Journal of Chromatography, 321 (1985) 185–197
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,356

DETERMINATION OF 1,3-DIPHENYLTRIAZENE AND AZOBENZENE IN D&C RED NO. 33 BY SOLVENT EXTRACTION AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

JOHN E. BAILEY, Jr.
Division of Color Technology, Food and Drug Administration, Washington, DC 20204 (U.S.A.)
(Received November 1st, 1984)

SUMMARY

Data are presented for the determination of trace levels of 1,3-diphenyltriazene (DPT) and azobenzene (AB) in D&C Red No. 33. The contaminants are extracted with chloroform from an aqueous solution of the color, and the chloroform is removed under vacuum. The residue is dissolved in acetonitrile and the solution is analyzed by reversed-phase high-performance liquid chromatography (HPLC) with detection at 365 nm. DPT and AB were determined at levels of $\leqslant 439$ ppb* and $\leqslant 2.2$ ppm, respectively, in samples of commercial D&C Red No. 33. Method precision for the DPT determination (five analyses) was 6.8% relative standard deviation. The UV–Vis spectra of the HPLC analytes, obtained by using a rapid-scan diode-array spectrophotometer, were used to confirm the identity of the DPT and AB responses produced during analysis of the D&C Red No. 33 extracts. The method is also useful for the separation of *cis*- and *trans*-AB.

INTRODUCTION

D&C Red No. 33 (Colour Index No. 17200) is a monoazo color additive that is permitted for use in ingested drugs, lipsticks and externally applied drugs and cosmetics in the United States[1]. The color is prepared by coupling diazotized aniline with H-acid (4-amino-5-hydroxy-2,7-naphthalenedisulfonic acid), which yields 5-amino-4-hydroxy-3-(phenylazo)-2,7-naphthalenedisulfonic acid, as shown in Fig. 1.

The commercially prepared color additive is impure and contains a variety of contaminating substances. Incomplete reaction during manufacture leads to the presence of the starting intermediates in the color. Side reactions that occur during manufacture form additional impurities. Impurities in the color intermediates also can contaminate the color directly or lead to additional side reactions. Finally, contaminating substances can form as a result of decomposition during processing or through reagents used during manufacture. The exact nature and levels of contam-

* Throughout this article the American billion (10^9) is meant.

0021-9673/85/$03.30

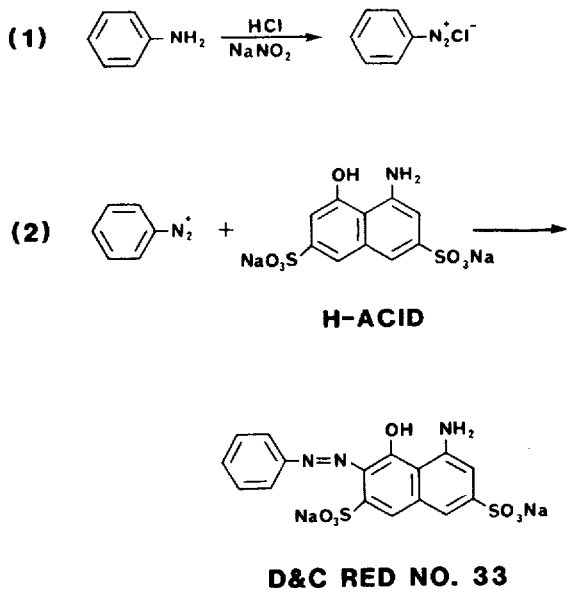


Fig. 1. Preparation of D&C Red No. 33.

inants vary from batch to batch because of different sources of intermediates and variations in the manufacturing process.

The composition of the color additive is of interest in evaluating the toxicological risk associated with human exposure to the color that occurs through its use in commercial products. The composition of samples of the commercial color should closely approximate the composition of the color used in the toxicity tests that are required to establish safety. Also, the levels of substances of known toxicological concern that are suspected of being contaminants of the color should be established so that a risk assessment can be performed. For example, evaluation of the chemistry of manufacture of D&C Red No. 33 suggested the presence of aromatic amines in the color since aniline is an intermediate. Recent investigations[2] have confirmed the presence of a number of aromatic amines and have established the levels that are present in samples of commercial D&C Red No. 33.

Azobenzene (AB) and 1,3-diphenyltriazene (DPT) are included among the possible contaminants of D&C Red No. 33 that are of toxicological concern. AB[3] and DPT[4] have been reported to be carcinogenic to animals. AB can arise either as a product of the oxidation of aniline[5] or, possibly, from the decomposition of diazoaniline during the coupling reaction[6]. DPT is readily formed by the coupling of diazoaniline with the nitrogen atom in free aniline[7]. However, DPT is unstable, especially in aqueous acidic media, in which it decomposes to 4-aminoazobenzene[8]. No data have been found in the literature that establish the levels of AB and DPT that are present in D&C Red No. 33 or in any of the other regulated color additives.

