Thin-Layer Chromatography of Certified Coal Tar Color Additives

By MELVIN H. PENNER

A comprehensive thin-layer chromatographic procedure has been developed for the systematic separation and identification of 19 water- and/or alcohol-soluble coal tar dyes currently permitted for use in pharmaceutical dosage forms. TLC on cellulose-coated chromatoplates and development in one of the following solvent systems: (A) ethyl acetate–*n*-butanol–pyridine–water (5:5:6:5); (B) ethyl acetate–*n*-butanol–concentrated ammonia (20:55:25); (C) ethyl acetate–*n*-propanol–concentrated ammonia–water (35:35:20:20); (D) *n*-propanol–ethyl acetate–concentrated ammonia (65:75:60) offers rapid, sharp separations into compact zones of the most common colorant combinations used to achieve special color effects. Quantitation of the colorants may then be achieved by densitometric scanning of the developed plates. The method has also been applied for the detection of dye incompatibility in a liquid preparation.

DYES ARE USED in the pharmaceutical industry to achieve esthetic color characteristics required for consumer acceptance of drug products and for the identification and differentiation of dosage forms. Only those colorants which have been certified by the Food and Drug Administration may be used in drug products marketed in the United States. The array of coloring agents previously available has been considerably reduced because of the delisting of a number of certified dyes, necessitating the reformulation of many products. Since colors are generally produced by blending two or more dyes to achieve a desired effect, a rapid chromatographic system for the separation and identification of the component dyes in a color was highly desirable for formulation studies. Since the separation of all dyes is more of theoretical than practical interest, this investigation was limited to 19 certified colorants most frequently encountered in pharmaceutical formulations.

Prior separation of dyes in mixtures is necessary for identification and quantitation in most cases, for absorption spectrophotometry usually presents a confusing picture of overlapping bands. Color analysis based on partition of the dyes between two immiscible solvents was reported by Koch (1). However, at best the separations are only fractional and several of the permitted dyes are discolored by the treatment.

The chromatography of coal tar colorants has been the subject of numerous investigations. Separation of colors by paper chromatography (2–9) gives sharper separations than partition or adsorption column chromatography (10–13), electrophoresis (14–16), or ion-exchange methods (17, 18). However, no one solvent can effect satisfactory separation of mixtures of dyes, particularly the red dyes which give almost the same R_f value with most solvents.

Absorption spectrophotometric methods have been most widely used for the quantitative estimation of separated colors. Stanley and Kirk (3) have also applied reflectance measurements to the isolated dyes on paper chromatograms and achieved better results.

This report describes a scheme for the separation, identification, and quantitation of the most frequently used certified water- and/or alcohol-soluble colorants by TLC in a limited number of solvent systems. An example of the use of this technique in studying dye incompatibility is presented.

EXPERIMENTAL

Chromatoplates—Commercially prepared plates, 20 × 20 cm., coated with microcrystalline cellulose[1] to a layer thickness of 250 μ and purchased from Analtech, Inc., were used in this study without prior activation.

Solvent Systems—Four monophasic solvent systems were found to be the most useful: A, ethyl acetate–*n*-butanol–pyridine–water (5:5:6:5); B, ethyl acetate–*n*-butanol–concentrated ammonia (20:55:25); C, ethyl acetate–*n*-propanol–concentrated ammonia–water (35:35:20:20); D, *n*-propanol–ethyl acetate–concentrated ammonia (65:75:60).

Dye Solutions—Stock solutions of the following certified dyes were prepared at a concentration of 1 mg./ml. of water: FD&C Red No. 2 (R2), FD&C Red No. 3 (R3), FD&C Red No. 4 (R4), D&C Red No. 19 (R19), D&C Red No. 22 (R22), D&C Red No. 28 (R28), D&C Red No. 33 (R33), FD&C Blue No. 1 (B1), FD&C Blue No. 2

Received May 22, 1968, from the Pharmaceutical Research and Development Labs., Warner-Lambert Research Institute, Morris Plains, NJ 07950

Accepted for publication August 19, 1968.

Presented to the Drug Standards, Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

The author wishes to express his gratitude to Mr. D. M. Schoeffel and his associates for their technical assistance, and to Drs. T. I. Fand and L. Chafetz for their invaluable aid in preparing this manuscript.

