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Simultaneous determination of methylene violet, halogenated methylene violet and their photoproducts in the presence of DNA by high-performance liquid chromatography using an internal surface reversed-phase column

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Abstract

A simple and rapid isocratic high-performance liquid chromatographic method for the analysis of methylene violet, a neutral phenothiazine dye, in the presence of DNA has been developed. These chromatographic conditions are also applicable to its N-demethylated, bromo and iodo analogs. The method utilizes an internal surface reversed-phase column and a mobile phase consisting of 20% acetonitrile in 50 m*M* phosphate buffer (pH 7.0) and detection at 280 nm. Under these conditions all five dyes are well resolved from one another and from the faster migrating DNA. The effects of organic modifiers, ionic strength and pH of the buffer on the capacity factors of the dyes have been investigated. The method has successfully been applied to detect the photoproducts of methylene violet and its bromo analog in the presence of DNA without removing the biopolymer from the reaction mixture. © 1997 Elsevier Science B.V.

Keywords: Methylene violet; DNA

1. Introduction

For more than 60 years, red light-absorbing dyes have been recognized as photoinactivating agents for bacteria and viruses. DNA is believed to be the major target for photosensitized damage of these microorganisms, though damage of other biological molecules such as proteins and lipids has also been observed [1]. Methylene violet (MV, Fig. 1) is a neutral dye that belongs to the family of phenothiazine dyes. Recently the dye has been investigated for its potential in the photosensitized inactivation of viruses in blood and its constituents [2]. Its absorption in the visible region makes it attractive for the photoinactivation of viruses in the presence of hemoglobin. Concomitant with its application in the decontamination of blood, we have studied MV to understand its mechanism of action, and to this end we have investigated its photochemical interaction with DNA in vitro. We found that MV and its synthetic analog, bromomethylene violet (BrMV), irreversibly binds to DNA when irradiated at $\lambda > 520$ nm [3]. The unimolecular photochemistry of MV is dominated by N-demethylation and reduction [T. Mohammad, H. Morrison, unpublished results].

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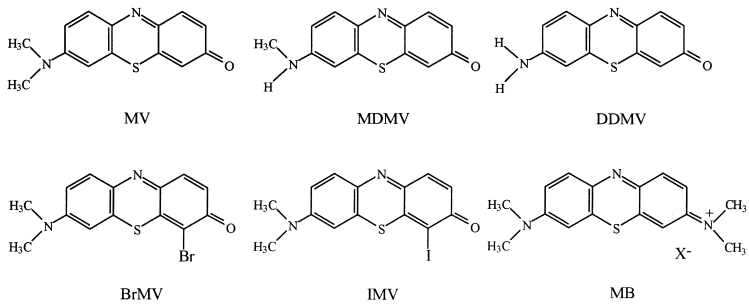

Fig. 1. Chemical formulae of methylene violet (MV), monodemethylated methylene violet (MDMV), didemethylated methylene violet (DDMV), bromomethylene violet (BrMV), iodomethylene violet (IMV) and methylene blue (MB).

Though we do not yet fully understand the mechanism of binding of MV and its halogenated derivative to DNA, there is some indication that binding and N-demethylation involve electron transfer chemistry which is influenced by the presence of DNA [T. Mohammad, H. Morrison, unpublished results]. In order to delineate the binding mechanism, one needs to analyze the MV photoproducts in the presence of DNA. We are unaware of an HPLC method for the analysis of MV. In this paper, we demonstrate that an internal surface reversed-phase (ISRP) column can be successfully adapted under suitable eluent conditions to analyze the photoproducts of MV and its derivatives in the presence of DNA without removing the latter before injection on to the column.

The unique concept of ISRP, developed and introduced by Pinkerton et al. about a decade ago [4], belongs to the family of restricted-access media (RAM) support [5]. The support was designed to analyze drugs and their metabolites in the untreated biological matrix. The ISRP support is achieved by bonding of the tripeptide, glycine–L-phenylalanine–L-phenylalanine (GFF), to a glycerylpropyl moiety on the silica surface [4]. By virtue of two surfaces, an external porous hydrophilic and internal hydrophobic surface, ISRP combines the characteristics of size-exclusion chromatography (SEC) and conventional reversed-phase chromatography (RPC). The support excludes proteins from the outer surface which pass through the column while low molecular weight compounds enter the pores and are separated from each other, based on the mechanism of RPC, from the fast moving biological molecules.