Methods of analysis for the determination of DPT and other triazene compounds in other matrices have been reported. DPT has been determined as a contaminant in 1-aryl-3,3-dialkyl triazene compounds by thin-layer chromatography[9]. Normal-phase high-performance liquid chromatographic (HPLC) columns have been

employed in the investigation of the rearrangement of 1,3-di-(4-methylphenyl)triazene[10]. Reversed-phase HPLC was used to investigate the formation of 1,3-di-(4-sulfamoylphenyl)triazene in gastric juice[11] and in the study of the metabolism of 1-(4-acetylphenyl)-3,3-dimethyltriazene[12]. Sulfonated diaryltriazenes have been determined in FD&C Yellow No. 5, FD&C Yellow No. 6[13,14] and FD&C Red No. 40[15] by using ion-exchange HPLC.

AB has been determined as a contaminant in phenylbutazone and sulfinpyrazone by using normal-phase HPLC[16]. Reversed-phase HPLC has been used to determine AB in atmospheric particulate matter[17] and as a metabolite of aniline[18]. Reversed-phase HPLC has also been used to separate *cis*- and *trans*-AB[19]. AB is sometimes used as an internal standard for gas chromatographic separations[20].

This paper describes the use of solvent extraction and reversed-phase HPLC for the determination of AB and DPT in D&C Red No. 33 and reports the results of analysis of several samples of the commercial color, including those used in the animal feeding studies.

EXPERIMENTAL

HPLC instrumentation

HPLC separations were performed on an Altex Model 322 MP gradient liquid chromatograph with Model 110A pumps and Model 420 controller (Altex, Berkeley, CA, U.S.A.). A Rheodyne Model 7125 loop injector (Rheodyne, Cotati, CA, U.S.A.) fitted with a 250-μl loop fabricated from 0.02 in. I.D. tubing was used. The column effluent was monitored with a Waters Model 440 UV detector with a 365-nm filter installed (Waters Assoc., Milford, MA, U.S.A.). A Shimadzu C-R1A integrator/printer-plotter (Shimadzu Scientific Instruments, Columbia, MD, U.S.A.) was connected to the 2-V output of the detector through a recorder attentuator set to provide a sensitivity of 0.02 a.u.f.s. The chromatograms were recorded at 5 mm/min. The separations were made by using a Bio-Sil ODS-5S column, 5-μm particle size, 25 cm $\times$ 4 mm I.D. (Bio-Rad Labs., Richmond, CA, U.S.A.).

Reagents

All chemicals were analytical reagent grade. HPLC grade solvents were used for the preparation of mobile phases. HPLC solvent A was prepared by dissolving 1.5 g of ammonium acetate and 0.5 ml of acetonitrile in 100 ml of purified water (Millipore/Continental Water Systems, Bedford, MA, U.S.A.); solvent B was acetonitrile.

Standards

AB (Eastman Organic Chemicals, Rochester, NY, U.S.A.) was recrystallized twice from methanol and purity was established by elemental analysis (Galbraith, Knoxville, TN, U.S.A.). DPT (Eastman) was purified by recrystallization from iso-octane, followed by preparative HPLC of the product and a second recrystallization from iso-octane[21]; purity was established by elemental analysis (Galbraith). Stock solutions were prepared by dissolving approximately 50 mg of each standard in 100 ml of methanol. Aliquots of the stock solutions were diluted with methanol to yield solutions containing 1.82 μg of AB/ml and 49.4 ng of DPT/ml.

Extraction

A 1-g portion of color was accurately weighed and transferred to a 250-ml separatory funnel containing 100 ml of a solution of 1 g of sodium chloride/100 ml of 0.01 *M* sodium hydroxide. The mixture was swirled and extracted (60 sec vigorous shaking) with 25 ml of chloroform. The layers were allowed to separate and the chloroform was drained through a washed (25 ml of chloroform) glass wool pledget in the constriction of a funnel into a 200-ml round-bottom flask. The aqueous solution of the color was extracted with two additional 25-ml portions of chloroform (30 sec vigorous shaking), which were also drained into the 200-ml round-bottom flask. The extract was transferred to a rotary evaporator and the chloroform was removed under aspirator vacuum at 30°C. Care was taken to ensure that the flask was removed from the evaporator as soon as the chloroform was distilled. The residual chloroform vapors were gently driven from the flask by using a 2-min air purge. A 2-ml portion of acetonitrile was added and the residue was dissolved with the aid of a capillary transfer pipet. The solution was then transferred to a 2-ml vial.

