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PURIFICATION OF D&C RED NO. 33 BY PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Preparative reversed-phase high-performance liquid chromatography (HPLC) was used to purify a composite sample of D&C Red No. 33. A number of contaminating impurities were isolated from the dye and the purified material was essentially free of residual aromatic amines. A comparison was made between purification by preparative HPLC and recrystallization.

INTRODUCTION

D&C Red No. 33 (Colour Index No. 17200) is an azo dye prepared by coupling diazotized aniline with H-acid (4-amino-5-hydroxy-2,7-naphthalenedisulfonic acid). It is permitted for use in the U.S.A. in ingested drugs, lipsticks and externally applied drugs and cosmetics[1].

The commercially prepared dye consists principally of the disodium salt of 5-amino-4-hydroxy-3-(phenylazo)-2,7-naphthalenedisulfonic acid (CAS No. 3567-66-6). The color is also contaminated with unreacted intermediates, impurities in the intermediates, side reaction products and inorganic salts.

Recent investigations of various colorants have raised questions as to whether observed toxicity can be attributed to the principal component of the color or to impurities present in the dye. For example, genotoxic activity observed for amaranth was suggested to be the result of aromatic amines and subsidiary colors present in the dye[2]. Commercial Red 2G (Colour Index No. 18050), which is structurally similar to D&C Red No. 33, was found to be mutagenic with metabolic activation[3]. Purified Red 2G was found to be less mutagenic, suggesting that the presence of a substance other than the main color is responsible for the genotoxicity[4]. Mutagenic properties of some benzidine dyes, benzidine congener dyes and related monoazo dyes were suggested to be due to impurities in the tested material rather than to the main dye component[5].

Studies by the Food and Drug Administration (FDA) have been aimed at determining the composition of color additives intended for use in foods, drugs and cosmetics. Included in these studies is the development of an analytical procedure for the determination of unsulfonated aromatic amines and other unsulfonated constitu-

ents in D&C Red No. 33[6]. As part of this study it was necessary to obtain a purified reference material for calibration purposes that was free of aromatic amines. This paper describes the use of preparative reversed-phase high-performance liquid chromatography (HPLC) for the preparation of an adequate reference standard of D&C Red No. 33 as well as the isolation of impurities from the color additive.

EXPERIMENTAL

Instrumentation

Analytical HPLC separations were performed on a Varian (Palo Alto, CA, U.S.A.) Model 5060 gradient liquid chromatograph with a Varian Vista 401 system controller/integrator. Detection was with a Waters Assoc. (Milford, MA, U.S.A.) Model 440 dual wavelength detector at 254 nm. A Rheodyne (Cotati, CA, U.S.A.) Model 7010 pneumatic injector fitted with a 20-μl loop was used. The separations were made with a Hibar-RT column, 25 cm × 4.0 mm I.D., packed with LiChrosorb RP-18, 5-μm particle size (E. Merck, Darmstadt, F.R.G.).

The preparative HPLC separation was achieved by using a Chromatopac (Instruments S.A., Metuchen, NJ, U.S.A.) Prep-100 liquid chromatograph with a 1 m × 80 mm, axially compressed column. Solvent was delivered by an Eldex (Menlo Park, CA, U.S.A.) Model BBB triple piston pump. Detection was at 254 nm with an Altex (Berkeley, CA, U.S.A.) Model 152 UV detector fitted with a 0.5-mm pathlength preparative flow-cell.

UV–vis absorption spectra were obtained on a Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 8450A spectrophotometer.

Proton magnetic resonance (^{1}H NMR) spectra were obtained on a Varian Model EM-390 90 MHz spectrometer in DMSO-d_6 with tetramethylsilane in C^2HCl_3 as an external reference.

Reagents

All chemicals were analytical reagent grade unless specified otherwise. HPLC grade solvents were used for the preparation of all mobile phases.