ISRP provides the unsurpassed advantage of analyzing directly biological samples containing small bioactive molecules, e.g. drugs and their metabolites in protein [6,7], plasma [8,9] and serum [10–12]. Recently, in a modified version, an ISRP guard column was combined with a column switching technique to analyze granisetron and its metabolites in plasma [13]. The use of an ISRP column eliminates lengthy conventional procedures of preclean-up and extraction involving protein precipitation, centrifugation, work-up of supernatants, evaporation and re-dissolution prior to injection on to the conventional HPLC column. In a previous paper we reported that the ISRP column works equally well and efficiently for the analysis of low molecular mass organic compounds in the presence of nucleic acids [14]. Because DNA is believed to be the major target for many skin sensitizing drugs [15], biologically active acrylic acid derivatives [16–18] and environmental pollutants [19], the fast and reliable analysis of such DNA mixtures using ISRP support certainly has several advantages as outlined above. In this paper, we present data to demonstrate that a totally different family of compounds, i.e. dyes (cf. Fig. 1), can be separated on an ISRP column in the presence of DNA. Further utility of the column is evident from the analysis of the MV photoproducts resulting from the interaction with DNA.

2. Experimental

2.1. Chemicals and reagents

Calf thymus DNA (type I: sodium salt, highly polymerized), Trizma base and hydrochloride were from Sigma (St. Louis, MO, USA). HPLC grade methanol and sodium phosphate (monobasic and dibasic) were procured from Mallinckrodt (Paris,

KY, USA). Sodium acetate and HPLC grade acetonitrile were purchased from Spectrum (New Brunswick, NJ, USA) and Fisher (Fair Lawn, NJ, USA), respectively. Water used in the preparation of buffer solutions was distilled from glass, employing a Corning MP-1 water still.

Methylene violet (Bernthsen) was purchased from Aldrich (Milwaukee, WI, USA); the dye content was reported by the supplier to be 87%. We found the sample to be 84% MV, with the contaminants being 13% monodemethylated methylene violet (MDMV) and 3% didemethylated methylene violet (DDMV), as revealed by HPLC analysis. Purification was achieved by employing either Chromatotron® (for quantities $<$100 mg) and/or flash chromatography on silica gel (230–400 mesh) for large quantities, using a gradient elution of 10% methanol in chloroform. The chemical purity was $>$99% as evidenced by HPLC analysis. Purified MV was characterized by spectral data including mass and 500 MHz ^{1}H NMR spectra [3]. Small quantities of MDMV and DDMV were separated and collected from the silica-gel chromatography of commercial MV (vide supra). The HPLC purity was 93 and 88% for MDMV and DDMV, respectively. On a large scale, MDMV was conveniently prepared by treating MV with 1-chloroethyl chloroformate in 1,2-dichloroethane at reflux temperature followed by flash chromatography on silica gel [20]. In the chemical ionization mass spectra molecular ions ($M+H^+$) were observed as the base peak at m/z 243 and 229 for MDMV and DDMV, respectively. The electron impact mass spectral data were as follow: m/z (rel. int.), MDMV: 242 (100, $M^{+\cdot}$), 241 (3), 229 (2), 228 (5), 214 (66), 200 (7), 199 (48), 185 (7), 172 (14), 140 (8), 107 (45), 69 (17), 63 (22), 55 (13); DDMV: 228 (94, $M^{+\cdot}$), 226 (2), 214 (3), 200 (100), 199 (21), 172 (11), 100 (34), 83 (13), 69 (29), 57 (32). The UV–Vis absorption spectra in water displayed absorption maxima at 620, 608 and 593 nm for MV, MDMV and DDMV, respectively. BrMV and IMV were synthesized from MV in these laboratories and characterized as previously reported [3].

2.2. Chromatographic system and operating conditions

HPLC analyses were performed on a Varian 5000 ternary solvent delivery system fitted with a Rheodyne 7125 injection port (Cotati, CA, USA) which carried a 0.2-ml injection loop. The column effluent was monitored with a Varian 2050 variable wavelength detector set at 280 nm (unless otherwise specified) and processed on a Perkin-Elmer LCI-100 computing integrator. In some experiments, a Waters (Milford, MA, USA) Delta Prep 4000 solvent delivery system equipped with a Waters 486 tunable absorbance detector and Waters 746 data module were used for the analysis. The reaction mixture was analyzed and separated on an ISRP GFF-S5-80 (250×4.6 mm I.D., 5 μm) column equipped with a guard precolumn (1.0 cm×3.0 mm I.D., 5 μm spherical GFF ISRP material) (Regis Technologies, Morton Grove, IL, USA). An Alltech (Deerfield, IL, USA) C_{18} Econosil column (250×4.6 mm I.D., 10 μm) fitted with a guard column of pellicular C_{18} material was used to analyze the dyes in the absence of DNA. The ternary solvent delivery capability system controlled the on-line mixing of the organic modifier. Unless otherwise mentioned, an isocratic solvent delivery program was used to elute the components of the mixture.

The capacity factor, k', was calculated as $t_R - t_0/t_0$ where t_R is the retention time of an individual component and t_0 is the retention time of an unretained compound (confirmed by injecting 3 *M* KCl solution) which was determined as the time from injection to the first distortion of the baseline. The separation factor, α, was calculated from the ratio of the capacity factor of the slow migrating component to that of the fast moving species. A value of $\alpha >$ unity indicates good selectivity of the mixture components to the column and acceptable resolution between the two peaks.

