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Preparative purification of tetrabromotetrachlorofluorescein and Phloxine B by centrifugal counter-current chromatography

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ABSTRACT

A centrifugal counter-current chromatographic method for preparative purification of commercial tetrabromotetrachlorofluorescein and Phloxine B (D&C Red Nos. 27 and 28, respectively) was developed. Ethyl acetate–*n*-butanol–0.01 *M* ammonium acetate (1:1:2) was used as the two-phase solvent system. Each purification trial involved 50 mg of sample and yielded 22 mg (±2 mg) of pure dye. The purity of the product was measured by high-performance liquid and thin-layer chromatography and was found to be 99.9%. The partition coefficients of these compounds were found to be highly concentration-dependent in the two-phase solvent system used. If this problem can be circumvented, then the counter-current chromatographic method can be extended for use with gram quantities of dye.

INTRODUCTION

Tetrabromotetrachlorofluorescein [**1**, Colour Index (1971) No. 45410:1, Fig. 1] and its disodium salt, Phloxine B (**2**, Colour Index (1971) No. 45410, Fig. 1], are dyes of the xanthene class. They are used primarily for coloring drugs and cosmetics [1], histological counter staining, and selective staining in some bacteriological and haematological techniques [2,3].

Tetrabromotetrachlorofluorescein is manufactured by condensing resorcinol with tetrachlorophthalic anhydride and brominating the reaction product [4]. Phloxine B is manufactured by alkaline hydrolysis of **1**. The reaction products (**1** and **2**) contain various organic impurities, including residues of the starting materials, side-reaction products and lower-halogenated subsidiary dyes. Compounds **1** and **2** are listed in the U.S. Code of Federal Regulations (CFR) for use in drugs and cosmetics and are designated as D&C Red No. 27 and D&C No. 28, respectively [1]. They are subject to batch certification by the Food and Drug Administration (FDA) to assure compliance with specifications and other requirements set forth in the CFR. The specifications include limitations for intermediates, subsidiary colors and organic

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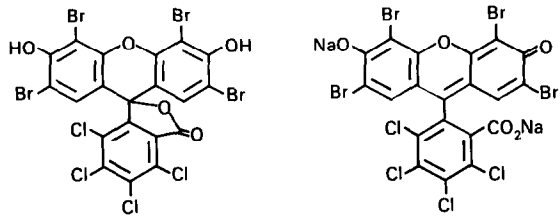

Fig. 1. Structures of tetrabromotetrachlorofluorescein (left, **1**) and Phloxine B (right, **2**).

side-reaction products. If present near the specification levels, these contaminants would represent a total of up to 8.3% of D&C Red No. 27 or 28.

In the development and validation of a high-performance liquid chromatographic (HPLC) method for analyzing batches of D&C Red Nos. 27 and 28 submitted to FDA for certification, pure samples of **1** and **2** were needed for use as standards. Pure dyes are desirable also for histological staining purposes to prevent the anomalous histochemical staining results explained by the presence of dye impurities as reported in the literature [5].

No preparative purification method was found in the literature for **1**; however, two methods were found for the purification of Phloxine B. One method uses acid precipitation [6], and the other uses gel chromatography [7]. Acid precipitation does not separate the lower-halogenated subsidiary colors from Phloxine B. The gel chromatographic method, while very effective, separates relatively small quantities (10–30 mg) of dye in each trial. For this study, a well-established method of purification, centrifugal counter-current chromatography (CCC) [8,9], was chosen as an alternative. CCC was previously shown to be successful in the preparative separation of polar compounds [10] and in the purification of other water-soluble dyes [11–13]. More significantly, two of these earlier studies [12,13] involved relatively large quantities of dyes (approximately 500 mg).

While preparative HPLC is a proven method for purification of gram quantities of compounds [14], it was not chosen for use in this case. From a study of the analytical-scale HPLC analysis of D&C Red No. 28 in progress at FDA, it was observed that the dye is adsorbed on the solid support of each of four different reversed-phase columns under evaluation. Since CCC involves liquid–liquid partition chromatography without a solid supporting matrix, the possibility of dye adherence to the column packing is eliminated.

