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THE ANALYSIS OF ROMANOWSKY BLOOD STAINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic procedure has been developed for separating and quantitating the components of thiazine dyes and compound blood stains. In terms of speed, quantitation, and component resolution, the assay reported here is superior to other reported chromatographic methods of cationic dye separation. The components present in commercial samples of thiazine dyes and LARC™ stain, a modified Wright-Giemsa stain, have been resolved in 25–40 min on a 5-μm microparticulate silica column using a methanol–water–glycine/acetic acid mobile phase. The mechanism of separation is demonstrated to be based predominantly upon the weak ion-exchange properties of silica.

INTRODUCTION

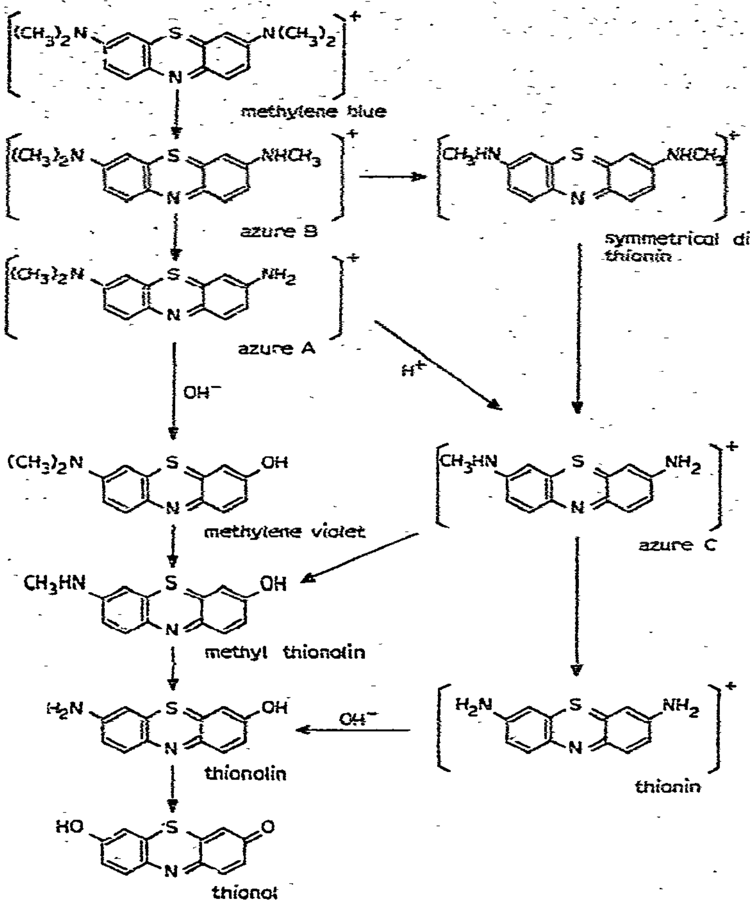
Since their introduction by Romanowsky in 1891[1], stains composed of methylene blue in combination with eosin Y have been used extensively for the routine dyeing of blood smears. These stains now include a complex variety of combinations of methylene blue and its closely related demethylated derivatives with eosin Y. Such variations in stain composition are exemplified by the staining properties of Giemsa's, Leishman's, MacNeal's, Wright's, and May-Greenwald's stains.

One major problem in using blood stains is the large variation in their staining properties from batch to batch, resulting from differences in stain composition[2–4]. In an automated leukocyte recognition system such as the LARC™ system developed by Corning Glass Works, cell color and density are required elements in the recognition sequence[5–7]. For such instruments reproducible stain is a key element[8].

Variation in stain composition is, in part, a result of the slow degradation of the thiazine dye components to their lower homologs[2,3,9–12] (Scheme I).

The major cause of stain component variation, however, is the unavailability of pure dyes used in stain construction and the lack of an adequate method for the separation and quantitation of the individual dye components.

Numerous procedures have been developed for analyzing the thionine-type dyes (methylene blue, azure B, azure A, azure C, symmetrical dimethyl thionin, and thionin), thionolin-type dyes (methylene violet, methyl thionolin, thionolin, and



Scheme I. Degradation of the thiazine dye components.

thionol), and eosin dyes. All are components of blood stains. Recent studies have employed paper chromatography[13–15], thin-layer chromatography (TLC)[16–19], electrophoresis[15], gel filtration chromatography[20], and adsorption chromatography[13,14,21]. When used to analyze basic thiazine dyes or blood stains, these techniques are either time-consuming and their separations are incomplete, or they are difficult to quantitate. This report describes a high-performance liquid chromatographic (HPLC) system for analyzing Romanowsky-type blood stains and thiazine dyes. The technique is rapid and quantitative, allowing its use in the quality control of dye and stain production and in the study of degradation and staining mechanisms.

MATERIALS AND METHODS

Dyes and stains

The dyes used as thiazine standards in this study are listed in Table I. All dyes were dissolved in methanol to a concentration of 0.1 % (w/v). They were sonicated for 5 min, filtered through Whatman No. 541 paper, and stored in the dark at 4°. LARC stain (No. MD-6-3A; Corning Glass Works, Medfield, Mass., U.S.A.), a modified

TABLE I

COMMERCIAL DYES USED AS STANDARDS

Dye	*Supplier*[*]	*Stock No.*	*Cert No.*	*Lot No.*
Methylene blue	Fisher	M-291	EA-17	700990-B
Azure A	Fisher	A-970	EAz-16	726236
Azure B	Harleco	137	LAb-7	3029 P
Azure C	Allied Chemical	NA-0451	NAc-9	1900451
Thionin	Harleco	360	CT-20	2245 P
Methylene violet (Berntsen)	MCB (Matheson, Coleman & Bell)	B-346	Clv 2	mx1030
Eosin Y	Fisher	E-511	EE 28	723894

[*] Addresses: Fisher Scientific, Fair Lawn, N.J., U.S.A.; Harleco, Philadelphia, Pa., U.S.A.; Allied Chemical, Morristown, N.J., U.S.A.; MCB, Norwood, Ohio, U.S.A.