2.3. Preparation of solutions

The stock solutions (1 m*M*) of MV, BrMV and IMV were prepared in DMSO and diluted in 50 m*M* phosphate buffer (pH 7.0) (unless otherwise specified) alone or containing DNA to the desired final concentration. For studies involving BrMV and DNA, it is important that the dye solution is added to DNA solution *dropwise with continuous agitation* to avoid the dye from being precipitated. Owing to the poor solubility of IMV in aqueous solvent, even this procedure provided irreproducible results.

The aqueous mobile phase of 100 m*M* sodium

acetate (pH 4.5) was prepared by dissolving 8.2 g of the salt in 1 l of water containing 8 ml of glacial acetic acid and 1 l of 50 m*M* sodium phosphate buffer (pH 7.0) contained 2.76 g and 4.26 g of mono- and dibasic sodium phosphate, respectively. The aqueous mobile phase was prepared fresh and filtered through a 0.45-μm nylon 66 membrane (Alltech).

2.4. Manipulation of DNA

The dry fiber of calf thymus DNA was dissolved in 10 m*M* Tris buffer (pH 7.2) by overnight incubation at room temperature in the dark. DNA was precipitated with 0.1 volume of 2 *M* sodium chloride followed by addition of 2 volumes of chilled absolute ethanol and stored in the freezer overnight. The DNA pellet obtained after centrifugation at 2000 *g* for 20 min was suspended in 50 m*M* phosphate buffer (pH 7.0). The concentration was adjusted from absorbance measurement (at 260 nm) of an appropriately diluted solution and using the molar absorptivity of 6.6×10^{3} M^{-1} cm^{-1} [21].

2.5. Irradiation procedure

The solutions of MV and its halogenated derivatives in 48 m*M* phosphate buffer (pH 7.0) containing 5% DMSO (v/v) were irradiated, in the absence and presence of calf thymus DNA, in matched quartz photolysis tubes. Prior to irradiation, the dye–DNA solutions were equilibrated at a slow speed on a wrist action shaker (Burrell Scientific, Pittsburgh, PA, USA) for 2–12 h at ambient temperature in the dark. The solutions were degassed with argon for ~20 min, stoppled and parafilm-sealed, and inserted in a turntable. The latter surrounded the light source of a Canrad-Hanovia (Newark, NJ, USA) 450 W medium pressure mercury lamp (Model 679A-36). In this configuration the solutions were ~8 cm from the lamp which was filtered through either a cylindrical Pyrex glass (for irradiation at $\lambda>280$ nm) or cylindrical uranium yellow glass (cut-off>330 nm) in combination with a 2% potassium dichromate solution (1.25-cm path-length). With the latter set-up, the dyes were essentially excited with the 546-nm (green) and 578-nm (yellow) mercury lines. After irradiation, the solutions were exposed to air for at least 2 h before HPLC analysis. This treatment was necessary to re-oxidize the photoreduced dye [T. Mohammad, H. Morrison, unpublished results]. The dark controls, wrapped in aluminum foil, were treated under identical conditions.

3. Results

3.1. Elution profiles of MV and its analogs from the C_{18} and ISRP columns

The chromatographic elution patterns for MV and its derivatives using a C_{18} and an ISRP column with 50% acetonitrile in 100 m*M* sodium acetate buffer (pH 4.5) are compared in Fig. 2a and b, respectively. The C_{18} column is quite effective in holding the dyes and resolves them well even at relatively higher flow-rate (cf. Fig. 2a). In Fig. 2a (chromatogram A), the commercial sample of MV contains the fast eluting peaks of DDMV (3.6 min) and MDMV (5.7 min). The approximate retention times for MV (chromatogram B), BrMV (chromatogram C) and IMV (chromatogram D) were 9.4, 12.3 and 15.0 min, respectively. Under these eluent conditions, the three peaks in the commercial sample of MV are unresolved and appear near the solvent front on the ISRP column (Fig. 2b, chromatogram A). BrMV and IMV are weakly retained and elute at 4.2 and 4.4 min, respectively (cf. Fig. 2b, chromatograms B and C, respectively). Decreasing the flow-rate did not improve the retention nor resolution of the dyes. Therefore, new conditions were sought for the effective use of the ISRP column.

3.2. Chromatographic behavior of MV, MDMV and DDMV on ISRP column as a function of acetonitrile composition

The effect of 10–30% acetonitrile in 100 m*M* sodium acetate (pH 4.5) on the capacity factors of MV and its demethylated analogs is presented in Fig. 3. By reducing the organic modifier in the aqueous mobile phase both the capacity and separation factors of the dyes became larger.