A 50-g composite sample of D&C Red No. 33 was prepared by measuring and combining 5-g portions from ten different samples of certified lots of commercial dye. The dyes selected were all from the same manufacturer.

For the analytical HPLC system, solvent A was prepared by dissolving 1.5 g of ammonium acetate and 0.5 ml of acetonitrile in purified water (Millipore, Milford, MA, U.S.A.) and diluting to 100 ml. Solvent B contained the same amount of ammonium acetate, but 50 ml of acetonitrile.

Analytical chromatographic parameters

HPLC analysis on the analytical column was performed with detection at 254 nm by solvent gradient elution controlled by the Varian Vista 401 data system. The program was started at 100% solvent A and increased linearly to 100% solvent B over a 30-min period. The column was equilibrated with a flow of 100% solvent A for 10 min between injections. The flow-rate was kept constant at 1 ml/min throughout the analysis.

Preparative HPLC procedure

The 50-g composite of D&C Red No. 33 was mixed with 1 l of distilled water and stirred for 30 min. The solution was then filtered by suction through Whatman No. 2 filter paper. A 900-ml portion of the filtered solution was placed in a 1-l graduated cylinder and diluted to 1 l with water; the remaining 100 ml was diluted to 350 ml. These solutions were then subjected to preparative HPLC.

The preparative HPLC column was packed with approximately 2 kg of LiChroprep RP-18 packing material, 25–44 μm particle size, slurried in methanol. The column-packing piston was pressurized at 10 bar to compress the packing material. The column was flushed with 4 l of methanol (flow-rate = 90 ml/min) and then equilibrated with 4 l of methanol–water (7.5:92.5). The flow-rate was adjusted to maintain the column head pressure at 8 bar [void volume = 1950 ml, flow-rate after 4 l of methanol–water (7.5:92.5) = 50 ml/min].

The dye was applied to the column in two 500-ml portions followed by a final 350-ml portion for a total volume of 1350 ml. The application of the dye was followed by pumping 4 l of methanol–water (7.5:92.5) through the column. The solvent was then changed to methanol–water (50:50) and 1900 ml of this solvent was pumped onto the column, followed by 4 l of methanol to flush the column.

The collection of fractions was started when the detector first indicated that solute was eluting. This occurred at an eluate volume of 1460 ml measured from the point at which the dye was applied to the column. The fractions were cut according to their physical appearance and the detector response.

Treatment of fractions

Fractions 5 and 6. Each of the collected fractions was examined by analytical HPLC. Fractions 5 and 6 consisted principally of D&C Red No. 33 and were treated to isolate the dye from solution. Fraction 5 was diluted to 1200 ml with water and 50 g of sodium chloride was added. When the solution was stirred to dissolve the salt, a thick precipitate formed. Fraction 6 was diluted to 1200 ml and treated in a similar manner. Both precipitates were recovered by filtration through the same 15-cm medium porosity glass filter. The precipitate was washed on the filter with two 250-ml portions of acetone, allowed to air-dry for 60 h, and then dried in a vacuum oven at 110°C for 2 h. Yield of dried product is 32.93 g. Percentages calculated for $C_{16}H_{11}N_3S_2O_7Na_2$ are C, 41.12; N, 8.99; S, 13.72. Percentages found are C, 30.18; N, 6.54; S, 10.49. Volatile matter is 21.0% (vacuum oven at 110°C to constant weight). Sodium chloride is 4.3% by potentiometric titration. Total material accounted for is 99.4%. Purity (average of C, N and S) is 74.1%. Molar absorptivities calculated from the electronic absorption spectra are 26,330 at 236 nm in 0.1 *M* hydrochloric acid; 11,700 at 309 nm in 0.1 *M* hydrochloric acid; 29,880 at 536 nm in 0.1 *M* hydrochloric acid; 24,800 at 234 nm in 0.1 *M* sodium hydroxide; 14,800 at 307 nm in 0.1 *M* sodium hydroxide; 20,340 at 522 nm in 0.1 *M* sodium hydroxide.