TABLE II

METHANOLIC MOBILE PHASE COMPOSITIONS

Mobile phase code[*]	*% Water*	*Modifier*	*Final modifier conc. (mM)*	*pH*
20–Cl–1	20	Sodium chloride	1	
20–Cl–2.5	20		2.5	
20–Cl–5	20		5	
20–Cl–20	20		20	
20–Cl–50	20		50	
20–Cl–100	20		100	
20–Cl–200	20		200	
20–Cl–400	20		400	
5–Cl–5	5		5	
5–Ac–10/6.5	5	Sodium acetate	10	6.5
5–Ac–10/5.5	5		10	5.5
5–Ac–10/4.5	5		10	4.5
5–Ac–10/3.5	5		10	3.5
5–Ac–10/2.9	5		10	2.9
10–G–10/2.3	10	Glycine	10	2.3
10–G–10/2.5	10		10	2.5
10–G–10/2.7	10		10	2.7
10–G–5/2.3	10		5	2.3
5–G–10/2.3	5		10	2.3
5–G–10/2.5	5		10	2.5
5–G–5/2.0	5		5	2.0
2–G–10/2.5	2		10	2.5

[*] See text (Mobile phase preparation).

Wright-Giemsa stain[8], was used as a stain standard. It was filtered and stored at 4° in the dark.

Reagents

All solvents and chemicals were of reagent grade or better.

Mobile phase preparation

The mobile phases examined contained methanol, water, and a modifier consisting of sodium chloride, formic acid, or an organic buffer. Table II lists the mobile phases examined and their compositions. The buffered mobile phases were prepared by titrating an appropriate volume of aqueous sodium acetate or glycine with glacial acetic acid to the desired pH and then diluting to 1 l with methanol. In the mobile phase code A–B–C/D used throughout this paper, A is the percent water, B is the modifier (Cl for sodium chloride, Ac for sodium acetate, and G for glycine), C is the concentration (m*M*) of modifier in the final solution, and D corresponds to the pH of the aqueous modifier before dilution with methanol. D is not presented for non-buffered mobile phases.

Liquid chromatography (LC)

A Model 830 liquid chromatograph (DuPont, Wilmington, Del., U.S.A.) fitted with a Model 835 filter photometer was used for all analyses. A 5-μm microparticulate silica column (Zorbax-Sil; DuPont), 0.21 × 25 cm, was used for all studies. All analyses were performed at 50° to facilitate molecular diffusion and to decrease solvent viscosity. A 10-μl sample of dye or stain was introduced into the column by means of a 6-port high-pressure injection valve. Unless otherwise stated, the flow-rate was adjusted to 0.5 ml/min, which required 2,200 to 2,800 p.s.i. The separations were monitored at 254 nm and recorded on a Corning Model 840 recorder. Peak areas were integrated on the recorder's analog integrator.

Gas chromatography

Analysis of the reaction between methanol and formic acid in the methanol–water–formic acid mobile phase was made on a Tracor Model 550 gas chromatograph equipped with a thermal conductivity detector. Two C00508, 80–100 mesh Chromosorb 101 glass columns (Supelco, Bellefonte, Pa., U.S.A.) were used for all analyses. A carrier gas (helium) flow-rate of 30 ml/min was maintained. The detector current was 130 mA. Analyses were temperature-programmed from 60° to 130° at a rate of 7.5°/min.

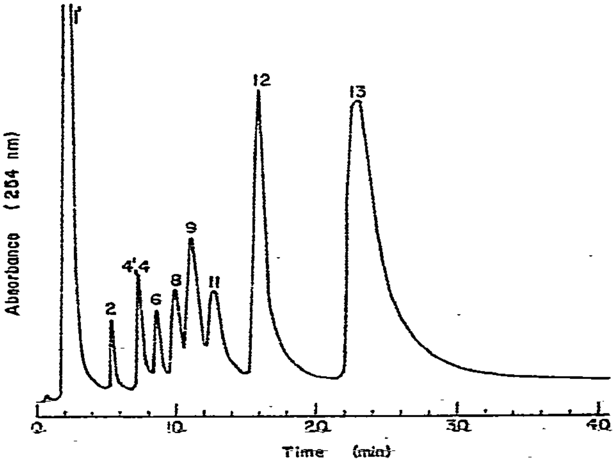


Fig. 1. Liquid chromatographic separation of the thiazine components in LARC stain. Mobile phase: 19.5% water and 0.5% formic acid in methanol. Flow-rate, 0.35 ml/min; temperature, 50°.

RESULTS AND DISCUSSION

Good separation of the components contained in commercial samples of thiazine dyes using TLC on silica gel plates was recently reported[18]. Our observations confirm that active solvent systems such as butanol or isopropanol in combination with water, salt, and an organic acid appear to offer the greatest resolution.

Using these solvents in HPLC mobile phases on microparticulate silica columns of 5-μm particle diameter has proved difficult due to the high viscosity of butanol and propanol and the resulting high pressures required for adequate flow-rates. Following numerous unsuccessful attempts at separating the components of LARC stain, reasonable resolution was achieved using a methanolic mobile phase containing 19.5% (v/v) water and 0.5% (v/v) formic acid at a column temperature of 50°. The flow-rate achieved at 2,200 p.s.i. was 0.35 ml/min. A chromatogram of this stain taken under these conditions is shown in Fig. 1. Three distinct classes of dyes are resolved with this system: eosin Y (2′,4′,5′,7′-tetrabromofluorescein), a xanthene dye used in combination with basic dyes for blood staining; the thionin-type dyes; and the thionolin-type dyes. The structures of each of the thionin-type and thionolin-type dyes (phenothiazine derivatives) are given in Scheme I. The tentative identities of each component resolved are listed in Table III. These assignments are based upon the analysis of a series of dye standards which is discussed below.

