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Post-column reaction for simultaneous analysis of chromatic and leuco forms of malachite green and crystal violet by high-performance liquid chromatography with photometric detection

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ABSTRACT

The chromatic and leuco forms of malachite green and crystal violet were readily separated and detected by a sensitive and selective high-performance liquid chromatographic procedure. The chromatic and leuco forms of the dyes were separated within 11 min on a C_{18} column with a mobile phase of 0.05 *M* sodium acetate and 0.05 *M* acetic acid in water (19%) and methanol (81%). A reaction chamber, containing 10% PbO_2 in Celite 545, was placed between the column and the spectrophotometric detector to oxidize the leuco forms of the dyes to their chromatic forms. Chromatic and leuco malachite green were quantified by their absorbance at 618 nm; and chromatic and leuco Crystal Violet by their absorbance at 588 nm. Detection limits for chromatic and leuco forms of both dyes ranged from 0.12 to 0.28 ng. A linear range of 1 to 100 ng was established for both forms of the dyes.

INTRODUCTION

Malachite green oxalate {N-[4-[[4-(dimethylamino)-phenyl]phenylmethylene]-2,5-cyclohexadien-1-ylidine]-N-methylmethanaminium oxalate} has been used to treat external fungal and protozoan infections in fish since 1933 [1]. Crystal violet (hexamethylpararosaniline chloride) has long been used to inhibit mold and fungal growth in poultry feeds, control fungal and intestinal parasites in humans, and combat microbial infections in domestic animals. Both chemicals belong to the triphenylmethane class of dyes, some of which are animal carcinogens [2]. Meyer and Jorgenson [3] demonstrated that malachite green caused significant developmental abnormalities when administered to eggs of Rainbow trout (*Oncorhynchus mykiss*) and to pregnant New Zealand white rabbits (*Oryctolagus cuniculus*). Crystal violet was determined to be mutagenic to *Bacillus subtilis*, *Escherichia coli*, and *Salmonella typhimurium* [4] and cytotoxic to mammalian cells [5].

Animals reduce malachite green and crystal violet to their leuco forms [6–8]. However, the leuco forms of these dyes are also their precursors during production and could be contaminants of commercial preparations of the dyes. Although the leuco forms of these dyes have no acute adverse effects in animals, Roybal *et al.* [9]

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quoting the National Center for Toxicological Research, stated that 'This leuco derivative is then structurally similar to the classical aromatic amine carcinogens'.

Concern about the health risks associated with the use of malachite green and crystal violet and their metabolites requires that methods be developed to monitor these residues. The chromatic and leuco forms of the dyes can now be analyzed simultaneously by high-performance liquid chromatography (HPLC) only with electrochemical detection [9]. Bauer *et al.* [10] analyzed for malachite green and leuco malachite green by splitting the sample and oxidizing half of the sample to all malachite green with PbO_2. The amount of leuco malachite green in the sample was determined by the difference in the malachite green found in the subsamples. We here present a method of separating the chromatic and leuco forms of two triphenylmethane dyes by HPLC, with oxidation of the leuco forms to the chromatic forms (Fig. 1), and detection of both forms by visible spectrophotometry. Detection in the visible region of the spectrum has the advantage of increased specificity as few potential interferences adsorb light in this area of the spectrum.