Other fractions. The solvent was removed from Fractions 1–4 and 10, and the solids were dried at 110°C in a vacuum oven for 2 h. Yields are Fraction 1, 0.4 g; Fraction 2, 0.9 g; Fraction 3, 0.7 g; Fraction 4, 0.2 g; Fraction 10, 0.2 g. These materials were transferred to vials and refrigerated for storage. Fractions 7–9 were discarded.

Recrystallization of D&C Red No. 33

A 10-g portion of composite sample was placed in a 1-l beaker and dissolved in 500 ml of hot water. Decolorizing carbon (10 g) was added and the mixture was heated and stirred for 1 h. The hot solution was filtered by suction and 25 g of sodium chloride was added. The solution was reheated to dissolve the solids, allowed to cool to room temperature and then chilled in an ice bath for 30 min. The precipitate that formed was recovered by filtration, washed with 250 ml of acetone (the acetone was not allowed to mix with the filtrate) and then dried in a vacuum oven overnight at 110°C. Yield is 6.29 g. The acetone wash yielded an additional 1.16 g of material when taken to dryness under vacuum and dried in a vacuum oven at 110°C for 1 h.

RESULTS AND DISCUSSION

Recrystallization of D&C Red No. 33

Fig. 1A–C shows the effectiveness of the purification of D&C Red No. 33 by recrystallization. Fig. 1A is the HPLC chromatogram of the dye before recrystallization. The chromatographic profile shows many responses in addition to the main dye peak. The HPLC chromatogram for the recrystallized dye, Fig. 1B, indicates that some purification was achieved, as noted by the alteration of the HPLC profile. The primary result was a significant reduction in the size of several of the contaminant peaks. The HPLC chromatogram for the material remaining in the recrystallization solution, Fig. 1C, shows that many of the contaminants were concentrated in this step. However, recrystallization did not effectively remove many of the impurities

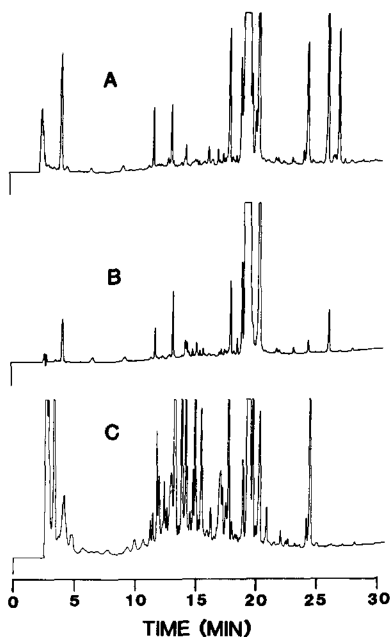

Fig. 1. Purification of D&C Red No. 33 by recrystallization. A, Starting material (0.25 g/100 ml); B, recrystallized dye (0.25 g/100 ml); C, mother liquor from recrystallization (with concentration adjusted to be approximately equal to A and B).

present in the commercial dye. Analysis for aromatic amines[6] also showed the presence of significant amounts of impurities, particularly of residual aniline. Since it was primarily the removal of the aromatic amines that was sought, preparative HPLC purification was attempted.

Preparative HPLC purification

The preparative HPLC purification of D&C Red No. 33 was accomplished by using a water–methanol solvent system that contained no added salts. The use of a relatively low level of organic modifier permitted the elution of the more lipophobic, sulfonated constituents of the dye while the unsulfonated, lipophilic impurities were retained on the column. Furthermore, the solvent system provided resolution of the various impurities of commercial D&C Red No. 33 from the main component.