Eosin Y is not retained in the column and elutes in the void volume. The thionin-type dyes are strongly retained by the silica and elute late, while the thionolin-type dyes are weakly retained and elute early. The degree of retention under these acidic conditions appears to be a function of the degree to which the dyes are methylated. The relationship between retention and the degree of methylation has been observed by others in TLC and LC separations on both silica gel and cellulose[13,19,22].

The analysis of thiazine dye standards under the above conditions has demonstrated that thionin (4) and methyl-thionolin (4′) elute at the same position. It was also observed that as the mobile phase aged, thionolin-type dye retention decreased and retention of the thionin-type dyes increased. This resulted in the eventual overlap of peaks 8 and 9. Gas chromatographic analysis of the solvent composition over a period of several days demonstrated that methyl formate was being produced at the

TABLE III

TENTATIVE COMPONENT IDENTITIES

Dye component	*Peak identification no.*
Eosin Y	1′
Thionol	1
Thionolin	2
Methyl thionolin	4′
Methylene violet	9
Thionin	4
Azure C	6
Symmetrical dimethyl thionin	8
Azure A	11
Azure B	12
Methylene blue	13

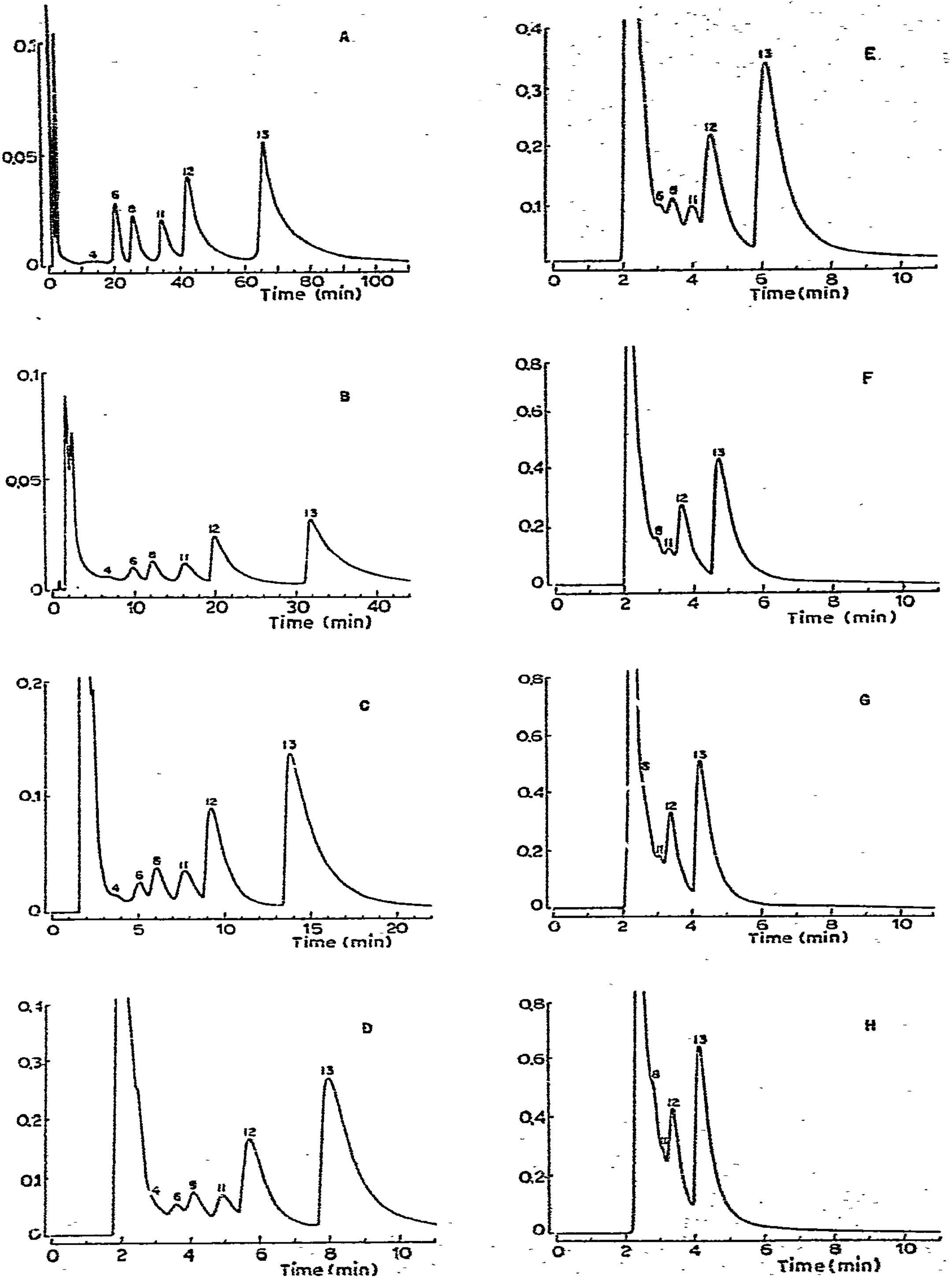


Fig. 2. Liquid chromatographic separation of the thiazine components of LARC stain Mobile phases: 20–Cl–X where X is (A), 1; (B) 2.5; (C), 5; (D), 20; (E), 50; (F), 100; (G), 200; (H) 400.

expense of the formic acid concentration. The resulting increase in solvent pH and decrease in ionic strength was altering retention. Each of these variables was examined in turn.

