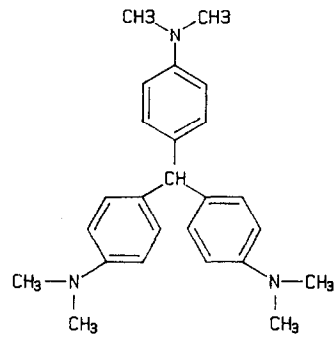


-methylpararosaniline chloride

	R_1	R_2	R_3	R_4
Hexa	CH_3	CH_3	CH_3	CH_3
Penta	CH_3	CH_3	CH_3	H
N′-1 tetra	CH_3	CH_3	H	H
N″-2 tetra	CH_3	H	CH_3	H



Leucogentian Violet
(Leuco Crystal Violet)
CAS 603-48-5

Fig. 1. Structures of Gentian Violet, its demethylated metabolites and Leucogentian Violet.

that it shall not contain less than 96% Crystal Violet[1]. The USP reference standard for GV was used for all the work reported here.

GV belongs to a class of compounds which have been recognized as animal carcinogens, the triphenylmethane dyes[2]. McDonald *et al.*[3] reported that demethylation of GV occurred in the uninduced liver microsomes of several species examined. The metabolic pattern was found to be similar for mouse, rat, hamster, guinea pig and chicken, regardless of sex. The major demethylated metabolites were PENTA and two isomers, N,N,N′,N′-tetramethylpararosaniline chloride (N′-1-TETRA) and N,N,N′,N″-tetramethylpararosaniline chloride (N″-2-TETRA) (Fig. 1). Intestinal microflora, under anaerobic conditions, are also capable of metabolizing GV; they reduce GV to its leuco derivative (Fig. 1)[4]. According to the National Center for Toxicological Research[5], "This leuco derivative is then structurally similar to the classical aromatic amine carcinogens.".

Methylene Blue (MB) [3,7-bis(dimethylamino)phenothiazin-5-ium chloride, CAS 61-73-4] (Fig. 2), also known as Basic Blue 9 (Colour Index No. 520125) is classified for veterinary use as an antiseptic and disinfectant and as an antidote for cyanide and nitrate poisoning. The Food and Drug Administration/Food Safety and Inspection Service Animal Drug Methodology Task Force expressed concern that MB is considered a mutagen[6–8].

A growing concern over the carcinogenicity of GV, its demethylated metabolites and Leucogentian Violet (LGV) and the mutagenicity of MB necessitates the development of methods to monitor these residues in tissues of food-producing animals. Two criteria of any good residue method are sensitivity and specificity.

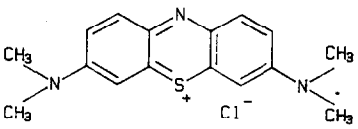


Fig. 2. Structure of Methylene Blue.

Because GV, its demethylated metabolites and MB are all highly colored compounds, detection in the visible range has been the method of choice[9–15]. However, working in the visible range does not provide the sensitivity required for trace/residue analysis (< 1 ppm). The leuco form of GV is not a colored species and gives very poor response in the visible region. As the UV absorptivity of GV, its demethylated metabolites and MB is low, simultaneous determination of all the above compounds using absorption detection is not possible.

This paper describes the development of a high-performance liquid chromatographic (HPLC) system using electrochemical detection (ED) for the simultaneous determination of LGV, GV and its demethylated metabolites. The response and chromatographic behavior of MB are also discussed.

EXPERIMENTAL

Chromatographic system

The HPLC system used in this study was modular in design. The system consisted of a Waters Assoc. (Milford, MA, U.S.A.) dual pump (Models 590 and 510) with a Waters automatic gradient control unit (Model 680) and a Waters universal liquid chromatograph injector (Model U6K). An Alltech Assoc. (Deerfield, IL, U.S.A.) CN reversed-phase, 250 × 4.6 mm I.D., 5-μm column (Stock No. 60138) was used to separate the methylpararosaniline compounds. The mobile phase was methanol–0.1 *M* sodium acetate (60:40) with the pH adjusted to 4.5 with aldehyde-free glacial acetic acid; it also contained 50 mg EDTA disodium salt per liter to help reduce background current and drift. The mobile phase flow-rate was maintained at 0.8 ml/min through the analytical column, which was operated at ambient temperature. The injection volume varied from 5 to 25 μl, depending on the level of analyte.

A Bioanalytical Systems (W. Lafayette, IN, U.S.A.) single channel LC-4B amperometric detector was used for ED determination. The detector cell consisted of a glassy carbon working electrode with a TG-2M thin-layer cell gasket. For this particular work the potential was varied from larger to smaller potentials as the need arose. The reference electrode was Ag/AgCl. The normal operating current range was set at 5 nA for full-scale deflection (f.s.d.).