[1] Trade marked as Avicel by American Viscose Div. of FMC Corp., Marcus Hook, Pa.

(B2), FD&C Yellow No. 5 (Y5), FD&C Yellow No. 6 (Y6), D&C Yellow No. 10 (Y10), D&C Orange No. 4 (O4), FD&C Violet No. 1 (V1), FD&C Green No. 3 (G3), and D&C Green No. 5 (G5). The stock solutions of D&C Yellow No. 11 (Y11), D&C Green No. 6 (G6), and D&C Orange No. 5 (O5) were prepared at the 0.1% concentration in alcohol since they are not water soluble.

Chromatographic Development—To determine the R_f values of the individual dyes under consideration and to determine the most effective solvent system to achieve the desired separation, 1 μl. of each stock solution, dispensed from a 1-μl. disposable pipet, was spotted to separate points on the origin on each of four chromatoplates, approximately 2.5 cm. from the bottom edge. The plates were then placed in the chromatographic chamber which has been equilibrated with the respective solvent system for 30–60 min. and the solvent allowed to travel a distance of 10–15 cm. from the point of origin. The plates were removed from the development chamber and air dried.

Detection—The developed chromatograms were viewed under long-wavelength UV light in addition to ordinary illumination. The colors and R_f values were noted.

Quantitation—A standard mixture containing four certified dyes, *i.e.*, FD&C Red No. 2 (0.5 mg./ml.), D&C Red No. 33 (0.5 mg./ml.), FD&C Yellow No. 6 (0.75 mg./ml.), and D&C Green No. 5 (0.1 mg./ml.), was prepared in aqueous alcohol. This stock solution was then serially diluted to contain 0.4, 0.3, 0.25, 0.2, 0.15, and 0.1 mg. of D&C Red No. 33/ml. solution. Five microliters of each solution, employing self-filling micropipets calibrated to deliver accurately known volumes, was spotted to alternate 1-cm. wide channels inscribed in a cellulose-coated chromatoplate (20 × 20 cm.) and allowed to air dry. The plate was developed in a chamber equilibrated with solvent system C to a distance of 12 cm. from the origin, removed from the chamber, and air dried. A Schoeffel spectrodensitometer model SD 3000, a double-beam ratio-type scanning instrument equipped with a high-intensity continuous xenon light source, quartz prism monochromator, and an integrating recorder was used for quantitation. Each dye was scanned and recorded with the monochromator set at the wavelength of maximum absorbance for the respective dye as determined on the chromatoplate.

RESULTS AND DISCUSSION

Qualitative Separations—The average R_f values of the 19 dyes considered in this investigation in each of the four solvent systems and the respective colors observed under long-wavelength UV light are recorded in Table I. The values reported in the parentheses represent minor impurities detected in the raw materials.

In conjunction with the R_f values, further verification of identities of the colorant mixture components may be ascertained by viewing the developed chromatogram under long-wavelength UV light and/or spraying the plate with various reagents noting the change in color as reported by numerous investigators (3, 4, 7, 19). It should be noted that FD&C Blue No. 2 has a tendency to fade in systems containing ammonia.

In 1944, Peacock (20) published colorant formulas

Table I—R_f Values of Certified Dyes by TLC

Dye	Solvent System A	B	C	D	Color Under UV Light
Blue					
FD & C No. 1	0.53	0.24	0.72	0.56	Violet
FD & C No. 2	0.33	0.09	0.47	—	Blue
Green					
FD & C No. 3	0.49	0.11	0.57	0.38	Pink
D & C No. 5	0.64	0.38	0.77	0.63	Blue
D & C No. 6	1.0	1.0	1.0	1.0	Blue
Orange					
D & C No. 4	0.76	0.55	0.89	0.79	Yellow-brown
D & C No. 5	0.74 (0.94)	0.28 (0.23)	0.63 (0.67)	0.55	Yellow
Red					
FD & C No. 2	0.28	0.00	0.28	0.10	Red
FD & C No. 3	0.94	0.46	0.79	0.68	Dull orange
FD & C No. 4	0.53	0.05	0.30	0.21	Reddish-orange
D & C No. 19	0.93	0.96	0.98	1.0	Brilliant orange
D & C No. 22	0.88	0.37	0.73	0.61	Yellow-green
D & C No. 28	0.79	0.51	0.83	0.74	Orange
D & C No. 33	0.43	0.12	0.54	0.39	Scarlet
Yellow					
FD & C No. 5	0.26	0.00	0.15	0.06	Deep yellow
FD & C No. 6	0.47 (0.26)	0.15	0.62	0.41	Red
D & C No. 10	0.62 (0.70)	0.29 (0.42)	0.72 (0.77)	0.58 (0.69)	Green
D & C No. 11	0.95	1.0	1.0	1.0	Green
Violet					
FD & C No. 1	0.61	0.38	0.80	0.66	Violet