Sodium chloride system

LARC stain was chromatographed in a methanolic mobile phase containing 20% water and increasing concentrations of sodium chloride from 1 to 400 m*M*. The resulting separations are displayed in Figs. 2A–H. Under these conditions, only the positively charged thionin-type dye components are retained and resolved on the column. Eosin Y and the thionolin dyes, which are not charged in neutral solvents, are not retained on the column. As the concentration of sodium chloride is increased to 400 m*M*, the thionin-type dye components progressively elute more rapidly and eventually merge with the non-retained components. Fig. 3 is a plot of retention volume *versus* sodium chloride concentration. The curves are similar to those obtained in classical ion-exchange systems in which decreased retention at increasing salt concentrations can be understood in terms of exchange equilibrium[23]. It appears, then, that silanol sites on the surface of the silica behave as weak cation-exchange sites under these conditions. Due to the extreme polarity of the solvent, it is difficult to imagine adsorption via hydrogen bonding is the mechanism of separation. Likewise, it is difficult to envisage a partitioning mechanism under these conditions.

To determine what effect water has on the separation, LARC stain was also chromatographed in a mobile phase containing 5% water in methanol and 5 m*M* sodium chloride. It was observed that decreasing the water concentration improved resolution possibly because of the lower viscosity, and thus improved mass transport.

Acetate buffer system

It is apparent that if the thionolin-type dyes are to be resolved, the pH of the mobile phase must be lowered such that the ionic equilibria of these dyes are shifted toward the cationic form. LARC stain was therefore chromatographed in a mobile

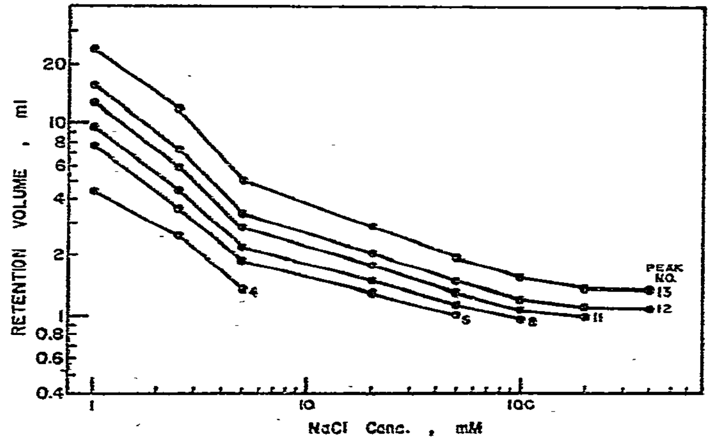


Fig. 3. Variation of the retention volumes of thiazine dyes as a function of sodium chloride concentration.