Fig. 2 shows the chromatogram obtained during preparative HPLC of D&C Red No. 33, and Table I summarizes the fractions collected. The elution pattern observed during preparative HPLC suggests that the dye was concentrated at the head of the column, since the volume of the fractions containing the separated components was less than the volume of the dye solution applied to the column. This occurred because the dye was applied to the column in a solvent (water) that was

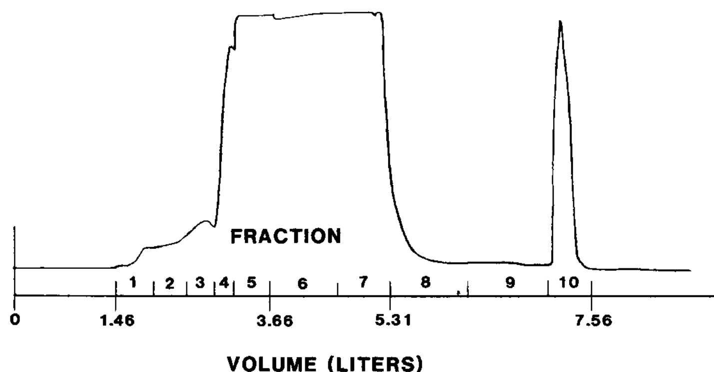

Fig. 2. Preparative HPLC chromatogram obtained for D&C Red No. 33.

TABLE I

PREPARATIVE HPLC FRACTIONS COLLECTED FOR D&C RED NO. 33

Fraction	*Volume of fraction (ml)*	*Total volume of eluate (ml)*	*Color of fraction*
1	500	1950	Light brown
2	500	2450	Light brown
3	450	2900	Brown
4	250	3150	Dark brown
5	500	3650	Red
6	900	4550	Dark red
7	750	5300	Dark red
8	1000	6300	Light red
9	950	7250	Light red
10	500	7750	Purple-violet

not strong enough to elute the dye. Subsequent elution with methanol–water (7.5:92.5) resulted in separation and collection of Fractions 1–9. Fraction 1 represents material excluded from the column since it eluted before the void volume.

Fractions 2 and 3 contained material which eluted with the water solvent in which the dye was applied to the column. Fractions 4–9 eluted with methanol–water (7.5:92.5). Fraction 10, which contained the material that was retained during elution with methanol–water (7.5:92.5), was subsequently flushed from the column with methanol–water (50:50). Flushing the column with 100% methanol did not elute any detectable amounts of impurities.

HPLC analysis of the fractions collected from the preparative HPLC purification procedure showed that most of the D&C Red No. 33 was contained in Fractions 5 and 6. Dilution followed by the addition of salt permitted the direct isolation of the dye from each of these fractions. Examination of the remaining fractions revealed that significant amounts of impurities were contained in Fractions 1–4 and 10. These fractions were concentrated, dried and retained for future investigations. Fraction 7 contained a relatively small amount of D&C Red No. 33 and the sub-