TABLE II—TLC SEPARATION OF SOME COLORANT MIXTURES

Color	Dye Mixture	Solvent System
Black	R2, Y6, B1	C, D
Black	R2, Y6, B2, Y5	C
Blue	R3, G5	B
Deep blue/grape	R2, B1	A, C, D
Caramel/chocolate	R2, Y5, Y6, B1	C
Mint-green	Y11, G6	A
Lime	B1, Y5	A, C
Reddish-orange	R2, Y6	C, D
Medium orange	R4, Y5	A, C, D
Amber	R2, Y5	C
Orangeade	R4, Y5, Y6	C, D
Wine	R2, Y6, B1	C, D
Cola	R2, Y5, B1	C
Maroon	R2, V1	A, C, D
Cherry	R2, R4	A, D
Strawberry	R2, R4, Y6	D
Pink	R3, Y6	A, B, C, D
Raspberry	R2, Y6	C, D
Egg-yellow/lemon	R2, Y5, Y6	C

for use in proprietary mixtures to obtain various color effects, such as mint-green, caramel, strawberry, *etc.* Since each of these formulas contained some proportion of FD&C Orange No. 1 or FD&C Green No. 2, which have since been delisted, new formulas had to be devised. Several such replacements were reported by Peacock (21) and Zuckerman (22). Table II lists the colorant mixtures used to produce some popular color effects and the solvent system(s) best suited in each case to resolve the dye components.

As evident from Tables I and II, solvent systems C and D are the best choice for general screening of all the dyes and the latter especially where the presence of one or more red colorants is indicated. The remaining two systems were developed for special cases as shown in the tables.

Quantitation—Both absorbance and reflectance measurements for the quantitation of the chromatographically resolved dyes have been reported by Stanley and Kirk (3). However, the quantity of dye required in their method was considered excessive, since repeated evaporation of a dilute dye solution may be accompanied by sufficient build-up of excipient materials to affect the migration of the dye material.

Spectrodensitometry offered a significant increase in sensitivity. A plot of the integrated area obtained *versus* the concentration applied is shown in Fig. 1. Statistically, the best straight line was constructed for each colorant by means of linear regression analysis. A linear relationship was obtained over the concentration range considered which is also evidenced by the calculated value of the correlation coefficient, r, for each dye being extremely close to 1. From Fig. 1, the standard error of estimate, $s_{y \cdot x}$, for each colorant was calculated. Table III summarizes the r and $s_{y \cdot x}$ values obtained in each case.

The lower limit of detection of 0.2 mcg. of D&C Green No. 5 was achieved utilizing a more sensitive recorder range than for the remaining three dyes.

Standard calibration curves were necessarily prepared for each dye since the slopes of the four lines varied which may be attributed to several

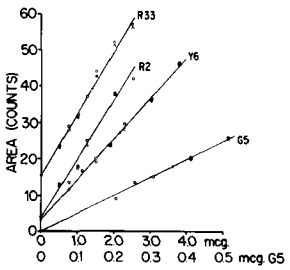

Fig. 1—Relationship of the spot area of various dyes to the concentrations applied. Slit width = 1 mm.; monochromator: R2 and R33 = 515 mμ, Y6 = 485 mμ, G5 = 628 mμ; recorder range: R2, R3, Y6 = 0–3 full scale, G5 = 0–0.5 full scale.

factors such as the intensity of stray light reaching the photocell detector, the purity of the standards, and variation in adsorbent layer thickness. Deviations from linearity resulting in curvature of the lines as the amount of dye spotted increases may be caused by saturation so that the peak density no longer increases proportionally with concentration. Differences in scans are then mainly caused by increases in spot size.