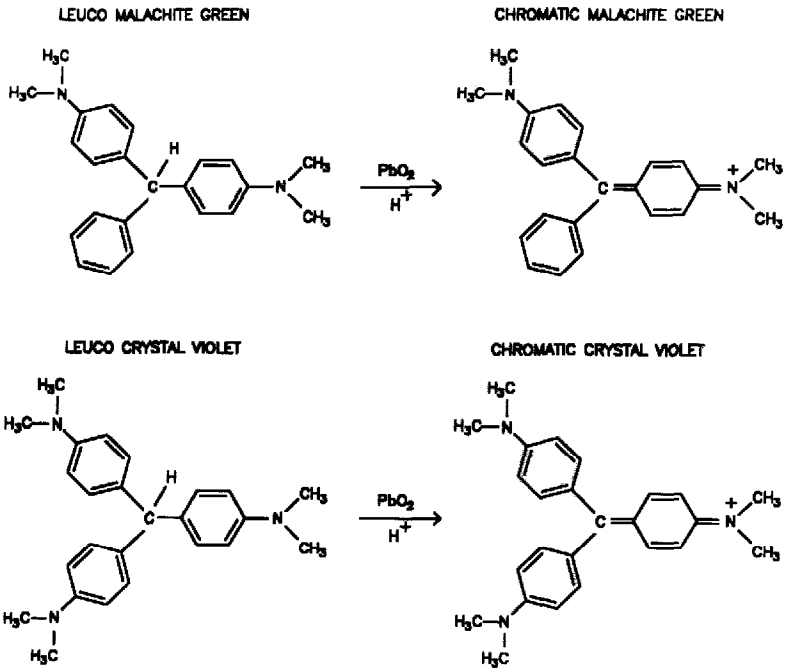

Fig. 1. Leuco malachite green and leuco crystal violet, when mixed with PbO_2 in the presence of acid, are oxidized to the chromatic dye forms.

EXPERIMENTAL[a]

Chemicals and reagents

All chemicalls were of reagent grade. Malachite green oxalate and leuco malachite green [4,4′-benzylidenebis-(N,N-dimethylaniline)] were obtained from Eastman Kodak (Rochester, NY, U.S.A.). Crystal violet {N-[4-[bis[4-(dimethylamino)-phenyl]-methylene]-2,5-cyclohexadien-1-ylidine]-N-methyl-methanaminium chloride}, leuco crystal violet [4,4′,4″-methylidynetris (N,N-dimethylaniline)] and anhydrous acetic acid, sodium salt, were obtained from Aldrich (Milwaukee, WI, U.S.A.). Glacial acetic acid, toluene sulfonic acid, and all solvents were of HPLC grade (Baker, Phillipsburg, NJ, U.S.A.). Celite 545 was obtained from Fisher Scientific and reagent grade lead dioxide (PbO_2, powder) was obtained from Mallinckrodt.

HPLC

Chromatographic separations of the triphenylmethane dyes and their reduced forms were achieved with a μBondapak C_{18} column (300 mm × 3.9 mm I.D., Waters, Milford, MA, U.S.A.), particle size 10 μm, and an isocratic, degassed mobile phase (flow-rate 1.5 ml/min) of methanol–water (81:19), buffered with 0.05 *M* sodium acetate and 0.05 *M* glacial acetic acid. The resulting solution had a pH of 6.0. A Beckman fixed-loop (10-μl) injector delivered the analyte to the column. A Waters Lambda-Max, Model 481 spectrophotometer was operated at 618 nm for malachite green analysis, 588 nm for crystal violet analysis, and 600 nm for analysis of a combination of two chemicals. Chromatographic data from injections were collected and analyzed with System Gold chromatographic software (Beckman, Arlington Heights, IL, U.S.A.). The detection limit was determined by using the 1984 U.S. Environmental Protection Agency (EPA) method [11]. Solutions of the triphenylmethane dyes and their leuco forms were prepared in 0.05 *M* toluenesulfonic acid in methanol. Solutions of the dyes and their leuco forms should be protected from light.

Oxidation of reduced forms of dyes

The leuco forms of the chemicals were oxidized to the chromatic forms in a post-column reactor (stainless-steel tube, 32 mm long, 4 mm I.D.) capped with guard column fittings and packed with 10% PbO_2 suspended in Celite 545. The post-column reactor was packed with the dry mixture of PbO_2 in Celite 545 by gently tamping with a glass rod and placed in line between the HPLC column and the spectrophotometric detector.