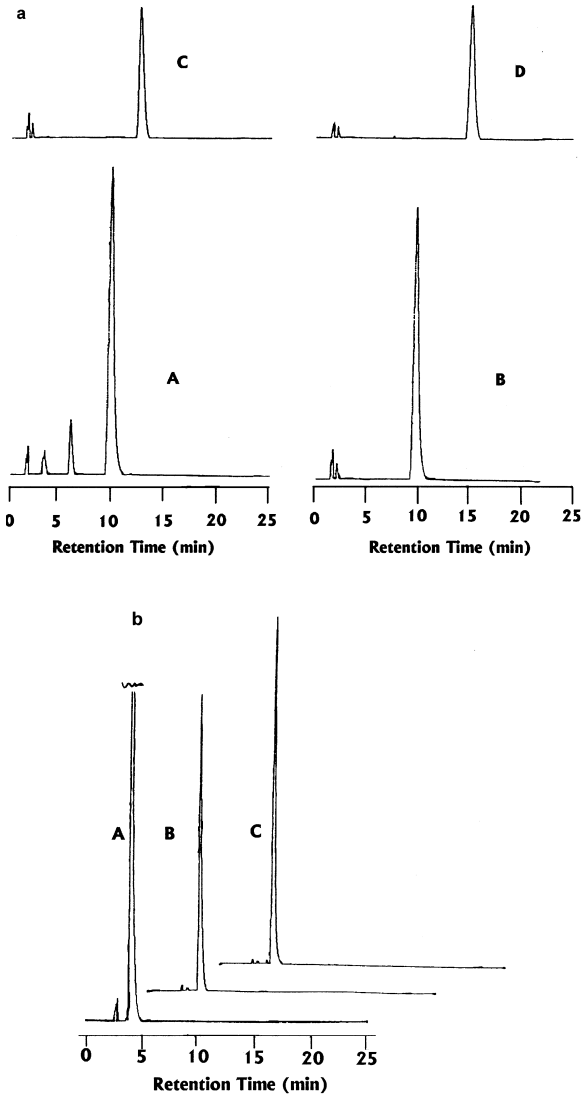

Fig. 2. Chromatographic separation of MV and its analogs. (a) Chromatographic conditions: column, C_{18}; mobile phase, 50% acetonitrile in 100 m*M* sodium acetate buffer (pH 4.5); flow-rate 1.6 ml/min; λ_{det}, 280 nm; sample, 25 μl of 50 μ*M* dyes in 50% aqueous ethanol containing 2.5% DMSO (v/v); chromatogram identification: A, commercial MV containing fast eluting MDMV and DDMV as impurities; B, pure MV; C, BrMV; D, IMV. (b) Chromatographic conditions: same as in (a) except that the column is ISRP and the flow-rate is 1.06 ml/min; sample, 25 μl of 25 μ*M* dyes in 50% aqueous ethanol containing 2.5% DMSO (v/v); chromatogram identification: A, commercial MV containing MDMV and DDMV; B, BrMV; C, IMV.

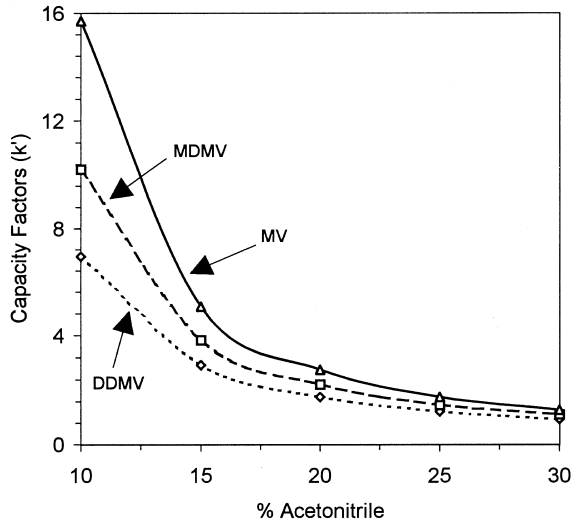

Fig. 3. Effect of acetonitrile composition on the capacity factors of MV, MDMV and DDMV on the ISRP column; chromatographic conditions: mobile phase, 10–30% acetonitrile in 100 m*M* sodium acetate buffer (pH 4.5); flow-rate 1.06 ml/min; λ_{det}, 280 nm, sample: 200 μl of 25 μ*M* commercial MV containing MDMV and DDMV in water containing 2.5% EtOH (v/v).

3.3. Effect of methanol on the selectivity of MV and its demethylated analogs towards ISRP support

Acetonitrile is a stronger solvent than methanol and elutes hydrophobic compounds faster from a RP column. The composition of methanol in 100 m*M* sodium acetate (pH 4.5) was varied from 35 to 50% for the analysis of MV and its demethylated analogs. At the highest methanol composition used (50%), the retention times for the dyes are in the same range as are observed for the same composition of acetonitrile using the C_{18} column (see Table 1). This shows that the bonded phase of an ISRP column is less hydrophobic than the C_{18} support.