HPLC analysis

The HPLC column was equilibrated with 100% solvent A for 10 min at a flow-rate of 1 ml/min with the injector in the "inject" position. After the first 8 min of equilibration, the injector was moved to the "load" position and 50 μl of the solution of the extract was loaded into the injector. At the end of the 10-min equilibration, the solution of the extract was injected onto the column. The solvent was continued at 100% solvent A for 2 min and then was changed to 55% solvent B in solvent A at 2 min and held at 55% solvent B in solvent A for 11 min. At 13 min, a linear gradient from 55 to 100% solvent B in 7 min was initiated. The solvent was held at 100% solvent B for 10 min to complete a 30-min chromatogram.

Measurements and calculations

The liquid chromatograph was calibrated by performing the determination with a color that had been spiked with the contaminants. A 1-g portion of the color was added to a separatory funnel containing 50 ml of a solution of 2 g of sodium chloride/100 ml of 0.02 *M* sodium hydroxide. Aliquots of DPT and AB were added along with sufficient water to bring the volume in the separatory funnel to 100 ml. The extraction and HPLC analysis were performed as described and the peak heights were measured by the integrator. The response for each component was measured at six different concentration levels. After correction for the color blank, the results were treated statistically to calculate the regression equation. The levels of DPT and AB in samples of commercial products were determined from the regression equation.

UV–Vis spectra of eluting solutes

The UV–Vis spectra of the contaminants were obtained as they eluted from the HPLC column by using a Hewlett-Packard Model 8450A diode-array spectrophotometer (Hewlett-Packard, Palo Alto, CA, U.S.A.) fitted with an HPLC flow cell (Model 178.32, $Z = 15$, 10-mm pathlength, 8-μl cell volume; Hellma Cells, Jamaica, NY, U.S.A.). The wavelength range (240–600 nm) was selected, and a reference baseline (balance) of the HPLC eluent was measured and stored in the memory of the spectrophotometer. The baseline was measured in a gradient blank run at the reten-

tion time of interest or in the vicinity of the response of interest during the HPLC analysis. When the HPLC detector indicated that solute was eluting, the spectrophotometer was instructed to store a 1-sec spectrum every 2 sec until five spectra of the component were stored in the memory. After the chromatogram was complete, each spectrum was examined and the spectrum with the best signal-to-noise ratio was selected and plotted for each component. In some cases, the extract was dissolved in less than 2 ml of acetonitrile to improve the signal-to-noise ratio in the spectrum.

RESULTS AND DISCUSSION

In the present work, AB and DPT were determined in D&C Red No. 33 by first extracting the analytes from an aqueous, alkaline solution of the color with chloroform. The chloroform was removed under vacuum at low temperature (30°C), and the residue was dissolved in acetonitrile. AB and DPT were then determined by reversed-phase HPLC analysis with detection at 365 nm.

Extraction

The extraction was performed by dissolving the color in an aqueous solution containing sodium hydroxide and sodium chloride and extracting the solution with chloroform. The sodium hydroxide was added to stabilize the DPT, which rapidly decomposes under acidic conditions. Sodium chloride was added to minimize the tendency of the chloroform–aqueous mixture to form emulsions. The organic and aqueous layers readily separated from each other in most of the extractions. However, some colors formed agglomerations, but these were broken by stirring the mixture with a 9-in. transfer pipet.

Filtration of the chloroform extract through a chloroform-moistened glass wool plug prevented the passage of water droplets into the collection flask. The chloroform was carefully distilled under vacuum at 30°C, and the flask was removed as soon as all of the chloroform had evaporated to ensure minimal loss of volatile components. Residual chloroform vapors were driven from the flask by using a gentle air purge for 2 min. The residue remaining in the flask was dissolved in a 2-ml portion of acetonitrile; the walls of the flask were washed with acetonitrile with the aid of a capillary transfer pipet. The contents of the flask were transferred to a vial for storage. The dissolution and transfer steps were performed as rapidly and consistently as possible to minimize loss of acetonitrile from evaporation. In most cases, the extracted material readily dissolved in acetonitrile. However, some colors yielded extracts which left a colored residue on the walls of the flask after treatment with acetonitrile. The acetonitrile solutions of the chloroform extract varied considerably in appearance. Some were essentially colorless, whereas others varied widely in color and intensity. They also fluoresced in a variety of colors when examined under a UV lamp.