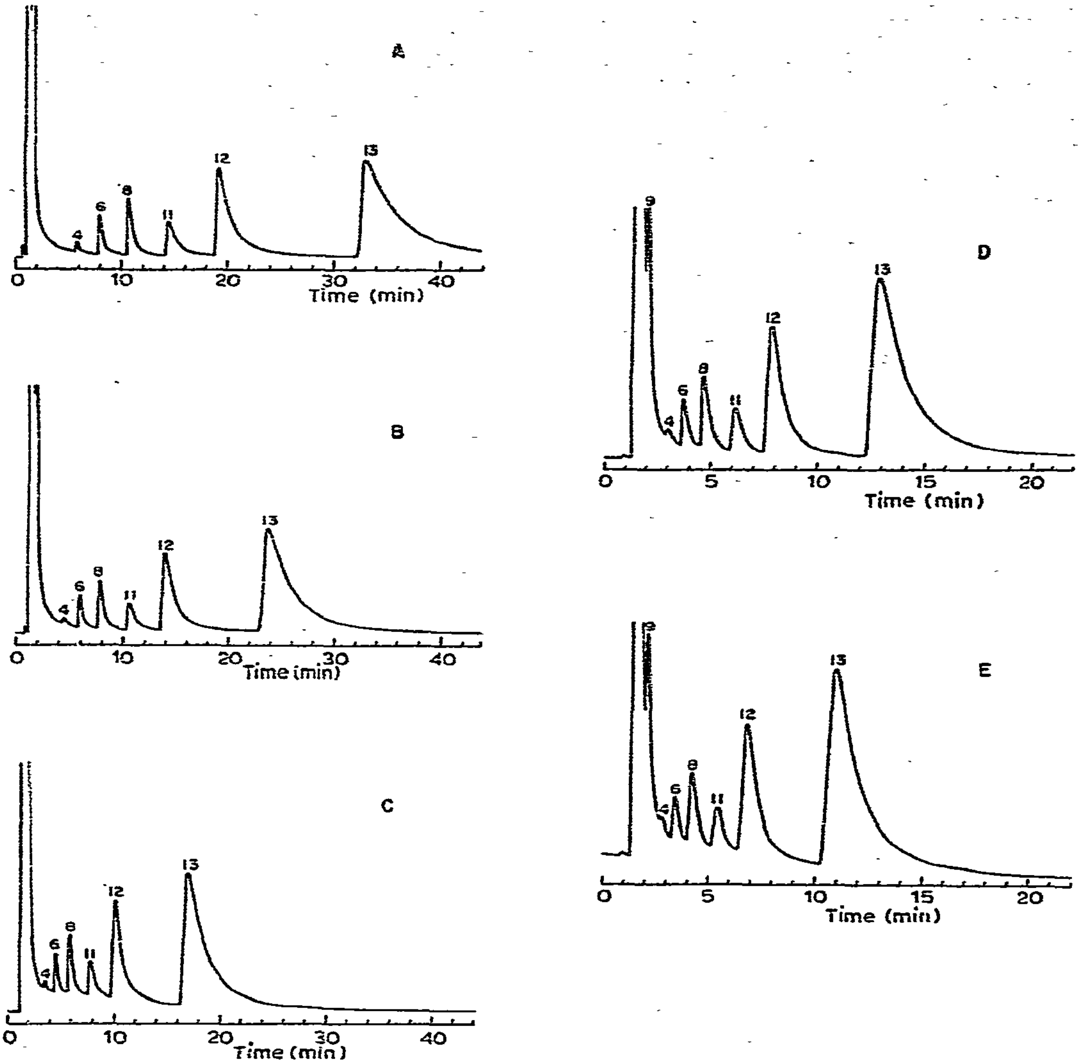


Fig. 4. Liquid chromatographic separation of the thiazine components of LARC stain. Mobile phases: 5–Ac–10/X where X is (A), 6.5; (B), 5.5; (C), 4.5; (D), 3.5; (E), 2.9.

phase containing methanol, 5% water, and 10 mM sodium acetate at decreasing pH's. It should be noted the ionic strength of these mobile phases was not constant but increased with increasing acidity. The small changes in the percent methanol concentration caused by increasing volumes of acetic acid titrant would not be expected to affect the chromatograms. The resulting separations are presented in Figs. 4A–E. As the pH of the mobile phase is lowered from 6.5 to 2.9, decreased retention of the thionin-type dyes is observed. This results from the progressively increasing ionic strength of the mobile phase as more acetic acid titrant was required to achieve lower pH's. Below a pH of 3.5, the thionolin-type dye methylene violet (9) begins to be retarded, but the protonation of methylene violet and concomitant retention is

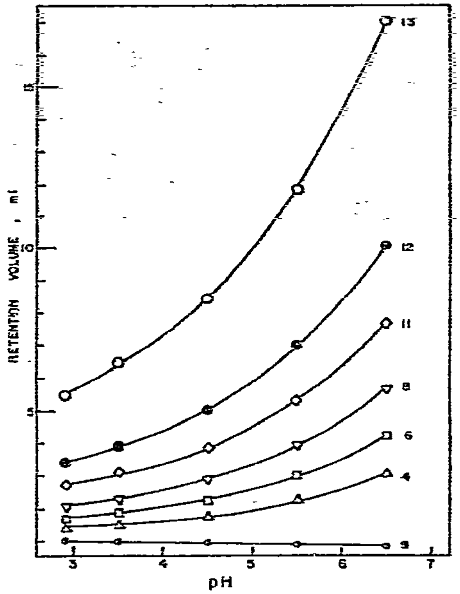


Fig. 5. Variation of the retention volumes of thiazine dyes as a function of the pH of the sodium acetate/acetic acid buffered mobile phase.

counteracted by the increased ionic strength of the mobile phase. A plot of retention volumes for the thionin-type dyes and methylene violet *versus* pH is shown in Fig. 5.

To completely resolve the thionolin and thionin-type dyes, lower pH and ionic strength solvents are required. A glycine/acetic acid buffer system was thus chosen for study.

Glycine buffer system

Mobile phases buffered with glycine were examined with respect to pH, percent water, and glycine concentration. As in the acetate buffer system, the ionic strength increased as the pH was decreased. Chromatograms of LARC stain taken in the various glycine mobile phases are given in Figs. 6A–H. The retention volumes of methylene violet (9), thionin (4), and methylene blue (13) are plotted as functions of the three variables in Figs. 7A–C.

The effect of simultaneously decreasing pH and increasing ionic strength can be seen in Figs. 6A, 6B, and 6C (solvent 10–G–10/X, where X = pH 2.7, 2.5, and 2.3, respectively) and in Figs. 6E and 6F (solvent 5–G–10/X, where X = pH 2.5 and 2.3, respectively). The effect of pH on retention volume is plotted in Fig. 7B. As the pH is decreased, the concomitant increase in ionic strength causes the retention of the thionin-type dyes to decrease. This is to be expected for an ion-exchange system where the ionic equilibrium of each species remains relatively unchanged at the different pH's. The thionolin-type dyes, however, exhibited increasing retention with decreasing pH, despite the increase in ionic strength. This is explained by the fact that their ionic equilibria are shifted more in favor of the cation at lower pH. The ionic strength increase was not able to counteract this effect.