3.4. Separation of MV, MDMV and DDMV as a function of ionic strength

The influence of 0–200 m*M* sodium acetate buffer (pH 4.5) on the capacity factors of MV, MDMV and DDMV with the ISRP column is presented in Table 2. As the buffer concentration increased there was a slight decrease in the capacity factors of all three

Table 1
Effect of methanol on the capacity factors[a] of MV, MDMV and DDMV on an ISRP column under isocratic elution[b]

MeOH (%)	DDMV	MDMV	MV	α_1	α_2
35	3.04	4.32	6.57	1.42	1.52
	(11.3)	(14.9)	(21.2)		
40	1.93	2.54	3.64	1.32	1.43
	(8.2)	(9.9)	(13.0)		
45	1.14	1.35	1.72	1.18	1.27
	(6.2)	(6.8)	(7.9)		
50	1.00	1.21	1.59	1.21	1.31
	(5.8)	(6.4)	(7.5)		

[a] The observed retention times in minutes are noted in parentheses.
[b] Chromatographic conditions: column, ISRP; mobile phase, 35–50% methanol in 100 m*M* sodium acetate (pH 4.5); flow-rate, 1.06 ml/min; λ_{det}, 280 nm; sample, 200 μl of 25 μ*M* commercial MV containing MDMV and DDMV; α_1 and α_2 are the separation factors for k'_{MDMV}/k'_{DDMV} and k'_{MV}/k'_{MDMV}, respectively.

dyes. This was accompanied by a decrease in the separation factors of the dyes with increasing buffer concentration. Elimination of the buffer altogether also provided good separation but produced relatively longer retention times. The latter conditions are attractive for preparative HPLC and eliminate the need for desalination after separation chromatography.

3.5. Effect of pH on the retention times of MV and its analogs

GFF-bound silica possesses selectivity towards both positively charged aromatic compounds and a variety of neutral molecules. Because MV and its analogs carry two basic nitrogens, it was reasonable to study the effect of pH on the elution profiles of the

Table 2
Effect of ionic strength on the capacity factors[a] of MV, MDMV and DDMV on an ISRP column under isocratic elution[b]

NaOAc (m*M*)	DDMV	MDMV	MV	α_1	α_2
0	3.33	5.03	5.85	1.51	1.16
	(14.3)	(19.9)	(22.6)		
10	1.97	2.76	3.30	1.40	1.20
	(9.8)	(12.4)	(14.2)		
20	1.14	1.35	1.72	1.34	1.21
	(9.5)	(11.6)	(13.4)		
40	1.79	2.33	2.85	1.30	1.22
	(9.2)	(11.0)	(12.7)		
80	1.67	2.12	2.64	1.27	1.24
	(8.8)	(10.3)	(12.0)		
100	1.67	2.09	2.61	1.25	1.25
	(8.8)	(10.2)	(11.9)		
150	1.58	1.97	2.45	1.25	1.24
	(8.5)	(9.8)	(11.4)		
200	1.54	1.91	2.36	1.24	1.24
	(8.4)	(9.6)	(11.1)		

[a] The observed retention times in minutes are noted in parentheses.
[b] Chromatographic conditions: column, ISRP; mobile phase: 20% acetonitrile in sodium acetate buffer (pH 4.5); flow-rate, 1.06 ml/min; λ_{det}, 280 nm, sample; 200 μl of 25 μ*M* commercial MV containing MDMV and DDMV; α_1 and α_2 are the separation factors for k'_{MDMV}/k'_{DDMV} and k'_{MV}/k'_{MDMV}, respectively.

dyes from the ISRP column. The effects of pH 4.5, 5.6 and 7.0 were investigated on the mobilities of MV, MDMV and DDMV. The retention times were 8.5, 10.0 and 12.0 min for DDMV, MDMV and MV, respectively in 20% acetonitrile in 100 m*M* sodium acetate (pH 5.6). These retention times were not significantly different at pH 4.5 or 7.0. The pH change also had no effect on the column back pressure. Under these conditions there was no variation in t_R on the same day; the day-to-day variation was $<\pm 5\%$.

3.6. Selectivity of MV, BRMV and IMV on the ISRP column in the presence of DNA

After analyzing the mobility of MV and its N-demethylated derivatives under a variety of eluent conditions we used the optimized conditions for the analysis of pure samples of MV and BrMV in the presence of DNA. Owing to the poor solubility of IMV in aqueous solvent, it could not be spiked with DNA (1.02 mg/ml). The elution profiles of the dyes using 20% acetonitrile in 50 m*M* phosphate buffer (pH 7.0) are presented in Fig. 4 where MV (chromatogram A), BrMV (chromatogram C) and IMV (chromatogram E) are seen to elute at 8.1, 14.0 and 18.9 min, respectively. The retention times for MV and BrMV were virtually unchanged in the presence of DNA (chromatograms B and D, respectively). The duplicate analysis provided consistent retention times and the detected peak areas varied $<\pm 6\%$. These results indicated that the dyes can be analyzed in the presence of DNA without any interference of the latter.