HPLC analysis

It was necessary to dissolve the extract in an organic solvent to ensure complete dissolution of the highly lipophilic AB and DPT as well as the other components present in the extract. Since DPT is unstable in aqueous media, mixed organic–aqueous solvents were avoided. However, the use of an organic solvent in reversed-phase HPLC analytical procedures can lead to the formation of distorted peaks that

are related to the strength of the solvent relative to that of the mobile phase[22]. This problem was overcome by partially filling the injection loop with the solution of the extract before injection. It is important to move the injector from the "inject" position to the "load" position and to load the solution of the extract into the injector at the same time during the equilibration. This ensures that the injection loop is well flushed with solvent A and that the solution of the extract has sufficient time to mix with the mobile phase before injection. The importance of mixing the solution of the extract with the mobile phase in the loop was determined when the analysis was attempted with a 50-μl loop flushed with the acetonitrile solution. Under these conditions, analysis using the HPLC system described above produced distorted responses for AB and DPT as shown in Fig. 2B. Injection of 50 μl of extract into a 250-μl injection loop filled with solvent A produced usable responses for DPT and AB as shown in Fig. 2C. No significant decomposition of DPT was observed during the short time that it was in contact with the aqueous mobile phase in the injection loop. An additional concern was the possibility of precipitation of the extract either in the injection loop or on the column during analysis. However, there was no indication of extract precipitation during the study. The HPLC gradient was designed so that the components, upon injection, are deposited on the head of the column. The solvent composition is then changed to elute some of the components in an isocratic-like manner, and a solvent gradient is employed to elute the more highly retained components.

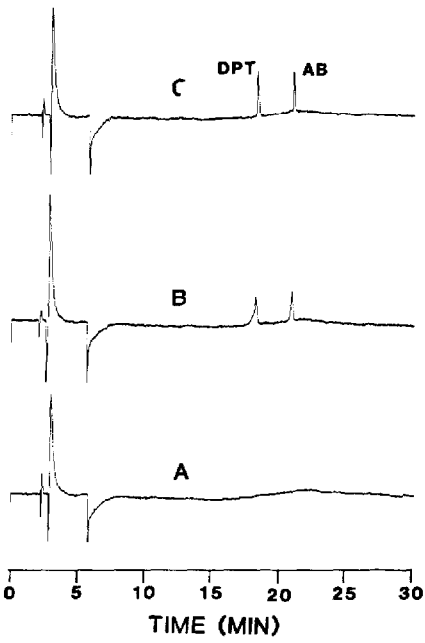
Fig. 3 shows HPLC chromatograms obtained by using the method described above. Fig. 3A represents a color blank obtained from analysis of commercial D&C Red No. 33 which contains no added DPT or AB. Fig. 3B is the chromatogram obtained from analysis of the color fortified with 494 ppb DPT and 5.5 ppm AB. The chromatogram for the color blank is free of responses at the retention times of AB and DPT and shows good peak shape for the responses present. The DPT response in the chromatogram for the fortified color is sharp and well resolved from the other peaks. AB also produces a sharp peak, but the response occurs in an area of the chromatogram where responses appear for a number of other components.

Calibration

The liquid chromatograph was calibrated according to the external standard method by analyzing separate weighings of commercial color spiked with AB and DPT. The liquid chromatograph was calibrated at six concentration levels ranging from 49–494 ppb for DPT and from 0.9–9.1 ppm for AB. The responses obtained were treated statistically to generate the regression equation and to evaluate the performance of the method[13]. The results of the regression analysis of the calibration data are shown in Table I. A linear relationship was obtained for the data points, and the intercepts were all close to zero. The use of a detection wavelength of 365 nm provided considerably greater sensitivity of the method for DPT than for AB.

Analysis of commercial samples

A total of eleven samples of commercial D&C Red No. 33 were analyzed for DPT and AB. The samples were selected so that all of the manufacturers of the color additive were represented by at least one batch of color. Included in the survey are the two samples (entries A and B in Table II) from the animal feeding studies that were used by the Food and Drug Administration (FDA) to evaluate the toxicity of



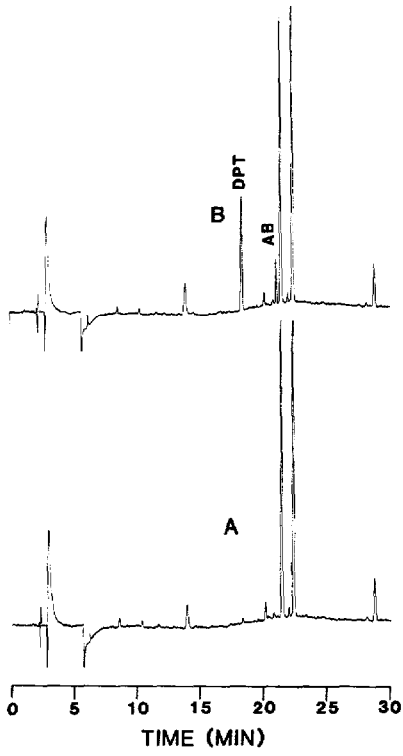


Fig. 2. HPLC chromatograms for (A) method blank (50 μl of acetonitrile injected); (B) injection of DPT and AB, using a flushed 50-μl loop; (C) 50 μl of DPT and AB injected into a 250-μl loop.