This paper describes a simple and rapid method for the preparative purification of commercial tetrabromotetrachlorofluorescein and Phloxine B, using centrifugal counter-current chromatography.

EXPERIMENTAL

Materials

Tetrabromotetrachlorofluorescein and Phloxine B were selected from samples of commercial batches submitted for certification to the FDA. The tetrabromotetrachlorofluorescein (D&C Red No. 27) samples were hydrolyzed with Na_2CO_3 in accordance with the method used for solubilizing the lactone forms of Rose Bengal and

Eosin Y [15]. For HPLC measurements, stock solutions of commercial dyes and of CCC-purified dyes were prepared in 0.1 *M* ammonium acetate–methanol (3:1) at concentrations of 1 mg/ml and 0.5 mg/ml. The stock solutions were stored in a dark environment. Ammonium acetate (HPLC reagent) and ammonium hydroxide (30%) were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.); 95% aqueous ethanol was obtained from Pharmco (Dayton, NJ, U.S.A.) and butylamine was purchased from Fisher Scientific (Springfield, NJ, U.S.A.). All other solvents used were HPLC or chromatographic grade.

Centrifugal counter-current chromatography

CCC separations were performed using a horizontal flow-through coil planet centrifuge equipped with three column holders. The basic design of the apparatus has been reported earlier [16]. The apparatus holds three sets of composite coiled-column assemblies around the rotary frame at a distance of 10 cm from the central axis of the centrifuge. Each coil assembly consists of eight identical coils, each prepared from 1.6 mm I.D. PTFE (polytetrafluoroethylene) tubing by winding it onto a 12 cm × 1.25 cm O.D. aluminum pipe, forming two layers of coils with approximately 70 helical turns and 9 ml capacity. These dual-layer coils are connected in series and mounted around the column holder parallel to, and at a distance of, 4 cm from the central axis of the holder. The three coil assemblies are connected in series on the rotary frame to make up a total column capacity of approximately 230 ml. The apparatus can be rotated up to 1000 rpm with a speed controller. Similar equipment is commercially available from either Peptide Technologies (Washington, DC, U.S.A.) or Pharma-Tech Research (Baltimore, MD, U.S.A.).

Based on the partition coefficient measurements, *n*-butanol–ethyl acetate–0.01 *M* ammonium acetate (1:1:2, adjusted to pH 9 by addition of ammonium hydroxide to the lower phase) was selected as the two-phase solvent system for purifying **1** and **2**. The solvent mixture was thoroughly equilibrated in a separatory funnel and the two phases separated shortly before being applied to the column. The upper and lower phases were each used separately as the mobile phase in different runs. In each separation, the column was first filled entirely with the stationary phase followed by injection of the dye solution (50 mg in 5 ml of the lower phase). The mobile phase was then pumped into the column at a flow-rate of 60 ml/h while the apparatus was rotated at 800 rpm. When the lower phase was used as the mobile phase, the effluent from the outlet of the column was continuously monitored with an ultraviolet detector (Uvicord S; LKB, Stockholm, Sweden) at 276 nm and fractionated (3 ml or 6 ml/test tube) with a fraction collector (Ultrorac, LKB). When the upper phase was used in that capacity, an air bubble in the flow cell disrupted the UV monitoring. Therefore, an aliquot of each fraction was mixed with 3 ml of methanol and the absorbance determined at 546 nm with a Zeiss PM6 spectrophotometer.

Recovery of the pure dye

After separating the fractions with the highest absorbance values (*e.g.*, fractions 85–96, Fig. 7), the solvent (upper phase or lower phase) was removed by rotary evaporation. The purity of the dye was confirmed by HPLC and thin-layer chromatography (TLC). A typical trial yielded 23 mg of purified material.