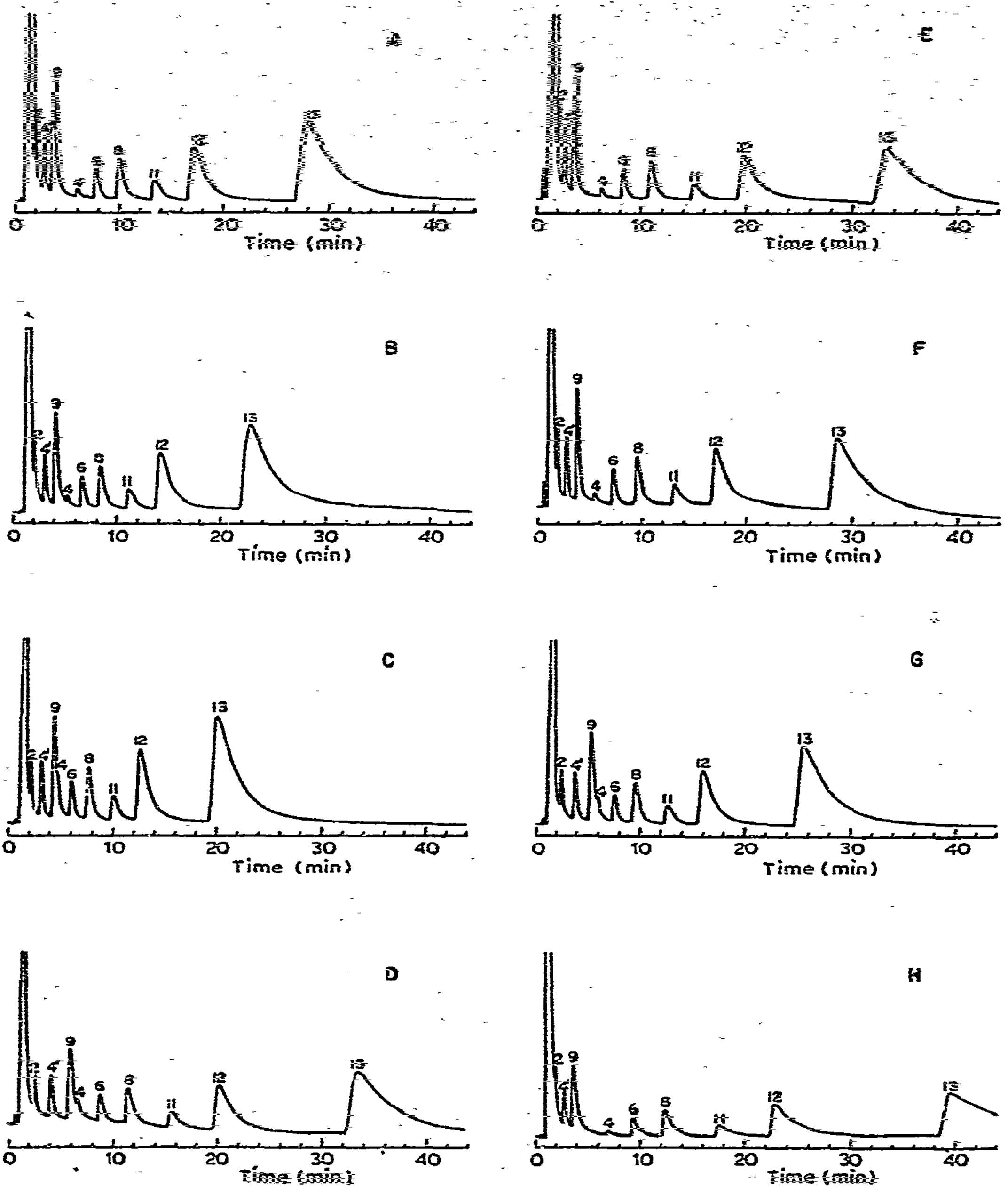


Fig. 6. Liquid chromatographic separation of the thiazine components of LARC stain. Mobile phases: (A), 10–G–10/2.7; (B), 10–G–10/2.5; (C), 10–G–10/2.3; (D), 10–G–5/2.3; (E), 5–G–10/2.5; (F), 5–G–10/2.3; (G), 5–G–5/2.0; (H), 2–G–10/2.5.

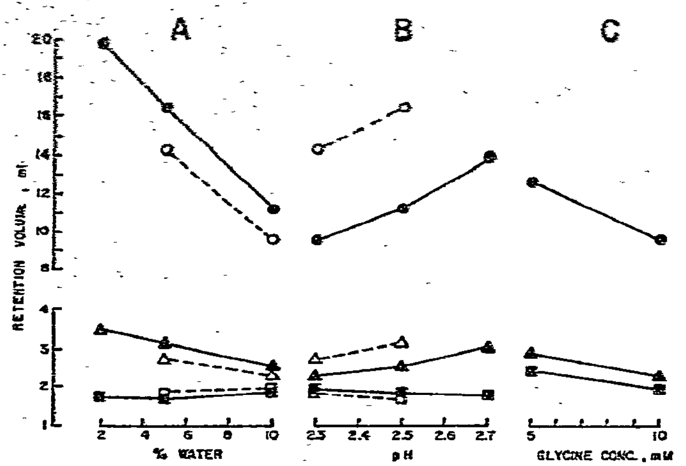


Fig. 7. Variation of the retention volumes of methylene blue (circles); thionin (triangles), and methylene violet (squares) as a function of % water, pH and glycine concentration. (A), X–G–10/2.5 (——); X–G–10/2.3 (– – –). (B), 10–G–10/X (———); 5–G–10X (– – –). (C), 10–G–X/2.3. (X is the % water, pH, or glycine variable).

The effect of increasing the percent water can be seen from Figs. 6B, 6E, and 6H (solvent X–G–10/2.5, where X = 10, 5, and 2%, respectively) and from Figs. 6C and 6F (Solvent X–G–10/2.3, where X = 10 and 5%, respectively). The effect of percent water on the retention volume is plotted in Fig. 7A. The retention of the thionin-type dyes decreases with increasing water, while that of the thionolin-type dyes increases. To understand this observation, one must remember that the pH of the aqueous buffer solution was adjusted before dilution with methanol. Increasing the water percentage of the mobile phase decreases the p*K* of the buffer system, increases acidity, and increases ionization of the buffer[23]. The resulting higher ionic strength causes the thionin-type dyes to be retained less, while the increase in H^+ activity shifts the ionic equilibrium of the thionolin-type dyes more in favor of the cation, thus causing them to be retained more strongly despite the increase in ionic strength.

The effect of increasing the glycine concentration can be seen in Figs. 6C and 6D (solvent 10–G–X/2.3, where X = 10 and 5 m*M*, respectively) and in Fig. 7C. As expected, all components are retained less as the ionic strength is increased.

The optimum assay

The optimum stain assay must utilize a mobile phase in which the pH is low enough that the thionolin-type dyes are protonated and retained and in which the ionic strength is low enough that the thionin-type dyes are more strongly retained. Particular care must be taken to allow sufficient resolution between thionin (4) and methylene violet (9). Respectively, these are the least and greatest retained members of their groups. Also, the total assay time must not be too long or band spreading and base-line drift may present problems.