Fig. 3. HPLC chromatograms for (A) color blank (unfortified D&C Red No. 33); (B) D&C Red No. 33 fortified with 494 ppb DPT and 5.5 ppm AB.

D&C Red No. 33. All samples, with the exception of sample F (Table II), were analyzed by FDA and certified as meeting the specifications required for use in commercial products. Sample F was submitted for FDA certification, but the request was later withdrawn by the manufacturer. Most samples were analyzed once. The reproducibility of the method was established by repetitive analysis of sample K. This sample was analyzed five times and the results were examined statistically. The results for DPT were 414, 485, 421, 422 and 455 ppb for the individual analyses and a mean value of 439 ppb with a standard deviation of 30 ppb and a relative standard devia-

TABLE I

REGRESSION ANALYSIS OF CALIBRATION DATA FOR DPT AND AB IN D&C RED NO. 33

Component	*No. of data points*	*Range*	*Regression equation*	*r*
DPT	6	49–494 ppb	$y = 0.7681x - 16.3953$	1.0000
AB	6	9–9.1 ppm	$y = 25.3227x + 3.9833$	0.9994

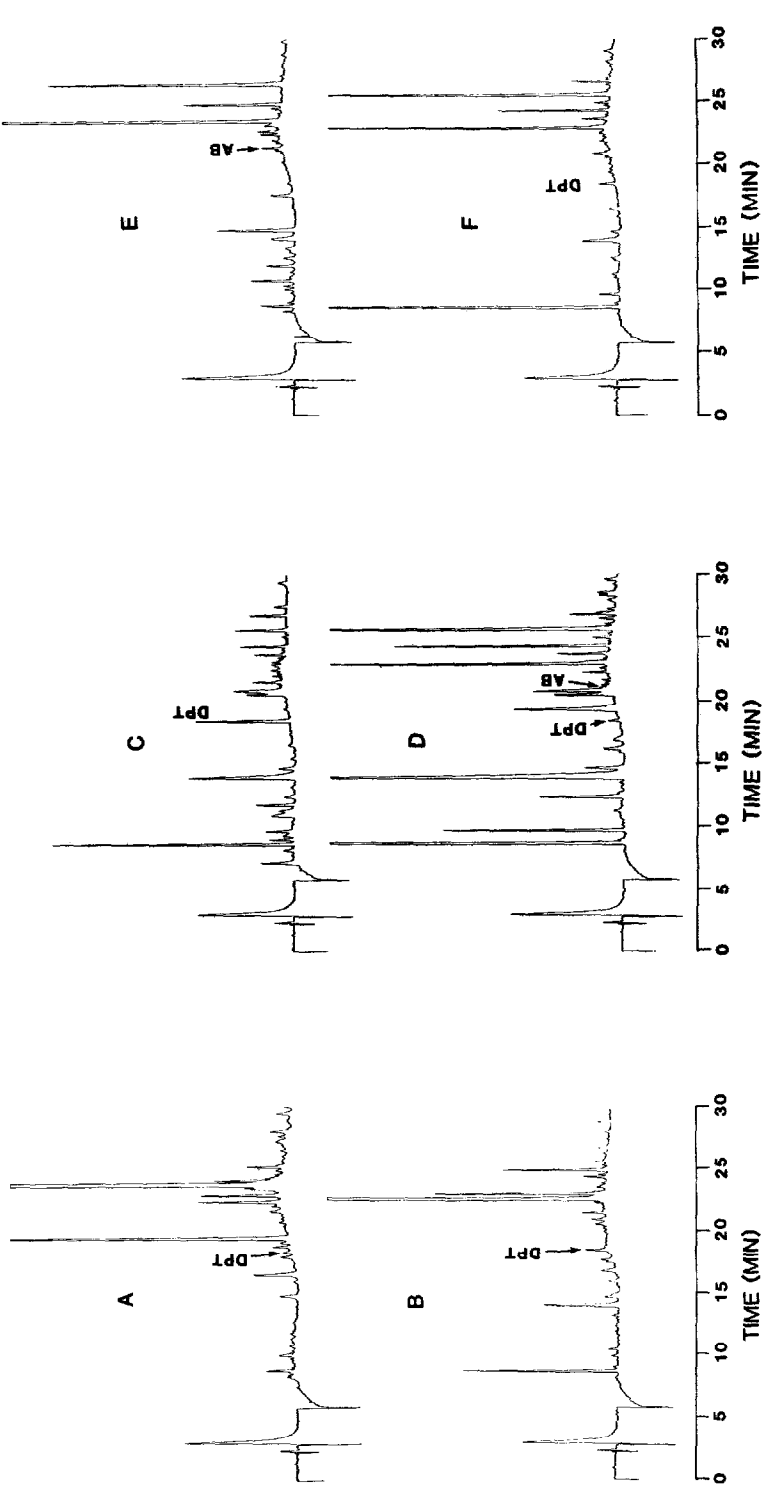


Fig. 4. HPLC chromatograms for (A) sample A; (B) sample B; (C) sample K; (D) sample J; (E) sample H; (F) sample C.

TABLE II

DETERMINATION OF DPT AND AB IN D&C RED NO. 33

Sample	*DPT (ppb)*	*AB (ppm)*
A*	68	ND**
B*	110	ND
C	94	ND
D	85	ND
E	75	ND
F	64	2.0
G	63	ND
H	ND	2.2
I	ND	ND
J	71	0.5
K	439***	ND

* Pharmacology sample.
** ND = none detected.
*** Average of five analyses.

tion of 6.8%. No AB was detected in this sample. The analytical results for the eleven samples of commercial product are shown in Table II. DPT was found in nine of the eleven samples analyzed at levels of up to 439 ppb (mean value for sample K). The overall average level of DPT found was 99 ppb.