High-performance liquid chromatography

The system consisted of a Model 8800 ternary pump, Model 4270 integrator, Model 8780 autosampler, Model 8500 dynamic mixer (all by Spectra-Physics, San Jose, CA, U.S.A.). The autosampler was equipped with a Model 7010 injector (Rheodyne, Cotati, CA, U.S.A.) with a 200-μl sample loop and Model 490 dual wavelength UV–VIS detector (Waters Assoc., Milford, MA, U.S.A.). A Hypersil MOS-1 RPC-8, 5 μm particle size column (250 mm × 4.6 mm I.D.) (Keystone, Bellefonte, PA, U.S.A.) was used throughout. A detector wavelength of 546 nm was used for monitoring the lower halogenated subsidiary dyes, and a wavelength of 254 nm was used for monitoring starting materials (uncombined intermediates) and side-reaction products. The solutions were filtered through LID/X syringeless filters AQOR.45 (Genex, Gaithersburg, MD, U.S.A.) prior to injection.

The chromatographic conditions were as follows: mobile phase, 0.1 *M* ammonium acetate (A)–methanol (B) with linear gradients from 25% to 90% B over 25 min and from 90 to 100% B over the next 5 min. This was followed by an isocratic column wash with 100% B for 6 min. The column was re-equilibrated with 25% B for 14 min. Other conditions were: injection volume, 50 or 100 μl; detector sensitivity, 1 a.u.f.s.; chart speed, 0.5 cm/min.

Thin-layer chromatography

The purity of the commercial samples and the CCC-purified fractions was determined by TLC according to the method used for the determination of lower-halogenated subsidiary dyes in D&C Red Nos. 27 and 28 [17]. Solid material (2 mg) from the original samples or obtained from the CCC separation was dissolved in 95% ethanol–water–30% ammonium hydroxide (5:5:0.1) (1 ml). A total of 1 mg of dye was streaked onto a silica gel G 20 × 20, 250 μm plate (Fisher Scientific). The plates were developed with acetone–chloroform–butylamine (66:24:4.5). After the band was scraped and the material corresponding to subsidiary dyes was extracted with 15% ammonium hydroxide (8–10 ml), the solution was examined spectrophotometrically (400–700 nm). No scraping was required for the CCC-purified fractions since no bands corresponding to lower-halogenated subsidiary dyes were observed.

RESULTS AND DISCUSSION

The results of the study demonstrate the ability of the CCC method to separate the impurities of **1** and **2**. Confirmation of adequate purification was provided by HPLC and TLC analyses. Each separation experiment involved 50 mg of commercial **1** and **2** and yielded 22 mg (±2 mg) of purified dye. Figs. 2 and 3 show HPLC chromatograms of typical samples of **2** and **1**, respectively, before (I) and after (II) purification. In both cases, integration of the HPLC–UV peak areas indicates that the desired separated dye is 99.9% pure (at 254 nm and 546 nm). TLC analyses corroborated the HPLC results in yielding only one spot each for purified **1** and purified **2**.

It should be noted that CCC purification was also attempted with larger quantities (210 mg and 2 g) of commercial Phloxine B. In each of the two trials, an anomalous double-peak elution curve was obtained, suggesting that the dye was separated into two different components (Fig. 4). HPLC analyses revealed that the two peaks (fractions 75 and 84, Fig. 4) of the elution curve represented identical, partially puri-

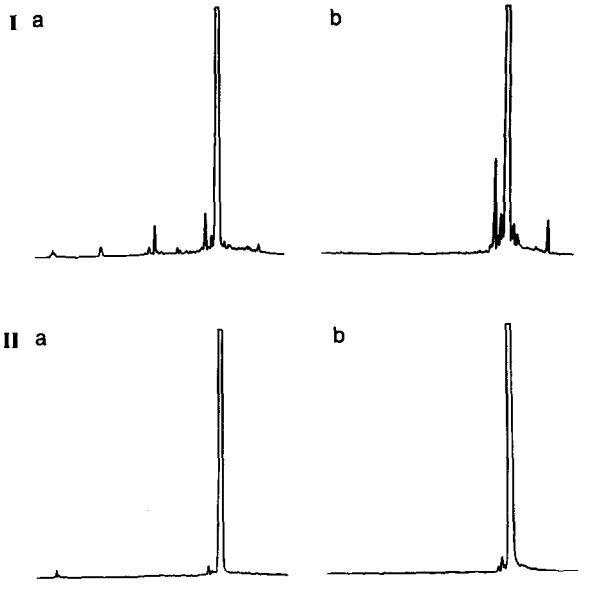

Fig. 2. HPLC chromatograms of typical commercial (I) and CCC-purified (II) Phloxine B. (a) 254 nm, (b) 546 nm. Each of the injections (100 μl) contained 50 μg of dye. Other chromatographic conditions are described in the Experimental section.