Of the mobile phases presented here, the 10–G–10/2.7 system was the best. An example of the separation achieved is found in Fig. 6A. Here, methylene blue, the most strongly retained component of the stain, had a retention time of 28 min.

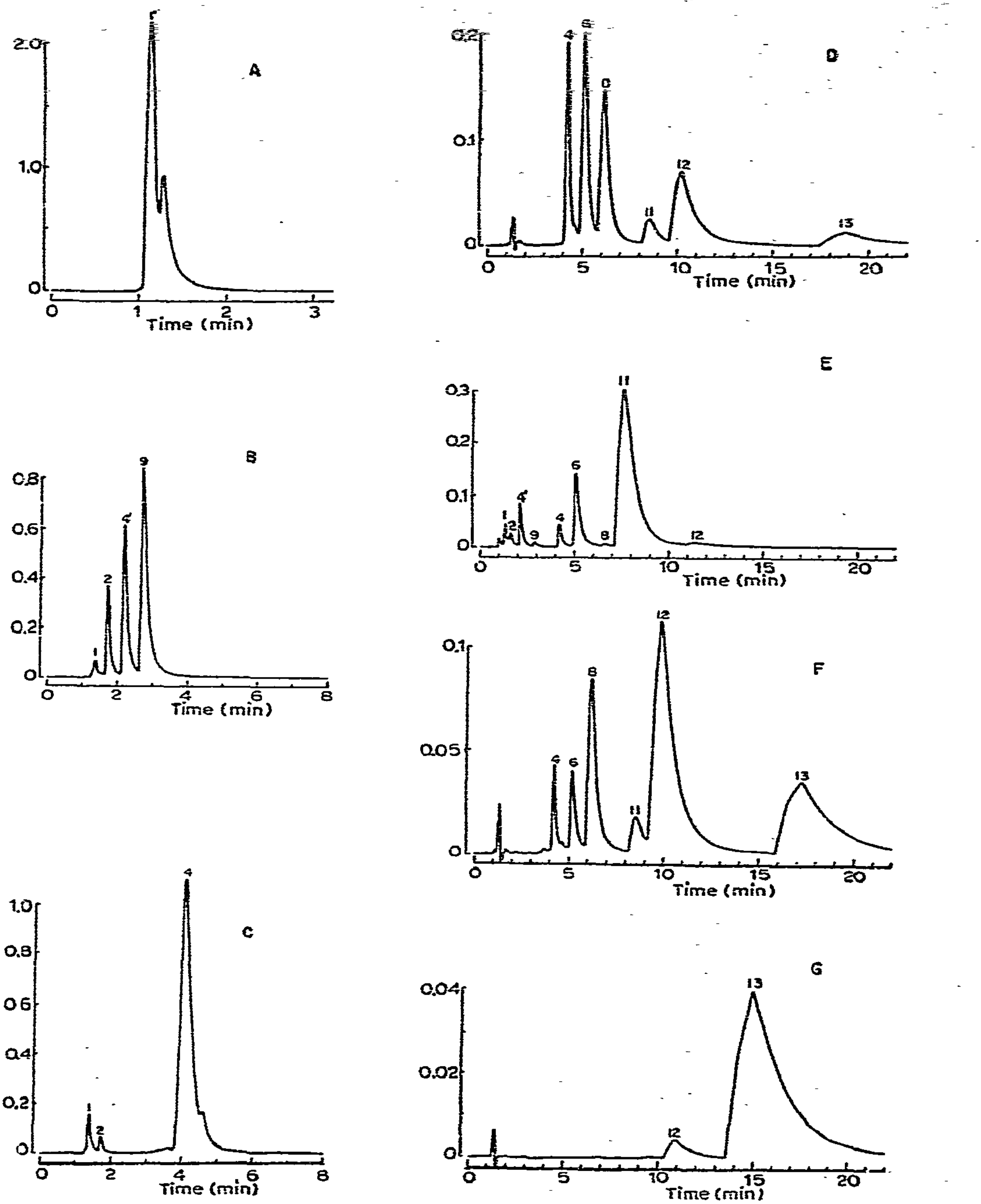


Fig. 8. Liquid chromatograms of commercial dye standards. Mobile phase, 10–G–10/2.7. (A), eosin Y; (B), methylene violet; (C), thionin; (D), azure C; (E), azure A; (F), azure B; (G), methylene blue.

With a recent Zorbax-Sil column the retention was cut to 14 min with similar resolution of all components. Variations in the mobile phase as described above, or the addition of another salt such as sodium chloride, may be utilized to adjust the separation as a column becomes less efficient or to accommodate silica gel of varying characteristics.

Dye standards

Methanolic solutions of eosin Y, methylene blue, azure B, azure A, azure C, thionin, and methylene violet were chromatographed in the formic acid mobile phase used in our initial studies and in the optimum 10–G–10/2.7 mobile phase. The chromatograms obtained with this mobile phase are given in Figs. 8A–G. It is immediately apparent that these commercial dye samples are not pure compounds and may contain as many as ten additional components.

Eosin Y, an acid dye utilized in combination with the thiazine dyes to form blood stains, elutes as two components slightly behind the void volume (Fig. 8A). The major component is assumed to be eosin Y since, in several samples examined, it was the only component present. The contaminant is most likely di- or tribromofluorescein[19]. No attempt was made to resolve the acid dyes further.