AB was found in three of the eleven samples analyzed at levels up to 2.2 ppm. The determination of AB was more difficult since that region of the chromatogram contained a greater number of responses. The three samples reported to contain AB were the only ones to produce a definite response that was measured by the integrator.

The physical appearance and composition of the extract varied from sample to sample. The colors of the acetonitrile solutions of the extracts ranged from yellow to orange to red to blue. This variation was paralleled by the fluorescence observed when the extract was irradiated with UV light. The variability of the composition of the extract is reflected in the HPLC chromatograms obtained during analysis of D&C Red No. 33 as shown in Figs. 4A–F. The chromatograms in Figs. 4A and B were obtained for the two pharmacology batches of D&C Red No. 33. Examination of these chromatograms shows that there are many responses present in addition to those corresponding to DPT and AB. The identities of these contaminants have not been established.

UV–Vis spectra of DPT and AB

The DPT and AB responses obtained during analysis of the D&C Red No. 33 extracts were characterized for the purpose of confirming peak identity by obtaining the electronic absorption spectrum of each component as it was eluting from the HPLC column. This was accomplished by using a rapid-scan diode-array spectrophotometer with an HPLC flow cell installed in the cuvette holder[23]. A reference spectrum of authentic material was first obtained under conditions of analysis. The reference spectrum was then compared with the spectrum for the corresponding component obtained during the analysis of commercial D&C Red No. 33. Figs. 5A and B show the reference spectrum *versus* the spectrum of the extract obtained for DPT