RESULTS AND DISCUSSION

The chromatic forms of malachite green and crystal violet are separated from their leuco forms by HPLC on a reversed-phase column. The high absorbance of the dyes in the visible spectrum and the lack of absorbance by contaminants allows easy and specific detection by visible spectrophotometry. The leuco forms of the dyes are colorless and not detectable by visible spectrophotometry. Oxidation of the leuco

[a] Reference to trade names or manufacturers does not imply U.S. Government endorsement of commercial products.

form of the dye to the chromatic form after HPLC separation allows simultaneous analysis of the chromatic and leuco forms of the dyes with excellent sensitivity and specificity.

The detector response was about equal on a mol/mol basis when both chromatic and leuco crystal violet were analyzed with the spectrophotometric detector set at 588 nm (Fig. 2). The relative response indicates near complete conversion of the leuco to the chromatic form of the chemical. We prepared separate calibration curves, using this system with both the chromatic and leuco forms of crystal violet, that were linear over the range, of 1 to 100 ng, $r^2 = 0.998$. The minimum detection limits for the HPLC systeme, as estimated by the EPA method, were 0.13 ng for chromatic crystal violet and 0.16 ng for leuco crystal violet.

The detector response was lower for chromatic malachite green oxalate than for its leuco form due to the different molecular weight (Fig. 3). The calibration curves for both the chromatic and leuco forms of malachite green were linear over the range of 1 to 100 ng, $r^2 = 0.998$. The minimum detection limit was estimated to be 0.28 ng for chromatic malachite green oxalate and 0.12 ng for leuco malachite green.

We attempted to separate crystal violet, malachite green oxalate, and their leuco forms. Although a good response was obtained for all four chemicals, the leuco forms were poorly resolved (Fig. 4).

Roybal *et al.* [9], using electrochemical detection, achieved good sensitivity and selectivity for both chromatic and leuco crystal violet. Although an electrochemical detector yielded good sensitivity and selectivity for a triphenylmethane dye, UV–visible detectors are readily available and widely used in HPLC analysis. Our use of a

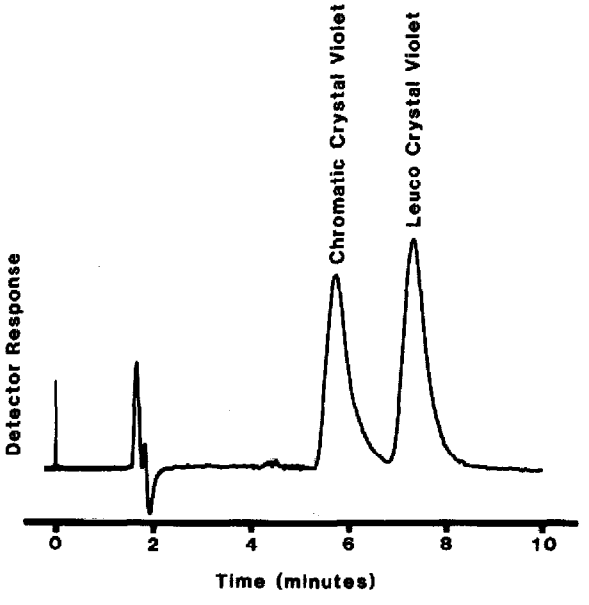

Fig. 2. Chromatogram of 10 ng each of chromatic crystal violet and leuco crystal violet on a C_{18} column with 1.5 ml/min of methanol–water (81:19) buffered with 0.05 *M* sodium acetate and 0.05 *M* glacial acetic acid at 588 nm and a sensitivity of 0.005 a.u.f.s., with post-column oxidation.