Reagents and materials

All solvents were distilled-in-glass (Burdick & Jackson Labs., Muskegon, MI, U.S.A.). The glacial acetic acid was ACS grade, aldehyde-free (J. T. Baker, Phillipsburg, NJ, U.S.A.). Water for HPLC was deionized, 18 MΩ, glass-distilled. All solvents used in the HPLC system were filtered through a 0.5-μm Fluoropore filter (No. FHLP 04700, Millipore, Bedford, MA, U.S.A.).

Standard stock solutions of GV, N′-1-TETRA and N″-2-TETRA were prepared in 95% ethanol. Standard stock solutions of LGV, PENTA and MB were prepared in methanol. All subsequent dilutions of standard stock solutions were prepared with methanol.

RESULTS AND DISCUSSION

The initial HPLC was performed by using parameters similar to those reported by Rushing and Bowman[2], which consisted of a Nova-Pak, 150 × 3.9 mm I.D., 5-μm

RP-C_{18} column and a mobile phase of methanol–buffer (85:15) (buffer = 0.01 *M* KH_2PO_4, adjusted to pH 3 with 0.33 *M* H_3PO_4), at a flow-rate of 1 ml/min. The effluent was monitored at 588 nm. This system gave an elution order, in increasing elution time, of N′-1-TETRA, N″-2-TETRA, PENTA and GV. LGV had the longest elution time but had to be monitored at 280 nm.

To apply ED to the chromatographic system described above, a tee was added at the outlet of the HPLC column and a make-up mobile phase was introduced at this junction. The make-up mobile phase was methanol–buffer (20:80) introduced at 0.5 ml/min. The resultant composition of the mixture through the electrochemical detector was methanol–buffer (60:40). Introduction of the make-up solvent was necessary to allow the electrode reaction to occur. A practical guideline was to maintain a minimum concentration of 35% electrolyte solution to convey charge through the ED cell. A modifier (*e.g.*, methanol, acetonitrile, etc.) impedes the charge.

Although this composition gave a good signal (ED response), mixing of the solvents at the tee generated gas which created spiking at the recorder output. After several unsuccessful attempts to remove the gas from the line, a cyano column was used. Higher polarity of this stationary phase allowed for the elution of the compounds at lower modifier content.

Both methanol and acetonitrile were evaluated as modifiers in the HPLC system (Table I). Acetonitrile gave better separation of LGV from the other compounds (GV, PENTA, N′-1-TETRA, N″-2-TETRA, MB) (Fig. 3). Acetonitrile increased the response factor, and therefore the sensitivity, by a factor of 3–8 over that of methanol. The operating column pressure was approximately 1300 p.s.i. with acetonitrile. The column pressure with methanol was approximately 2500 p.s.i. Methanol, on the other hand, resolved not only the LGV from the other compounds but also the N′-1-TETRA from the N″-2-TETRA isomer (Fig. 4).

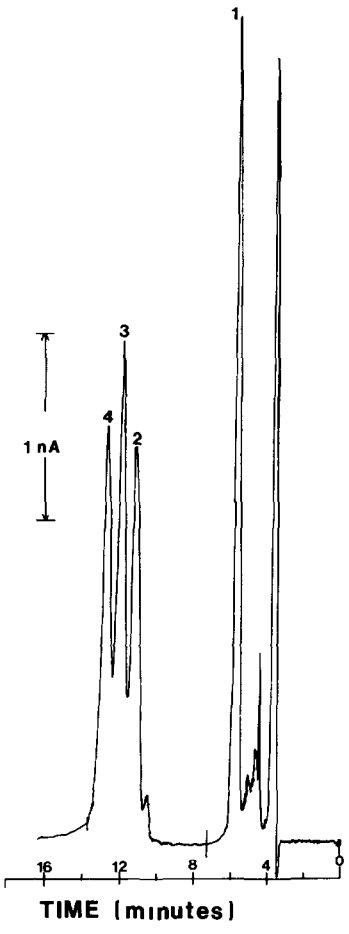
The main reasons for selecting methanol as the modifier for our system were that it allowed slightly longer retention times for LGV and it resolved the two tetramethyl isomers. For the detection of MB, acetonitrile was preferred because of the increase in sensitivity.

TABLE I

EVALUATION OF METHANOL AND ACETONITRILE AS MODIFIERS

Abbreviations: t_R = retention time; RRT = relative retention time with respect to LGV; RF = response factor; ND = not determined; GV = Gentian Violet; PENTA = pentamethylpararosaniline chloride; N′-1-TETRA = N,N,N′,N′-tetramethylpararosaniline chloride; N″-2-TETRA = N,N,N′,N″-tetramethylpararosaniline chloride; LGV = Leucogentian Violet; MB = Methylene Blue.

