CHROM. 5455

THIN-LAYER CHROMATOGRAPHIC SEPARATION OF METHYLENE BLUE AND RELATED THIAZINE DYES

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(Received April 5th, 1971)

SUMMARY

Methylene blue, azures A, B and C, thionine and their oxidation- and photo-degradation products can best be separated by triple development with 95% ethanol–chloroform–acetic acid (85:10:5) on commercial Silica Gel G thin-layers. Serious errors are caused by photo-degradation of the dyes during development; the chromatograms must be carefully shielded from light. At least ten components can be clearly separated. Other sources of error and uncertainty are also described.

INTRODUCTION

Methylene blue and related thiazine dyes are often used as analytical reagents[1–11] and as biological stains[12]. It is known that commercial samples are usually mixtures (either deliberate or accidental) of several related dyes[13–20]. A rapid chromatographic method for the separation of closely related thiazine dyes would be helpful in their identification, assay and quality control. Many of the published procedures appear to give incomplete resolution of complex mixtures or separate them very slowly[14–19, 21–25]. The need for a separation of complex thiazine dye mixtures has lead to the development of the following thin-layer chromatography (TLC) system and the definition of several major and minor sources of error.

EXPERIMENTAL

Commercial samples of dyes were used as test materials; their specifications are listed in Table I. After preliminary trials had established the best conditions and had defined sources of error (see RESULTS section), the following conditions were chosen for best separations; these will be referred to as "standard conditions" hereinafter. Samples were spotted in 5, 10 or 20 μl volumes of 0.1% w/v methanolic solution in plastic-backed silica gel layers (Brinkmann MN SIL-N-HR/UV (254) 20 × 20 cm) and separated in glass tanks (28 × 8 × 25 cm) lined with Whatman 3MM paper liners. The liners were soaked in fresh solvent mixture and equilibrated with the closed tank for 30 min before development. The tanks were wrapped and covered

TABLE I

DESCRIPTION OF DYE SAMPLES[a]

No.	*Name*	*Color index No.*	*Batch number*[b]	*Dye content*[b]
I	Methylene blue	C.I. 52015	NA 47	84%
II	Azure B	C.I. 52010	NA b7	85%
III	Azure A	—	NAz19	88%
IV	Azure C	—	NAc9	71%
V	Methylene violet BERNTHSEN	—	NLv10	78%
VI	Thionine	C.I. 52000	NT 27	86%
VII	Giemsa Stain	—	NGe19	—

[a]All samples made by the National Aniline Division of Allied Chemical Corporation; other similar dye samples from other companies were also examined, but the results did not differ substantially from those discussed here.

[b]National Biological Stain Commission values.

with aluminum foil and held in a darkened fume-hood to exclude light. The sheets were fully developed three times in 95% ethanol–chloroform–acetic acid (85:10:5) (ECA) with 30 min drying periods in the dark between developments.

RESULTS

Solvent systems

Preliminary trials, on silicic acid coated slides, showed that non-polar solvents, pure or mixed, gave no separation of thiazine dye mixtures. The lower aliphatic alcohols, acetone, formamide, N,N-dimethyl formamide and N,N-dimethyl acetamide showed some promise of separation. However, acetone tended to promote streaking and the amides gave no better separations than the alcohols and were less volatile, so these were not examined further. Alkaline solvents appeared to encourage degradation and discoloration of the dye components. Good separations were found with alcohol–chloroform–acetic acid mixtures and these were investigated further, using commercial silica gel layers.

The best separations were eventually obtained with alcohol–chloroform–acetic acid (85:10:5) mixtures containing either methanol (MCA) or 95% ethanol (ECA). Solvents with high chloroform content ($\geqslant$ 50% chloroform) gave poorer separations and tended to cause blistering of the thin layers. The proportion of acetic acid was not critical. Replacement of acetic by formic acid gave no benefit. Addition of water up to 10% of the mixture had no marked effect, although separation was slightly poorer at the higher water contents. Higher alcohols gave a slower development and no improvement in separation.

Solvent mixtures more than two days old gave poorer separations, presumably as a result of esterification, so only freshly mixed reagent-grade solvents were used, within 24 h of mixing.

Multiple development

Single development gave moderately good separations but with some overlapping of spots. Resolution was very much improved by repeated development.