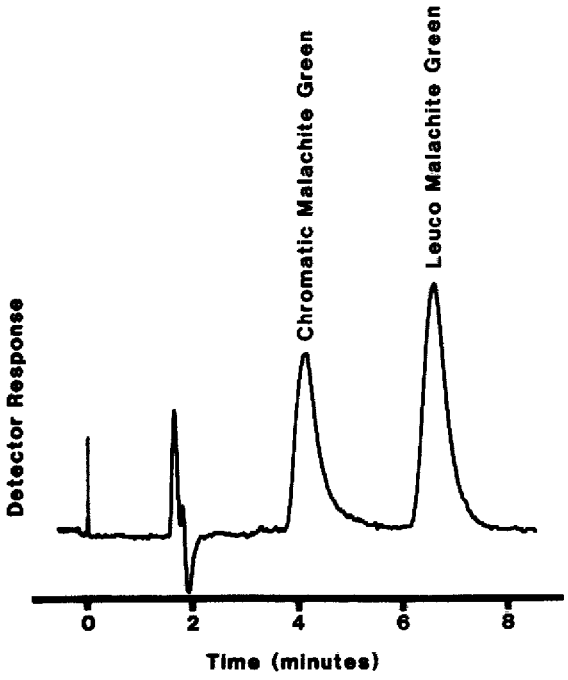

Fig. 3. Chromatogram of 10 ng each of chromatic malachite green and leuco malachite green on a C_{18} column with 1.5 ml/min of methanol–water (81:19), buffered with 0.05 *M* sodium acetate and 0.05 *M* glacial acetic acid at 618 nm and a sensitivity of 0.005 a.u.f.s., with post-column oxidation.

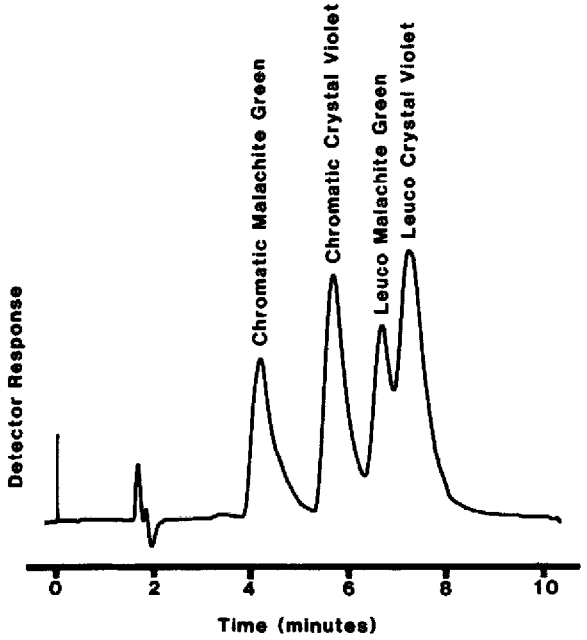

Fig. 4. Chromatogram of 10 ng each of chromatic malachite green, leuco malachite green, chromatic crystal violet, and leuco crystal violet on a C_{18} column with 1.5 ml/min of methanol–water (81:19), buffered with 0.05 *M* sodium acetate and 0.05 *M* glacial acetic acid at 600 nm and a sensitivity of 0.01 a.u.f.s., with post-column oxidation.

reaction chamber, containing PbO_2 between the HPLC column and the spectrophotometric detector, permitted simultaneous analysis of the parent dyes and their leuco forms at a single wavelength. Bauer *et al.* [10] used PbO_2 oxidation to analyze fish tissue for chromatic and leuco malachite green, but both the quantitative and qualitative information were obtained from the difference of two injections. The calibration curves indicate that the oxidation in the reaction chamber is reproducible over the range of 1 to 100 ng for the leuco forms of both dyes with the chromatographic conditions used. Although the reaction chamber increased the deadvolume of the chromatographic system, we achieved good sensitivity for all four components. The reaction chamber is able to convert these dyes form the leuco to the chromatic form after 400 injections; however, the efficiency of the conversion should be checked periodically using standards of the leuco forms of the dyes to ensure that the PbO_2 has not ben depleted.

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