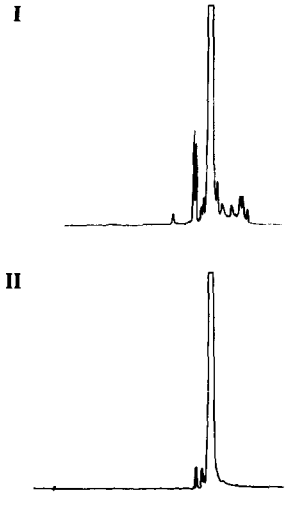

Fig. 3. HPLC chromatograms of typical commerical (I) and CCC-purified (II) tetrabromotetrachlorofluorescein at 546 nm. Each injection (100 μl) contained 50 μg of dye. Other chromatographic conditions are described in the Experimental section.

fied dye. It is suggested that this unusual result is related to the fact that the partition behavior of the dye is highly concentration-dependent. The effect of concentration on the partition coefficient in the two-phase solvent system of *n*-butanol–ethyl acetate–0.01 *M* ammonium acetate (1:1:2) is illustrated in Fig. 5, where the abscissa indicates the dye concentration and the ordinate, the partition coefficient expressed as the solute concentration in the upper phase divided by that in the lower phase or $K(C_u/C_l)$, both plotted on a logarithmic scale. As shown in the diagram, at a high concentration the dye is partitioned primarily into the lower phase; when diluted, it moves into the upper phase. This non-linear isotherm is considered to produce a significant effect on the elution curve. Introduction of the two highly concentrated dye solutions into the CCC column thus yielded the double-peak elution profile.

Fig. 6 shows a chromatogram of **2** obtained by on-line monitoring of the effluent at 276 nm using the described CCC centrifuge. The separation was performed by eluting the lower aqueous phase at a flow-rate of 1 ml/min at 800 rpm. A 50-mg dye sample was eluted in approximately 3 h. Because of the non-linear isotherm of the partition coefficient mentioned above, the peak of the pure dye is markedly skewed toward the left while most of the impurities are fairly well separated from the pure dye because they eluted near the solvent front or remained on the column.

Fig. 7 shows a chromatogram of the same sample of **2** as that used for Fig. 6. In Fig. 7, the separation was obtained by eluting the upper non-aqueous phase. Elution