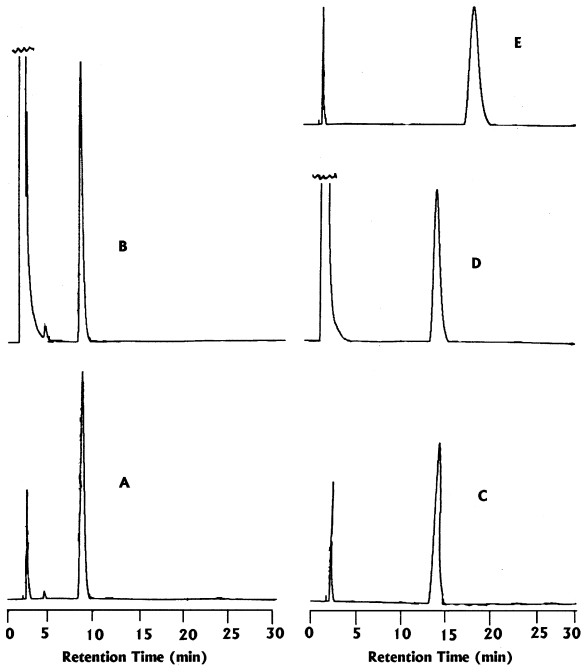

Fig. 4. Elution profile of MV, BrMV and IMV from ISRP column. Chromatographic conditions: mobile phase, 20% acetonitrile in 50 m*M* phosphate buffer (pH 7.0); flow-rate, 1.6 ml/min; λ_{det}, 280 nm; sample, 30 μl of 50 μ*M* MV, BrMV and IMV in 50% aqueous ethanol containing 5% DMSO (v/v) in the absence and presence of DNA (1.02 mg/ml) in 48 m*M* phosphate buffer (pH 7.0); chromatogram identification: A, MV; B, MV–DNA; C, BrMV; D, BrMV/DNA; E, IMV.

3.7. Analysis of the photoproducts of MV generated in the absence and presence of DNA

A 2-ml degassed solution of MV (50 μ*M*) in 48 m*M* phosphate buffer (pH 7.0) containing 5% DMSO (v/v) was irradiated at $\lambda > 520$ nm for 3 h. HPLC analysis on ISRP column showed two new peaks eluting before MV (Fig. 5, chromatograms A and B). The duplicate analysis provided consistent retention times and the detected peak areas varied $\pm 3\%$. The fast and slow migrating peaks were identified as DDMV and MDMV, respectively, by comparison of their retention times to those of the authentic samples and by co-injection. Under these irradiation conditions, a 50% loss of MV resulted in the 44 and 4% formation of MDMV and DDMV, respectively. When MV was irradiated in the presence of 3.24 m*M* native calf thymus DNA for 3 h, MDMV and DDMV were again produced but at a reduced rate (Fig. 5, chromatograms C and D). For example, the production of MDMV and DDMV was decreased by ~36 and 81%, respectively. In both cases no photoproducts were observed in the absence of light.

3.8. Evidence for photodebromination of BRMV in the presence of DNA

Degassed solutions (6×2 ml) of BrMV (50 μ*M*) and calf thymus DNA (1.07 mg/ml) in 48 m*M*

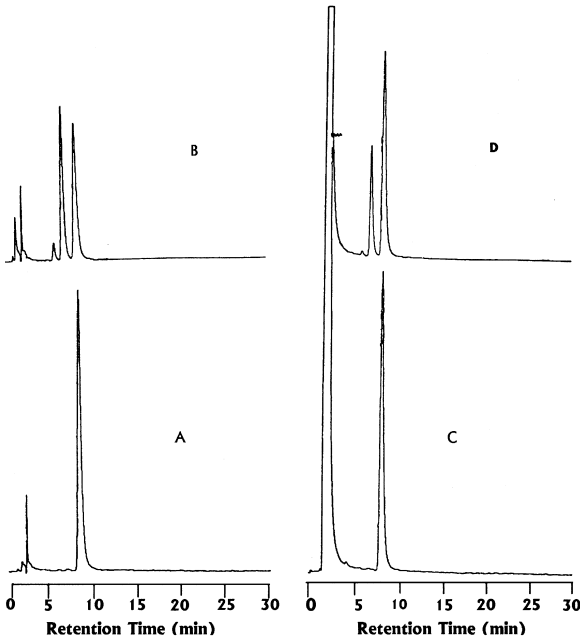

Fig. 5. Chromatographic elution profile of 50 μ*M* MV irradiated in the absence and presence of 3.24 m*M* calf thymus DNA in 48 m*M* phosphate buffer (pH 7.0) containing 5% DMSO (v/v) at λ>520 nm under anaerobic conditions at 11°C for 3 h; chromatographic conditions: column, ISRP; mobile phase, 20% acetonitrile in 50 m*M* phosphate buffer (pH 7.0); flow-rate, 1.6 ml/min; λ_{det}, 280 nm; analyte volume, 25 μl; chromatogram identification: A, non-irradiated MV; B, irradiated MV; C, non-irradiated MV–DNA; D, irradiated MV/DNA.

phosphate buffer (pH 7.0) containing 5% DMSO (v/v) in quartz photolysis tubes were irradiated at λ>280 nm at 15°C. One photolysis tube was wrapped with aluminum foil as a dark control and the irradiation of remaining solutions was interrupted after 1, 2, 4, 5 and 6 h. The photolysates were analyzed on the ISRP column and the results of this time course study are shown in Fig. 6.