In order to ensure reproducibility and accuracy of the densitometric quantitation, several experimental parameters were found to be critical. The spectrodensitometer employed is designed to minimize errors due to moisture and to variations in thickness of the adsorbent material by scanning the sample channel *versus* the adjacent strip untouched to serve as the reference. Since the reference and sample beams are closely adjacent, positioning of the well-defined strips evenly under the sample and reference beam is important. The scribe line must be positioned evenly between the two light beams for reproducible results. Although the width of the channels is approximately 1 cm., care should be taken when depositing the sample to minimize diffusion of the sample laterally toward the edges of the strip. If this occurs, the subsequently chromatographed strip may exhibit the edge phenomenon which will seriously affect the results. The slit covers virtually the entire channel which has the advantage that the full width of the absorbing spot is scanned and all the material in the spot contributes to absorption of light. This eliminates errors due to small changes in shape and centering of the spot. Care should be taken, however, to deposit the initial spot as uniformly as possible. Excessive tailing or overlapping of zones decrease the precision of area measurements. The densitometry error, calculated from repetitive scanning of the same spot, was 1.3%. The micropipets used for spotting the dyes are capable of delivering a known volume with an accuracy of ±3%.

Dye Incompatibility—The present chromatographic procedure has also been applied to deter-

TABLE III—CALCULATED VALUES OF r AND $s_{y \cdot x}$

Dye	r	$s_{y \cdot x}$, %
R2	0.988	±6.1
R33	0.993	±4.1
Y6	0.997	±3.3
G5	0.992	±3.8

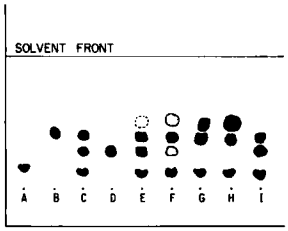

Fig. 2—Dye incompatibility study. Solvent system: ethyl acetate–n-butanol–pyridine–water (5:5:6:5). Plate: microcrystalline cellulose. Key: A, FD & C Red No. 2; B, FD & C Red No. 4; C and I, mixture of reds 2, 4, and 33; D, D & C Red No. 33; E, sample after 4 days at 60°; F, sample after 12 days at 60°; G and H, sample after 18 months at room temperature (3 and 5 μl., respectively).

mine the cause of color darkening observed visually in a liquid dosage upon aging at room temperature. The proposed formula contained a mixture of FD & C Red No. 2, No. 4, and D & C Red No. 33. A 100-ml. aliquot was extracted according to the procedure reported by Bandelin and Tuschhoff (7) and redissolved in 5 ml. alcohol. Standard solutions of the individual dyes and the sample extract were spotted on a microcrystalline cellulose plate and chromatographed in solvent system A. The resulting TLC pattern indicated the depletion of D & C Red No. 33 with the concomitant formation of a violet-colored species. Further experiments demonstrated that a reaction between D & C Red No. 33 with a flavoring component, anise oil, in the presence of formaldehyde was responsible for the formation of the violet moiety. This was clearly demonstrated when a synthetic mixture of the three red colorants with anise oil and formaldehyde were heated at 60° for 4 and 12 days and then chromatographed. The TLC pattern obtained is shown in Fig. 2.

SUMMARY

A thin-layer chromatographic procedure has been described which allows the rapid screening of 19 water- and/or alcohol-soluble certified colorants frequently used in the pharmaceutical industry. Since colorants are usually present in minute quantities in dosage forms, TLC is ideally suited due to the sharp separations and compact zones which result in its use.

A standard sample containing four certified dyes was scanned with a spectrodensitometer after thin-layer chromatographic separation. Plots of area *versus* concentration resulted in reasonably linear relationships with high sensitivity allowing for direct quantitation of the colorants on the chromatoplate.

The present chromatographic procedure has also been used to determine the cause of color darkening in a liquid preparation. Chemical reaction of one of the dyes with a flavor component resulted in the formation of a violet species responsible for the color change upon storage.

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Keyphrases

Color additives, coal tar—separation, identification
TLC—separation, identity
Chromatographic development systems—coal tar dyes
UV light—spot visualization
Densitometric scanning—color quantitation