Compound	*Methanol*			*Acetonitrile*		
	t_R *(min)*	*RRT*	*RF (nA/ng)*	t_R *(min)*	*RRT*	*RF (nA/ng)*
GV	11.2	1.44	0.59	12.6	2.25	2.21
PENTA	9.8	1.26	ND	11.8	2.11	ND
N′-1-TETRA	9.2	1.18	ND	11.2	2.00	ND
N″-2-TETRA	8.6	1.10	ND	11.0	1.96	ND
LGV	7.8	1.00	1.14	5.6	1.00	6.50
MB	11.2	1.44	0.036	12.0	2.14	0.291



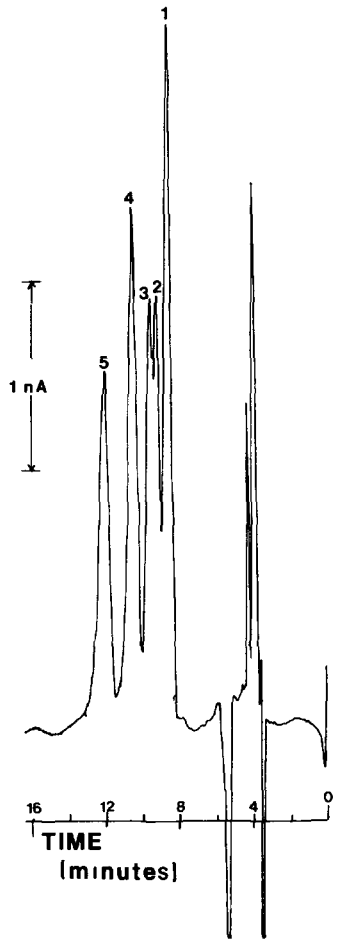


Fig. 3. Typical HPLC chromatogram obtained by using acetonitrile as the modifier, a 5-μl injection of standard mixture, a current-range setting of 5 nA f.s.d. and a potential of +1.000 V *versus* Ag/AgCl reference electrode. Peaks: 1 = Leucogentian Violet (0.101 μg/ml); 2 = N,N,N′,N′-tetramethylpararosaniline chloride (0.102 μg/ml) and N,N,N′,N″-tetramethylpararosaniline chloride (0.076 μg/ml); 3 = pentamethylpararosaniline chloride (0.114 μg/ml); 4 = Gentian Violet (0.107 μg/ml).

Fig. 4. Typical HPLC chromatogram obtained by using methanol as the modifier, a 20-μl injection of standard mixture, a current-range setting of 5 nA f.s.d. and a potential of +1.000 V *versus* Ag/AgCl reference electrode. Peaks: 1 = Leucogentian Violet (0.101 μg/ml); 2 = N,N,N′,N″-tetramethylpararosaniline chloride (0.076 μg/ml); 3 = N,N,N′,N′-tetramethylpararosaniline chloride (0.102 μg/ml); 4 = pentamethylpararosaniline chloride (0.114 μg/ml); 5 = Gentian Violet (0.107 μg/ml).

The elution order from the cyano column with methanol in the mobile phase was LGV, N″-2-TETRA, N′-1-TETRA, PENTA and GV (Fig. 4). MB had essentially the same retention time as GV.

A literature review showed no documentation on the ED of these compounds. Therefore, after establishing the HPLC conditions, the ED characteristics of the compounds of interest were evaluated. It should be noted that equilibration of the

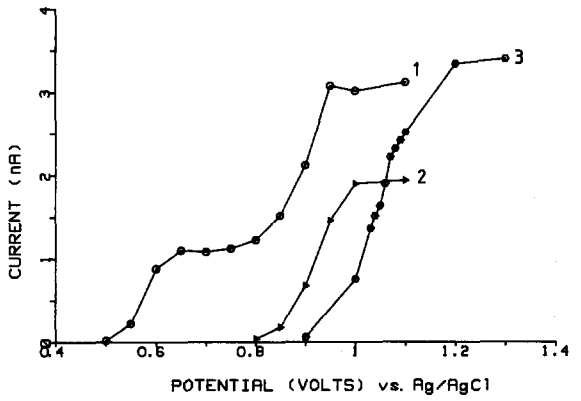


Fig. 5. Voltammogram of (1) Leucogentian Violet, (2) Gentian Violet and (3) Methylene Blue.

mobile phase with the column, for at least 16 h (overnight), was required for proper resolution when the HPLC system was initially set up.