The non-irradiated sample showed only one peak due to BrMV (peak 1). Irradiation for 1 h produced three new peaks labeled as peaks 2, 3 and 4 which corresponded to an unidentified photoproduct, MV and MDMV, respectively. The new peaks increased proportionally after 2 h irradiation at the expense of BrMV. Further irradiation (4 h) also produced an additional product (peak 5) which was identified as DDMV. The peaks continued growing with irradiation time. The identities of MV, MDMV and DDMV

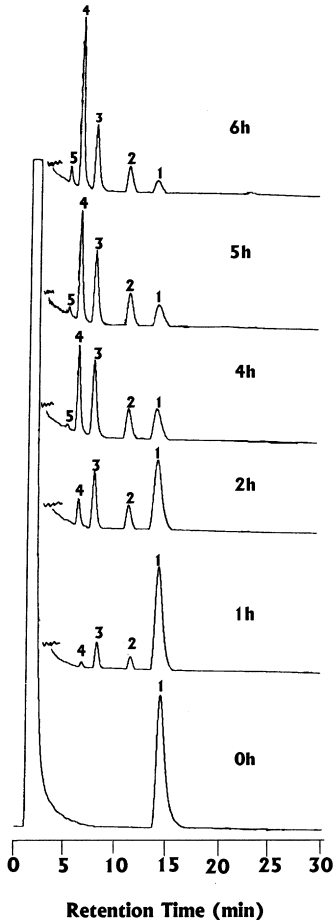

Fig. 6. Chromatographic analysis of a time course irradiation of BrMV (50 μ*M*) and calf thymus DNA (1.07 mg/ml) in 48 m*M* phosphate buffer (pH 7.0) containing 5% DMSO (v/v) at λ>280 nm, under anaerobic conditions at 15°C. The photoreaction time is indicated on the chromatograms. Chromatographic conditions: column, ISRP; mobile phase, 20% acetonitrile in 50 m*M* phosphate buffer (pH 7.0); flow-rate, 1.6 ml/min; λ_{det}, 280 nm; analyte volume, 25 μl; peak identification: 1, BrMV; 2, unidentified photoproduct; 3, MV; 4, MDMV; 5, DDMV.

were confirmed by direct comparison with authentic samples, both separately and through co-injection.

4. Discussion

MV has two absorption maxima both in the UV and visible regions. The low energy absorption band is attributed to a twisted intramolecular charge

transfer state and shows a solvatochromic effect [22]. Upon excitation with the visible light MV undergoes N-demethylation and reduction [T. Mohammad, H. Morrison, unpublished results]. A likely consequence of these reactions is the photoconjugation of MV and BrMV to DNA [3]. This irreversible modification of DNA may be responsible, at least in part, for the photovirucidal activity associated with MV, MDMV and DDMV. It has been shown that MV, in contrast to the structurally related charged dye methylene blue (MB, Fig. 1) (currently used for photosterilization of blood [2,23]), is capable of killing the intracellular viruses [2]. The latter property is attributed to the hydrophobic nature of MV which helps in dye transport across the cell membrane. Additionally, along with several other phenothiazine dyes, MV has been investigated for its phototoxicity against human carcinomas [24]. We believe that DNA modification might be one of the mechanisms through which MV exerts its phototoxic effects. We have also synthesized and studied bromomethylene violet (BrMV) and iodomethylene violet (IMV) (Fig. 1) to potentially enhance the photodamage of DNA. The halogenated derivative, BrMV, also covalently binds to DNA upon photoactivation [3].

In order to understand the mechanism of the photochemical reaction of MV and its analogs with DNA, we needed to monitor the course of reaction by HPLC. We used a C_{18} column and a mobile phase consisting of 50% acetonitrile in 100 m*M* sodium acetate (pH 4.5) for the determination of dye purity as shown in Fig. 2 [3]. The C_{18} column provided all the desired requirements, i.e. selectivity, separation, and efficiency (Fig. 2a). It was not feasible to use these analytical conditions for the analysis of MV in the presence of DNA. Presumably this caused precipitation of the biopolymer on the column because of the high percentage of the organic solvent and the relatively low pH of the mobile phase. We, therefore, turned to the ISRP column which combines the properties of both size-exclusion and reversed-phase chromatography. The eluent conditions developed for C_{18} support were ineffective for the ISRP column since all the dyes eluted near the solvent front and no selectivity was observed. Poor resolution resulted even when the flow-rate was reduced from 1.6 ml/min (C_{18} column) to 1.06 ml/min (Fig. 2b). Thus, it became necessary to first optimize the eluent conditions for the selective retention of MV and its analogs in the absence of DNA. Several variables were investigated, i.e., nature and composition of organic modifier, pH and ionic strength of the aqueous mobile phase, in order to achieve the desired selectivity of MV and its analogs.