Four components have been resolved in this commercial sample of methylene violet (Berntsen) as seen in Fig. 8B. The major fraction (9) has been tentatively identified as methylene violet by spectral analysis of the purified material[24]. In order of decreasing retention, the contaminants include: methyl thionolin (4′), thionolin (2), and thionol (1). Similar separations have been achieved on TLC plates in-house and by others[19], and component identification was similarly based upon the observation that retention is a function of the extent of methylation[13]. These identities were verified by oxidizing purified samples of each component in aqueous 0.06 M $NaHCO_3$ and observing the order of product formation. Similarly, peak 2 was verified as being thionolin by oxidizing thionin to thionolin and observing its reaction products[13].

As shown in Fig. 8C, thionin is one of the purest commercial dyes yet examined. Thionin (4) is contaminated with thionolin (2), thionol (1), and an unknown species of slightly greater retention. Its identity has been established by spectral analysis of the nearly pure material and by noting that it is oxidized in basic solution to form thionolin[13].

All commercial samples of azure C appear to be highly contaminated with other thiazine dyes (Fig. 8D). Based upon the postulate that retention is a function of methylation, peak 6 is tentatively identified as azure C. Its contaminants include methyl thionolin (4′), which was discussed above, azure A (11), azure B (12), and methylene blue (13). Peak 8 has been tentatively identified as symmetrical dimethyl thionin since its two methyl groups would result in a slightly greater retention than azure C.

Azure A, like azure C, is highly contaminated with other thiazine dyes (Fig. 8E). Peak 11 has been tentatively identified as azure A since, in all samples examined, it comprises greater than 75% of the total area observed. Its contaminants include azure B, symmetrical dimethyl thionin, azure C, thionin, methylene violet, methyl thionolin, thionolin, and thionol.

The sample of azure B, like the other azures, is highly contaminated with the other thiazine dyes (Fig. 8F). The major fraction (12) has been tentatively identified

TABLE IV

RELATIVE COMPOSITIONS OF THE THIAZINE DYE STANDARDS AND LARC STAIN

Dye	*Relative area (254 nm)*									
	Peak no.									
	1	*2*	*4'*	*4*	*6*	*8*	*9*	*11*	*12*	*13*
Methylene blue									4.6	95.4
Azure B				3.3	4.0	13.9		3.5	44.7	30.6
Azure A	1.0	1.3	3.6	1.8	12.4	0.4	0.2	78.6	2.5	
Azure C				13.1	21.5	24.9		5.3	25.9	9.4
Methylene violet	1.1	10.2	24.6				64.2			
Thionin	3.4	1.7		94.9						
LARC stain	*	4.1	4.0	1.2	3.2	5.9	9.7	3.8	17.9	50.2

* Eosin Y and thionol not included.

as azure B. It was the major fraction in all samples examined, it elutes between di- and tetramethyl thionin, and the spectra of purified samples correspond to those of azure B (ref. 24). Its major contaminants include methylene blue, azure A, symmetrical dimethyl thionin, azure C, and thionin.

The chromatogram of methylene blue is presented in Fig. 8G. It is the purest of all dyes examined and has been conclusively identified by spectral analysis of the purified material[24]. Methylene blue (13) is contaminated by a small amount of azure B (12).

Table III lists the tentative identities of each of the dyes examined. Absolute confirmation of dye identity must await chemical analysis of the purified components. Similarly, absolute quantitation of each component within the dye mixture must await the evaluation of extinction coefficients in the mobile phase used in the separation. Quantitation of the stain and dye components may be made in terms of the percent total area observed for each component at the wavelength in question. If we assume the extinction coefficients of the thiazine dyes at 254 nm to be nearly equal, we may express the relative areas as relative molar concentrations. The areas of each component peak, expressed as a percent of total area, are tabulated in Table IV for

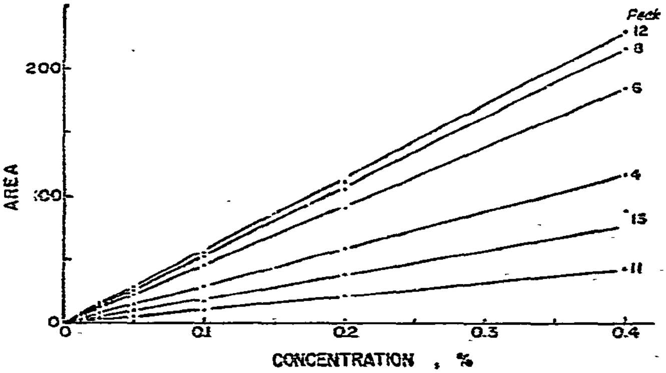


Fig. 9. The dependence of individual peak area upon total concentration. Increasing concentrations of azure C were chromatographed in 10–G–10/2.7 solvent at 50°.

all of the dye standards and for LARC stain. A comparative examination of commercial dyes and stains will appear in a subsequent paper[25].

The linearity of this assay technique was examined by analyzing multiple dilutions of azure C in mobile phase 10–G–10/2.7 (Table II). The peak areas for each of the six major components, expressed as integrator counts times detector sensitivity are plotted *versus* concentration in Fig. 9. Excellent linearity is indicated by the good fit of the points to the linear least square lines.

CONCLUSIONS

All of the major thiazine dyes composing Romanowsky-type blood stains have been resolved in a quantitative manner by HPLC on a 5-μm microparticulate silica column. The assay technique is useful for the quantitative analysis of blood stains and the thiazine dyes from which they are constructed.

The behavior of the system in mobile phases of varying ionic strength, pH, and percent water strongly suggests that the silica packing is behaving as a weak cation exchanger. The resolution of dye components is probably based upon their degree of charge localization. This distribution of charge is a function of the degree of methylation of each dye type and affects the pK_a of each species.

This technique has proved indispensable in the evaluation of dye purity, in the precise construction of stains for use in pattern recognition systems, in the studies of the effects of compositional changes on staining, and in quantitating the demethylation reactions which result in stain instability.

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