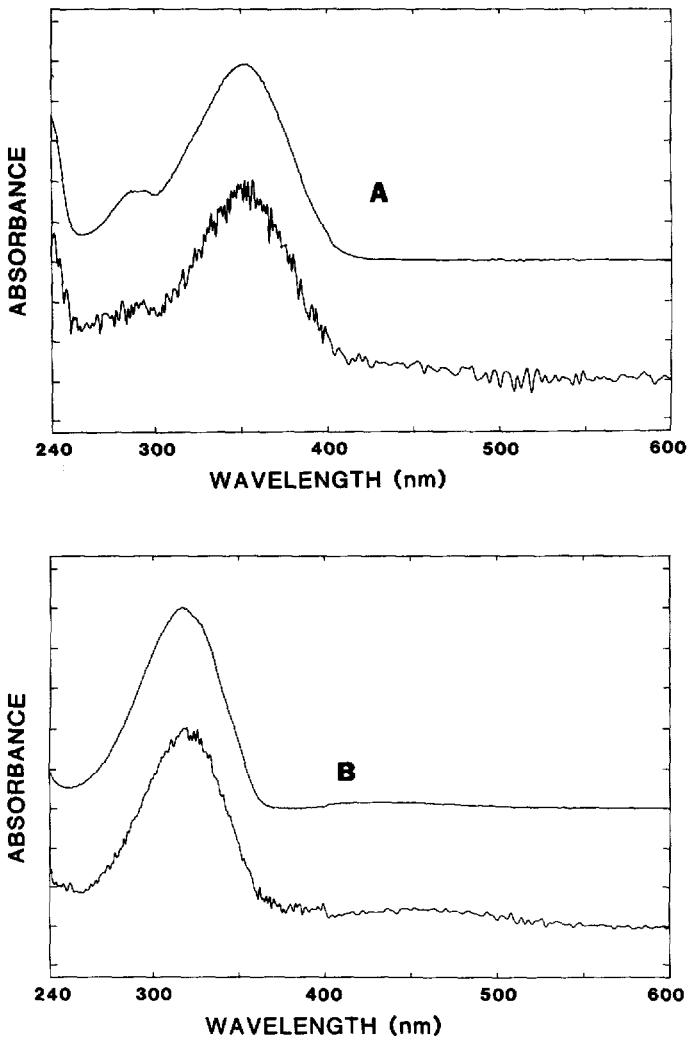


Fig. 5. UV–Vis spectral scans obtained during HPLC analysis: (A) DPT (sample K); (B) AB (sample H). Plotted spectra compare (top) authentic material with (bottom) corresponding component from analysis of the color extract.

and AB, respectively. The DPT spectrum (Fig. 5A) was obtained from sample K, which was found to contain the highest concentration of this component. The AB spectrum (Fig. 5B) was obtained from sample H. However, in order to obtain a usable spectrum for AB, it was necessary to concentrate the extract to approximately 0.25 ml and to inject 50 μl of the concentrate. In addition, the resolution was improved by using an isocratic solvent system in place of the gradient. The spectra for each of the components are identical to the spectra for the authentic material treated in a similar manner, thereby confirming the identity of the HPLC responses in these samples.

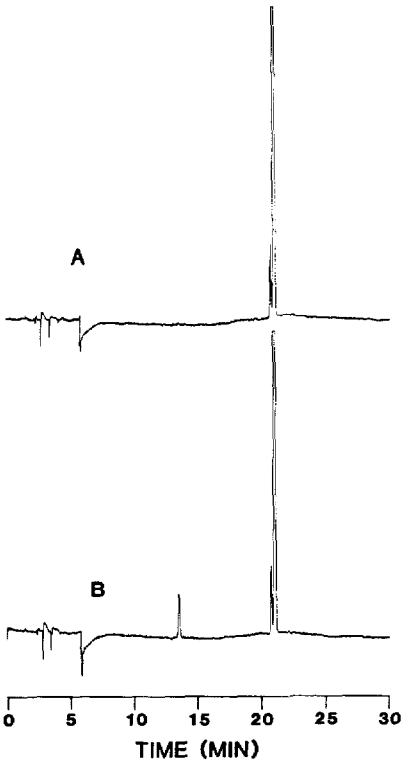


Fig. 6. HPLC traces for (A) 5-μl injection of freshly prepared stock solution of AB in methanol (67.4 mg/50 ml); (B) 5-μl injection of aged stock solution of AB in methanol.

Cis–trans isomerism of AB

During the course of this investigation it was noted that the HPLC profiles of the AB stock solution changed over a period of time. A freshly prepared stock solution of recrystallized AB in methanol produced one predominant chromatographic response. However, analysis of the same solution several days later showed an additional chromatographic response, which maintained a fairly constant level in subsequent chromatograms. Solutions of crude AB (before purification by recrystallization) produced a similar pattern of chromatographic responses. It is well known that AB can exist as *cis*- and *trans*-isomers, which can be separated from one another[24]. Therefore, the response that appeared during the analysis of solutions of AB was thought to arise from the formation of *cis*-AB. Solutions of AB reach equilibrium with the relative concentration of each isomer dependent on the solvent, temperature and presence or absence of light[24,25]. Reversed-phase HPLC has been reported to separate the isomers from one another[19]. Fig. 6A and B are chromatograms obtained from the HPLC analysis of a freshly prepared stock solution of AB and a stock solution aged in ambient light. The electronic absorption spectra obtained for these components during HPLC analysis (Fig. 7A and B) closely match the spectra reported for *cis*- and *trans*-AB in 95% ethanol[26]. The analytical HPLC separation of *cis*- and *trans*-AB was scaled to preparative level and the two components were

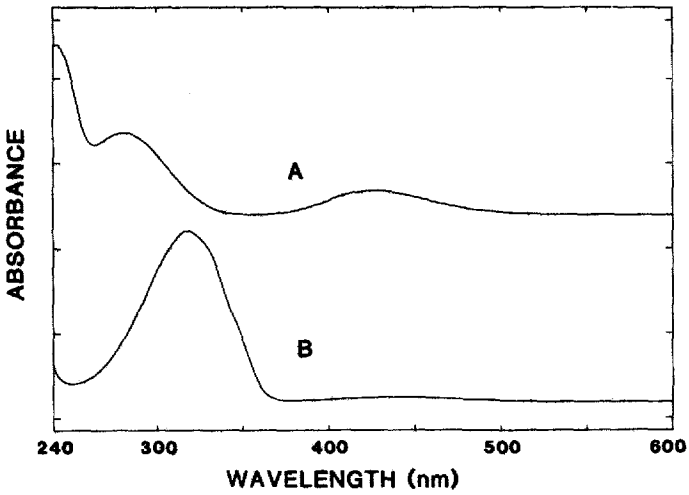


Fig. 7. UV–Vis spectral scans obtained during HPLC analysis of AB stock solution: (A) spectrum corresponding to *cis*-AB response; (B) spectrum corresponding to *trans*-AB response.