As shown in Fig. 3, ~20% acetonitrile in sodium acetate buffer provided good separation of MV and its N-demethylated analogs. This relatively small composition of acetonitrile proved advantageous for the analysis of dyes in the presence of DNA. Increasing the acetonitrile composition to >20% resulted in the decrease of the capacity factors of the dyes and led to poor selectivity. Substitution of methanol for acetonitrile also gave good retention of the dyes (Table 1) and allowed the use of relatively higher organic solvent composition, but would be of no use when DNA is present. When 65% buffer was replaced with water we observed an increase in the capacity factors by 22, 45, and 32% for DDMV, MDMV and MV, respectively. This modification makes it attractive for preparative chromatography where one would not need desalination of the collected pure fractions; these conditions, however, slightly increase the run time.

It was of interest to examine the influence of the concentration of the buffer on the retention times of the dyes with respect to preparative HPLC. There was a slight but progressive decrease in the capacity factors of the dyes with increasing buffer concentration (Table 2). The peaks were much resolved at lower buffer concentration. The increase in buffer concentration did not have a significant effect on the column back pressure (data not shown). The resolution was much improved in the absence of buffer, but at the cost of peak broadening and longer run time. Thus, for preparative purpose, 10 m*M* buffer (or even water) can be employed to minimize desalting after the purification. The 100 m*M* buffer can be used to expedite the analytical work. Substitution of phosphate buffer for acetate buffer did not have an effect on the elution profiles of the dyes. No significant effect of pH on the t_R of DDMV and MDMV was noticed; however the t_R of MV was ~5% longer at higher pH (5.6 and 7.0) than at pH 4.5. The retention times were reproducible and the variation was only $<\pm 5\%$ from day-to-day analysis.

Following the exploratory work on the chromato-

graphic conditions for the separation of MV and its analogs, we found that 20% acetonitrile in 50 m*M* phosphate buffer (pH 7.0) would be a good choice. This level of organic solvent should keep the dyes in solution and not precipitate DNA. This expectation proved true as is evident from Fig. 4. The dyes elute almost at the same retention volume alone and also when spiked with DNA. All the dyes are well separated from each other, and the DNA does not interfere in the elution of the dyes in terms of the detected area.

We adopted these conditions to study the photochemical reactivity of MV and BrMV in the presence of DNA. Upon excitation, MV undergoes N-demethylation to produce MDMV. The latter, upon sufficient build-up, further yields DDMV (see Fig. 5). The most likely mechanism of demethylation is via electron transfer (intra- or intermolecular pathway). The presence of DNA clearly suppresses the demethylation process and this inhibition may be by as much as 80% in the early stages of the reaction [T. Mohammad, H. Morrison, unpublished results]. The inhibition of demethylation could be associated with the interception of the MV excited state via electron transfer from the DNA. This would produce a radical ion pair which could ultimately give the MV-DNA covalent adduct(s) [3].

In fact, we have shown that irradiation of MV and BrMV in the presence of DNA and subsequent purification of the biopolymer (to remove the unreacted dyes) provides colored pellets of the DNA. The UV–Vis absorption spectra of both DNA adducts showed absorption maximum at 620 nm which corresponds to the absorption maximum of MV. We were interested in determining if MV is produced when BrMV/DNA are co-irradiated. From the chromatographic results of a time course reaction of BrMV/DNA presented in Fig. 6 there is clear evidence that MV is produced from BrMV and that its formation increases with the absorbed dose (see also Fig. 6). In the early stages of the reaction, both MV and MDMV result from debromination and debromination/demethylation of BrMV, respectively. At longer irradiation times, and as MV accumulates, there is competitive light absorption by MV and BrMV and therefore photochemistry from both MV and BrMV. The likely mechanism for debromination is intramolecular and/or intermolecular (from DNA) electron transfer to the excited dye. There are ample precedents for photodehalogenation of several different classes of compounds in the presence of DNA through electron transfer mechanisms. The resultant radicals initiate DNA damage either through covalent bond formation and/or chain scission involving oxygen-independent mechanisms [25–28].

5. Conclusions

(1) C_{18} column is the ideal choice for the analysis of MV, MDMV, DDMV, BrMV and IMV under isocratic elution conditions using 50% acetonitrile in 100 m*M* sodium acetate buffer (pH 4.5).

(2) For the analysis of the dyes in the presence of DNA the ISRP column and a mobile phase of 20% acetonitrile in 50 m*M* sodium phosphate buffer (pH 7.0) provide excellent selectivity and resolution for all the dyes.

(3) Using the ISRP column, we have demonstrated that DNA quenches the unimolecular photochemistry of MV, as evidenced by the suppression of MV photoproduct formation, and that the MV is formed from BrMV in the presence of nucleic acid.

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