Comparable results were achieved by four developments in MCA or three developments in ECA. MCA required about 80 min for each development and 20 min drying times; the total separation therefore required 6 h. ECA required about 140 min for each development and 30 min drying times; the total separation therefore required 9 h. MCA was faster but required more manipulation; ECA was slower but required fewer manipulations and gave a somewhat better separation because it distributed the spots over a greater length of chromatogram. MCA may be better for preliminary surveys requiring a more rapid method; ECA is better if high resolution is required.

Photodecomposition

Even in the best systems, separation was never satisfactory at first. Multiple development produced increasing separation but poorer resolution of the spots; the distance of separation of spots increased but they became more blurred and less clearly distinct.

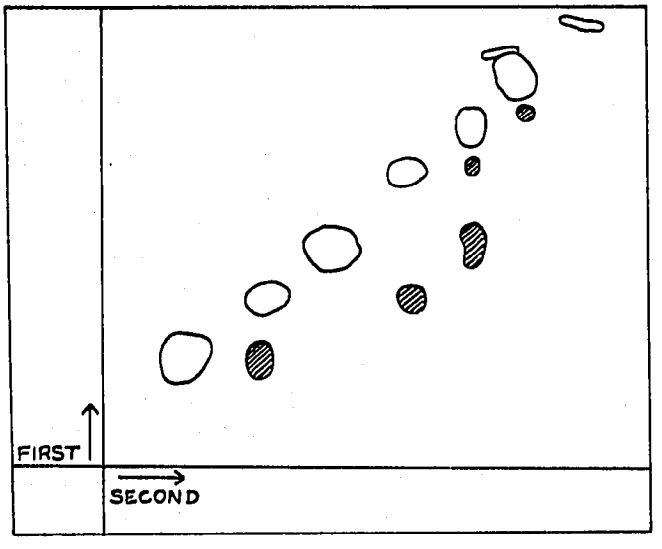


Fig. 1. Thin-layer chromatogram of mixture of methylene blue, azure B, azure A and thionine samples developed in silica gel in both directions with 95% ethanol–chloroform–acetic acid (85 : 10 : 5). The off-diagonal shaded spots were found only when the chromatogram was exposed to light between the first and second developments (smallest spots very faint).

Photodecomposition was suspected. This was tested by diagonal two-dimensional chromatography according to HAIS[26]. Ten micrograms samples of methylene blue–azure B–azure A–thionine test mixture were spotted at the origins of two thin-layer sheets. Each was developed under standard conditions in one direction. After the third development, one sheet was stored overnight in the dark and the other was left on a laboratory bench exposed to fluorescent lights overnight and daylight the next morning. The two sheets were then redeveloped three times in the second direction. On the unexposed sheet, the developed spots fell on the expected diagonal line away from the origin. However, on the exposed sheet, in addition to the diagonal spots, other spots had separated (see Fig. 1). The new spots had obviously formed by photodecomposition of the dyes during irradiation by light. After this finding care was always taken to protect dye samples from exposure to light.

TABLE II

R_{TH} VALUES FOR THIAZINE DYES: MEAN VALUES AND RANGES

Spot no.	*Probable identity*[a]	R_{TH}*(mean)*[b]	R_{TH}*(range)*	*No. of spots measured*[c]
1	Methylene blue	24	18– 28	29
2	Azure B	39	34– 48	34
3	Azure A	52	45– 58	27
4	*sym*-Dimethylthionine	71	63– 78	24
5	Azure C	84	76– 90	29
6	Methylene violet Bernthsen	94	92– 97	4
7	Thionine	100	—	23
8	Methyl thionoline	107	103–113	14
9	Thionoline	114	111–120	10
10	Thionol	118	115–124	5

[a] Reasons for these assignments are given in the discussion.

[b] $R_{TH} = \frac{\text{migration distance of spot}}{\text{migration distance of thionine}} \times 100$.

[c] Measurements are based on five chromatograms and forty samples.