collected and analyzed. The two fractions, each of which contained one predominant component, yielded similar ratios of the two components after standing several days, thereby adding further evidence to support the conclusion that *cis*-AB is formed in stock solutions of AB. Thus the HPLC procedure described above may be a useful tool for the study of the isomerization of AB derivatives.

ACKNOWLEDGEMENT

The author thanks C. J. Bailey for her assistance in this study.

REFERENCES

1 *Code of Federal Regulations,* U.S. Government Printing Office, Washington, DC, 1984, Title 21, Sec. 81.1(b).
2 J. E. Bailey, Jr., *Anal. Chem.,* (1985) in press.
3 *IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man,* Vols. 1, 3, 4, 6–13, 17, Internal Agency for Research on Cancer, Lyon, 1974–1978.
4 A. H. M. Kirby, *Brit. J. Cancer,* 2 (1948) 290–294.
5 J. J. Sanchez Saez, C. A. Onecha Lopez, M. D. Herce Garraleta and M. L. Folgueiras Alonso, *Bol. Cent. Nac. Aliment. Nutr. (Spain),* (1981) 24–34; *C.A.,* 98 (1983) 33237j.
6 D. F. Detar and M. N. Turetzkey, *J. Amer. Chem. Soc.,* 77 (1955) 1745–1750.
7 K. H. Saunders, *The Aromatic Diazo Compounds,* Edward Arnold and Co., London, 2nd ed., 1949, pp. 157ff.
8 H. Zollinger, *Azo and Diazo Chemistry,* Interscience, New York, 1961, Ch. 8.
9 V. Zverina, J. Diva and M. Matrka, *Collect. Czech Chem. Commun.,* 20 (1977) 3109–3112.
10 Y. Ogata, Y. Nakagawa and M. Inaishi, *Bull. Chem. Soc. Jpn.,* 54 (1981) 2853–2854.
11 H. Endo, H. Noda, N. Kinoshita, N. Inui and Y. Nishi, *J. Natl. Cancer Inst.,* 65 (1980) 547–551.
12 P. Farina, A. Gescher, J. A. Hickman, J. K. Horton, M. D'Incalci, D. Ross, M. F. G. Stevens and L. Torti, *Biochem. Pharmacol.,* 31 (1982) 1887–1892.
13 C. J. Bailey, E. A. Cox and J. A. Springer, *J. Ass. Offic. Anal. Chem.,* 61 (1978) 1404–1414.

14 J. E. Bailey and E. A. Cox, *J. Ass. Offic. Anal. Chem.*, 58 (1975) 609–613.
15 J. E. Bailey and E. A. Cox, *J. Ass. Offic. Anal. Chem.*, 59 (1976) 5–11.
16 F. Matsui, E. G. Lovering, N. M. Curran and J. R. Watson, *J. Pharm. Sci.*, 72 (1983) 1223–1224.
17 R. M. Riggin, C. C. Howard, D. R. Scott and R. L. Hedgecoke, *J. Chromatogr. Sci.*, 21 (1983) 321–325.
18 L. A. Sternson and W. J. DeWitte, *J. Chromatogr.*, 137 (1977) 305–314.
19 Y. Fujita, K. Nara, A. Ito and O. Manabe, *Bunseki Kagaku, 26 (1977) 862–867; C.A.*, 88 (1978) 182081z.
20 J. D. Ramsey, T. D. Lee, M. D. Osselton and A. C. Moffat, *J. Chromatogr.*, 184 (1980) 185–206.
21 J. E. Bailey, *J. Chromatogr.*, submitted for publication.
22 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley-Interscience, New York, 2nd ed., 1979, pp. 791ff.
23 Hewlett-Packard Publication No. 23-5953-4725, Palo Alto, CA, Feb. 1980.
24 G. S. Hartley, *J. Chem. Soc.*, (1938) 633–642.
25 R. J. W. LeFevre and J. Northcott, *J. Chem. Soc.*, (1953) 867–870.
26 J. H. Collins and H. H. Jaffee, *J. Amer. Chem. Soc.*, 84 (1962) 4708–4712.