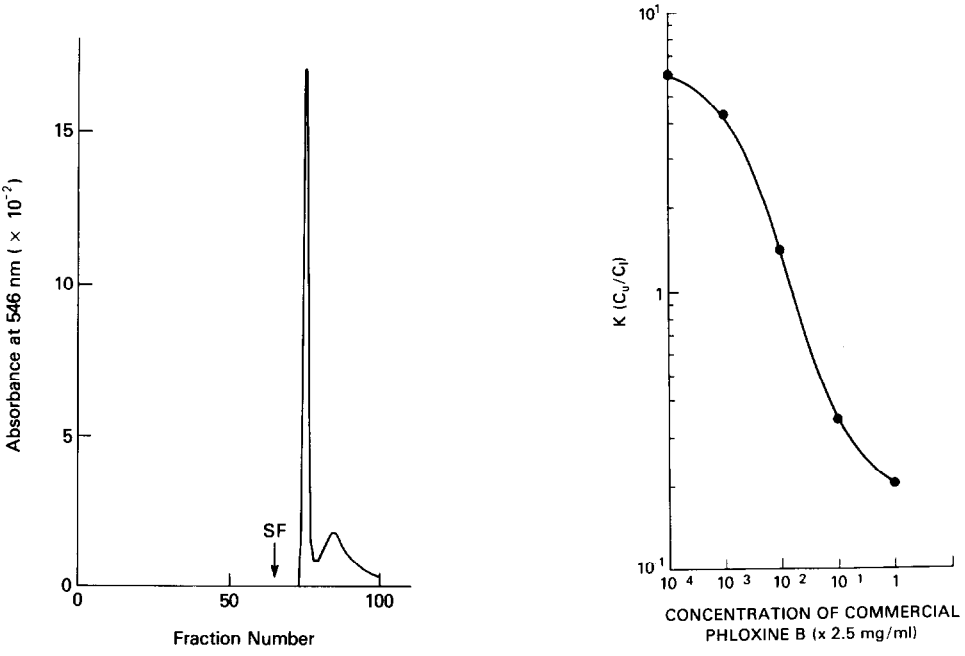

Fig. 4. Double-peak CCC elution curve obtained in a purification trial involving a 2-g sample of commercial Phloxine B. SF = Solvent front.

Fig. 5. Partition coefficient at various concentrations of commercial Phloxine B in *n*-butanol–ethyl acetate–0.01 *M* ammonium acetate (1:1:2).

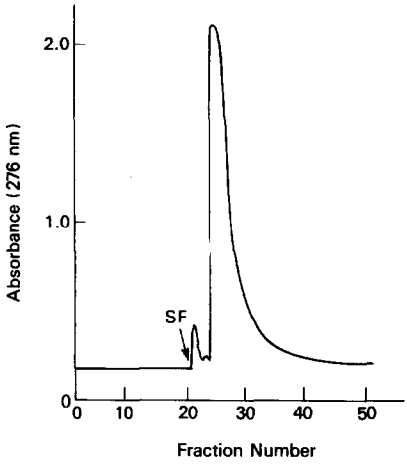

Fig. 6. Typical CCC elution curve for commercial Phloxine B when the lower aqueous phase of the *n*-butanol–ethyl acetate–0.01 *M* ammonium acetate (1:1:2) solvent system was used as the mobile phase. SF = Solvent front. Other experimental conditions are as follows: flow-rate: 1 ml/min; fractionation: 6 ml/tube; revolution: 800 rpm; retention of the stationary phase: 28%; pressure: 300 p.s.i.

with the upper phase, however, produced a problem in on-line monitoring of the effluent. Introduction of the effluent into the flow cell from the bottom in an upward direction trapped the carried-over stationary phase in the flow cell. By reversing the flow, air bubbles in the flow cell were trapped. For this reason, the elution curve was manually drawn by measuring the absorbance of each fraction at 546 nm with a PM6 Zeiss spectrophotometer. This reversed elution mode provides an advantage over the normal elution mode (Fig. 6) in that the dye is retained longer in the separation column, yielding fractions of higher purity.

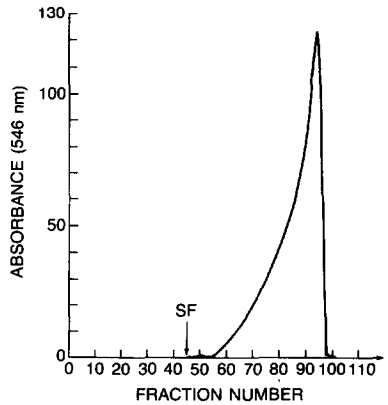

Fig. 7. Typical CCC elution curve for commercial Phloxine B when the upper non-aqueous phase of the *n*-butanol–ethyl acetate–0.01 *M* ammonium acetate (1:1:2) solvent system was used as the mobile phase. SF = Solvent front. Other experimental conditions are as follows: flow-rate: 1 ml/min; fractionation: 3 ml/tube; revolution: 800 rpm; retention of the stationary phase: 27%; pressure: 300 p.s.i.

CONCLUSIONS

The CCC method was demonstrated in this study to be an effective method for purifying dyes of the xanthene class. It is therefore worthwhile to determine a means of circumventing the double-peak problem so that the CCC method can be applied to the purification of larger quantities of dyes. Specifically, a different solvent system must be found, and investigations toward this end are currently in progress. Research efforts are also being directed toward the identification and quantification of the various impurities found in commercial tetrabromotetrachlorofluorescein and Phloxine B. The data characterizing the impurities, together with improved purification techniques, will upgrade the quality and efficiency of FDA's certification process and contribute to more accurate histochemical staining uses of the dyes.

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