Migration properties under optimum conditions

Once the effects of solvent age and photodecomposition were discovered and corrected, a number of separations were carried out using standard conditions. R_F values did not reproduce well from run to run. However, the relative migration value R_{TH} did reproduce quite well, where

$$R_{TH} = \frac{\text{migration distance of spot}}{\text{migration distance of thionine}} \times 100$$

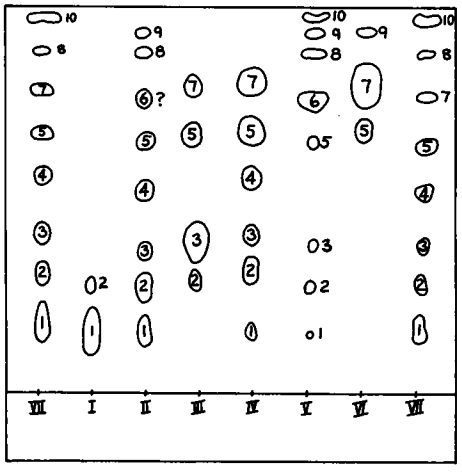


Fig. 2. Thin-layer chromatogram showing separation of thiazine dye samples by vertical development on silica gel with 95% ethanol–chloroform–acetic acid (85:10:5). Roman numerals refer to dye samples as in Table I; arabic numerals refer to spot identities as in Table II.

Accordingly R_{TH} values were measured for forty samples and are shown in Table II. (The identifications of the spots are tentative; the reasons for these assignments are given in the discussion.) A typical chromatogram is shown in Fig. 2. In general, the resolution of the spots was very satisfactory; the only spots that tended to overlap were Nos. 6 and 7 (methylene violet BERNTHSEN and thionine) and these could be distinguished by the bluer shade of the first relative to the second. (However, distinction by color tended to be difficult when the spots were small and the colors weak.)

Comparison with other thin-layer media

All the above separations were done on Brinkmann MN SIL-N-HR/UV (254) sheets. Separations were also tried on four other silica gel thin-layer media from other commercial sources. None of these media gave as good a separation as the Brinkmann sheets. It is clear that the thin-layer media must be chosen carefully and substitutions should not be made without standard trial separations.

Comparison with other solvent systems

Separations were also attempted with previously described solvent systems which have been applied to the TLC of thiazine dyes: *n*-butanol–acetic acid–water (2:1:5) (ref. 19), chloroform–methanol–formic acid (15:10:1) (ref. 27), butanol–water–acetone (20:20:7 with 0.5% diethylamine)[27] and *n*-propanol–*n*-butanol–ammonium hydroxide–water (4:4:4:1) (ref. 25). None of these gave as good a separation and resolution as the present systems, even after multiple development.

Effects of heavy metal ions

It is possible for thiazine dyes to be heavily contaminated with metals derived from the manufacturing process. These include zinc, copper, iron, manganese and chromium[20,28–30].

The effects of metal contaminants on the thin-layer separations were examined. Replicates of 0.01 μmoles of standard dye-mixture were spotted and overlaid with 0.05 μmoles spots of each of the above metals (as sulfate, nitrate or chloride) and separated under standard conditions. No differences could be seen between these and samples separated without added metals.

DISCUSSION

Structures and identities of the thiazine dyes

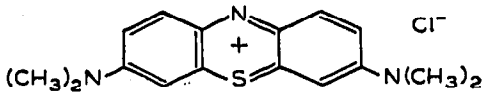
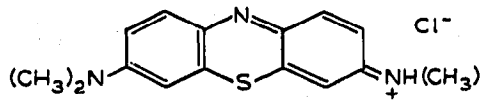
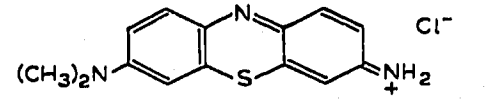
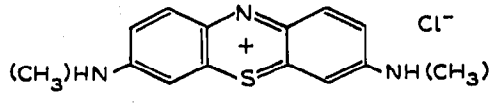
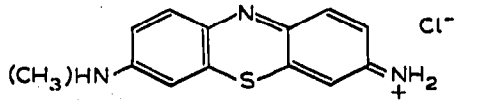
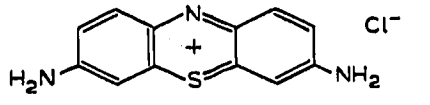
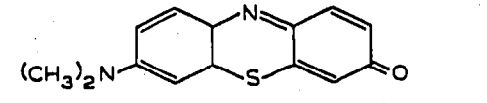
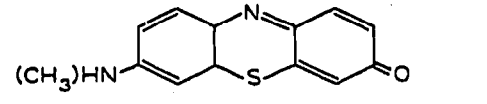
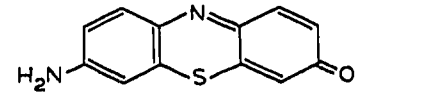
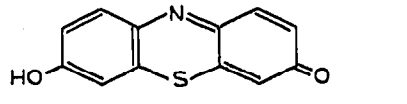
There appears to be no comprehensive, critical and recent discussion of the structures and relationships of the thiazine dyes. Most discussions are based on the classical studies of BERNTHSEN[31,32] and KEHRMANN[33,34]. Later authors have depended on their work mainly and most of the dyes have not been subjected to modern critical tests of purity or structure, with the exception of methylene blue itself.