Voltammograms were prepared by injecting standard solutions of each compound and recording the detector response at intervals of 0.050 V over a range of +1.300 to +0.500 V (Figs. 5 and 6). The plots show a very narrow range of oxidation potential, from +0.800 to +1.100 V for GV and its demethylated metabolites, with all of these compounds reaching a maximum at about +1.000 V. LGV showed a greater range of oxidation potential, with an apparent double plateau. This was verified by preparing a voltammogram using smaller increments of +0.020 and +0.010 V for LGV and GV, respectively (Fig. 7), which clearly indicates a two-phase oxidation (double plateau) for LGV. It also verifies that GV undergoes a single oxidation within the potential range of +1.100 to +0.500 V.

Although the reaction scheme is beyond the scope of this paper, a possible mechanism is suggested. The initial (phase one) or more easily oxidized state occurs at +0.650 V; at this potential the leuco (white) base is oxidized to the carbinol (colored)

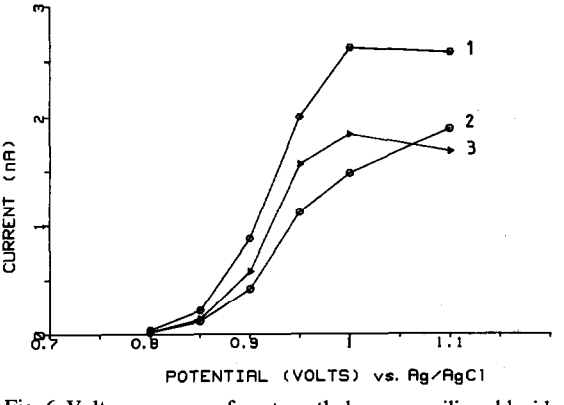


Fig. 6. Voltammogram of pentamethylpararosaniline chloride and two tetramethylpararosaniline chloride isomers. 1 = PENTA; 2 = N′-1-TETRA; 3 = N″-2-TETRA.

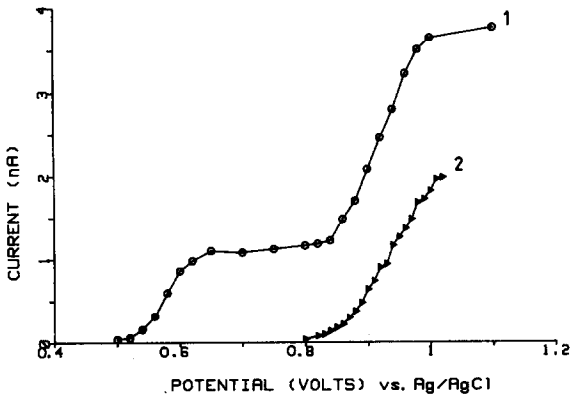


Fig. 7. Voltammogram of (1) Leucogentian Violet and (2) Gentian Violet.

base. Normally, once the triphenylmethane is oxidized to the triphenylcarbinol derivative, the reaction stops because the tertiary alcohol cannot be oxidized further without destruction of the benzene ring. Unlike the triphenylmethane, however, the N,N-dimethylaniline derivatives can be oxidized further under acidic conditions, leading to a loss of a methyl group on the dimethylaniline part of the molecule[16]. This reaction is reflected by phase two at +1.000 V.

In the case of MB, oxidation is more difficult. Although the reduction of MB to its leuco base is well documented[17], oxidation of MB has not been reported. We suspect that a demethylation of the dimethylaniline group similar to that of the methylpararosanilines is involved, but is occurring at the higher potential of +1.200 V (Fig. 6).

Linearity plots of GV, LGV and MB were prepared. Data for these plots were obtained using different current-range settings, from 2 to 50 nA f.s.d., to accommodate the wide range of amounts injected. The response was shown to be linear over the range 0.5–25 ng for GV and LGV and 5–300 ng for MB. From these plots, it was evident that the response of LGV is approximately twice that of GV, and the decreased response of MB with respect to GV and LGV is demonstrated (Table I). Linear regression data and correlation coefficients (r) for these plots are listed in Table II.

In summary, an HPLC–ED system was developed which can simultaneously detect, resolve and quantitate GV, its demethylated metabolites and LGV within 14 min. The detection limits of GV, its demethylated metabolites and LGV were

TABLE II

LINEAR REGRESSION DATA ($y = mx + b$) AND CORRELATION COEFFICIENTS (r) FOR PLOTS OF GV, LGV AND MB

Compound	m (ng^{-1})	b	r
GV	0.6475	−0.2438	0.9975
LGV	1.3008	−0.4305	0.9978
MB	0.0318	0.1973	0.9925

determined to be 0.1 pmol at a 3 × noise level at +1.000 V and a current range of 0.5 nA f.s.d. Additionally, the system has the versatility to detect and reliably quantitate MB at levels as low as 3 pmol. The system meets the requirements of sensitivity and specificity described earlier.

In future work we plan to apply this system to the determination of the residues of these compounds in animal tissues.

ACKNOWLEDGEMENTS

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