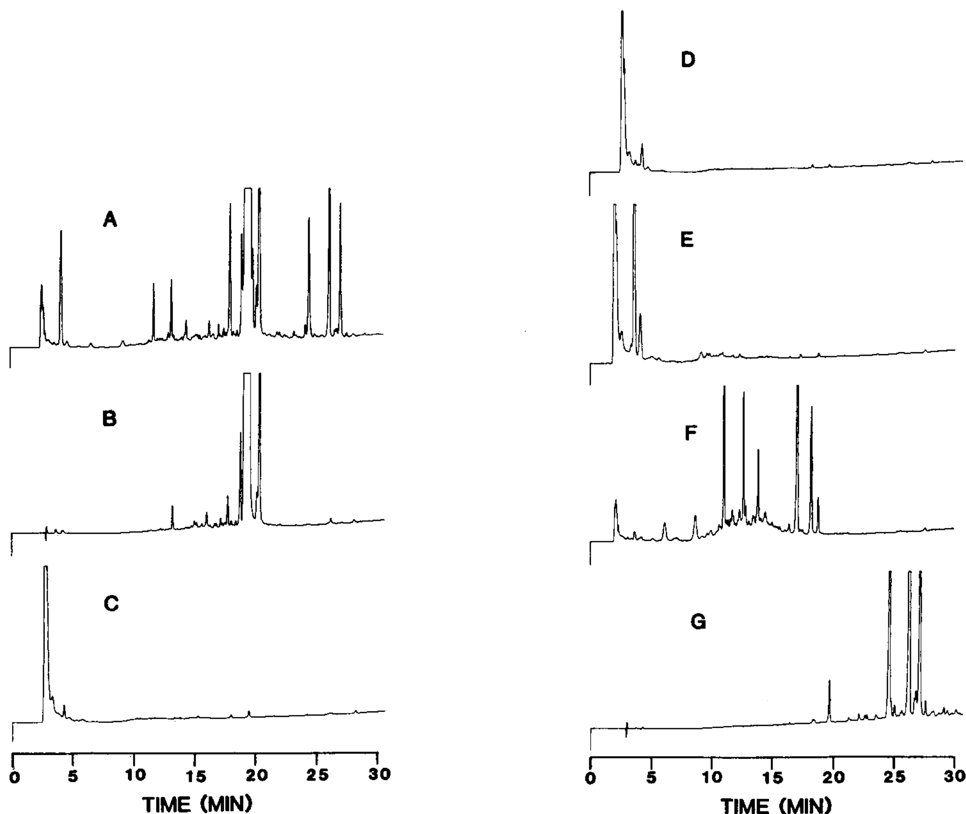

Fig. 3. Purification of D&C Red No. 33 by preparative HPLC. A, Starting material (0.25 g/100 ml); material isolated B, from Fractions 5 and 6 (0.25 g/100 ml); C, from Fraction 1 (5.4 mg/10 ml); D, from Fraction 2 (5.4 mg/10 ml); E, from Fraction 3 (5.3 mg/10 ml); F, from Fraction 4 (5.4 mg/50 ml) and G, from Fraction 10 (5.4 mg/100 ml).

sidiary color eluting just after the main dye component in the analytical HPLC system. Fractions 8 and 9 contained impurities eluting between the main dye component and the major late eluting components. Analysis suggested that these fractions contained very little detectable material and they were discarded.

Fig. 3A–G shows the analytical HPLC chromatograms for the dye before preparative HPLC, for the D&C Red. No. 33 isolated from Fractions 5 and 6 and for the material isolated from Fractions 1–4 and 10. It can be readily discerned that the preparative HPLC procedure separated the impurities in groups on the basis of their relative retention in the reversed-phase HPLC system. This separation is actually based on the relative lipophilicity of the components since retention has been shown to be related to the relative lipophilicity of compounds in a group[7–10]. The D&C Red No. 33 isolated from Fractions 5 and 6 is significantly purer than either the starting material or the recrystallized dye, although the HPLC chromatogram still contains responses in addition to those for the main dye component. Virtually all of the impurities eluting after the main dye component were removed after preparative HPLC purification of the dye. Analysis of the purified dye demonstrated that the amounts of aromatic amines present in the starting material were greatly reduced with only low levels of aniline detected[6].

The D&C Red No. 33 isolated by preparative HPLC was characterized by ^{1}H NMR spectrometry (DMSO-d_6) and by obtaining the UV–vis absorption spectra in 0.1 M sodium hydroxide and 0.1 M hydrochloric acid. The ^{1}H NMR spectrum indicated a complex pattern for aromatic protons. A singlet shifted strongly downfield (δ 15.4) is appropriate for a proton that is strongly hydrogen-bonded. This probably corresponds to the phenolic proton, which can be hydrogen-bonded with the amino nitrogen or can be involved in the azo–hydrazo tautomerism characteristic of azo dyes[11]. There was no signal that could be assigned to the amine group. This signal may be obscured by the broad multiplet for the aromatic protons (δ 7.0–8.0) or may be too broad to be observed.

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