In particular, the interrelations of the dyes do not seem to be satisfactorily established. Methylene blue can be oxidatively demethylated and deaminated to the various dyes in Table III. There is little known of the reaction routes or mechanisms of transformation from one dye to the next.

In passing, it should be noticed that there is no consistent nomenclature for the dyes. They are variously indexed in *Chem. Abstr.* as derivatives of thionine,

TABLE III

METHYLENE BLUE AND RELATED THIAZINE DYES

Spot No.[a]	*Common names*	*Structure*	*Systematic names*[b]
1	Methylene blue, C.I. Basic Blue 9, C.I. 52015		3,7-Bis(dimethylamino) phenazathionium chloride
2	Azure B, Trimethyl-thionine, C.I. 52010		7-(Dimethylamino)-3-(methylimino)-3-H-phenothiazine hydrochloride
3	Azure A, *asym.*-Dimethylthionine		7-(Dimethylamino)-3-imino-3-H-phenothiazine hydrochloride
4	*sym.*-Dimethylthionine		3,7-Bis(methylamino) phenazathionium chloride
5	Azure C, Monomethylthionine		3-Imino-7-(methylamino)-3-H-phenothiazine hydrochloride
7	Thionine, Lauth's Violet, C.I. 52000		3,7-Diaminophenazathionium chloride
6	Methylene violet BERNTHSEN		7-(Dimethylamino)-3-H-phenothiazine-3-one
8	Methyl thionoline		7-(Methylamino)-3-H phenothiazine-3-one
9	Thionoline		7-Amino-3-H-phenothiazine-3-one
10	Thionol		7-Hydroxy-3-H-phenothiazine-3-one

[a]Spot numbers as in Table II and Fig. 2.
[b]Variations in the systematic nomenclature are found; see discussion.

phenazathionium, phenothiazine-5-ium, 3-H-phenothiazine or 3-H-phenothiazine-3-one or they are indexed by the trivial names or C.I. names (see Table III).

Assignments of chromatogram spot identities

There are ten dyes in the thiazine series from methylene blue to thionol. It is significant that ten distinct spots could be distinguished on the chromatograms.

Accordingly, the spots were identified to correspond with the ten expected compounds using the following criteria: (a) the principal spot in each separated commercial sample was assumed to be the nominal compound; (b) thionol was identified as the reddest spot (spot 10) because this could be expected to differ the most in color from methylene blue; (c) the remaining three spots were assigned on the assumption that the R_{TH} values should be direct functions of the degree of substitution of the amino groups in each of the thionine and thionoline sub-series; decreasing substitution is correlated with increasing R_{TH} values.

The above assignments are clearly tentative; confirmation or disproof awaits the availability of sufficient material of proven identity and purity of each of the dyes to allow measurement of reliable spectral and chromatographic properties and similar criteria and thus allow the confirmation of structural identities.

It should be emphasized that dye properties reported in the literature are usually not trustworthy because in most instances inadequate proof has been offered that the material was pure and uncontaminated with dye and non-dye impurities. Chromatography has often been applied and offered as proof of purity but in most instances the chromatographic system was inadequately specified or no proof was offered that full separation had been achieved or that the system was capable of separating known mixtures of the full range of dyes. In the most careful studies[14,18] good separation and resolution were achieved. However, no provision seems to have been made for the prevention of photodegradation or for checks of sample purity and identity after measurement of spectral and other properties (to prove degradation had not occurred after separation and isolation of the components).

Until reliable methods of structure determination, proofs of purity and standard spectral data are available, identifications and assays of thiazine dyes (and similar compounds) will always contain an element of uncertainty. Particularly noteworthy in this respect is the present evidence for the probable occurrence of symmetrical dimethylthionine in commercial thiazine dye samples. This appears to be the first time its occurrence has been claimed, although it appears to be a normal constituent of commercial samples.

ACKNOWLEDGEMENT

I wish to thank Miss LINDA MARIE CLARK for technical assistance in much of the laboratory work while she was supported by a National Science Foundation Teaching Internship.

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