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SEPARATION OF BILE PIGMENTS BY THIN LAYER CHROMATOGRAPHY*

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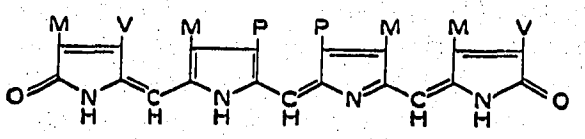
SUMMARY

Separation of bile pigments by ascending thin layer chromatography is described. Unesterified pigments separate on polyamide layer with methanol–water (3:1), the esterified ones on Silica Gel G with benzene–ethanol (100:8). Pigments inseparable in the above systems are separated: free violins and verdins on polyamide TLC with methanol–10% ammonia–water (9:1:2), esterified urobilin and stercobilin on Silica Gel G with chloroform–ethanol (100:2).

Bile pigments are open chain tetrapyrryl compounds[1,2] of which many have been isolated in nature or synthesized[1,3–5]. All, with very minor exception, are of IX, *α* configuration[6,7]. The parent bile pigment, biliverdin (I), is a totally unsaturated triene; hydrogenation in varying degree and position[2] is responsible for differing characteristics including chromatographic behavior. All members of the bile pigment family possess two carboxylic groups and four nitrogens of pyrrolenine (—N=) and/or pyrroline (—NH—) type[8]. For convenience we shall use the generally accepted common names as the corresponding systematic names are too cumbersome[2,5].



I

Thin layer chromatography (TLC) has but recently been employed for bile pigments, the methods thus far used being designed for identification or purification of specific compounds such as phycocyanobilin[9–11], phycoerythrobilin[12], or aplysioviolin[13]. These were separated on TLC in the form of dimethyl esters and comparatively large quantities have been used[9,12,13].

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Most naturally occurring bile pigments are isolated as the free base or hydrochloride, hence a TLC method for separation in this form would be advantageous. Also, we have found that changes may be induced on esterification which in part counterbalance the advantages of working with esters. In the present study, the existing TLC methods have been examined and modified for application to most of the bile pigments. In addition, certain new methods have been devised. Both hydrochlorides and methyl esters have been used.

EXPERIMENTAL

Materials

Silica Gel G, from E. Merck, AG, Darmstadt, Germany.

Polyamide ITLC, from Gallard-Schlesinger Chemical Manufacturing Corp., 15 × 15 cm, coated on both sides.

Chloroform, ethanol, benzene, methanol, ammonia—analytical grade reagents.

Water—deionized and distilled.

METHODS AND RESULTS

Preparation of TLC plates

For preparation of 5 plates 20 × 20 cm, with 0.25 mm layer thickness, 30 g of Silica Gel G were placed in an Erlenmeyer flask to which 40 ml of distilled water was added with constant stirring until the slurry had a uniform consistency and was free of air bubbles. An additional 20 ml of water was then added with further stirring. The slurry is at once placed into a Desaga applicator adjusted for 0.25 mm layer thickness for even distribution over the plates. When thicker layers are desired, the amounts are increased proportionately. The plates are allowed to dry for 2 h in the air after which their treatment varies according to the solvent system to be used. For benzene–ethanol the plates are activated for 1 h at 120°, then stored in a desiccator over calcium chloride; for chloroform–ethanol, the plates are allowed to dry in the air for 24 h and are then ready for use, or may be stored in a desiccator over calcium chloride.

Esterification of bile pigments

Three methods were studied, *i.e.*, methanol–sulphuric acid (4:1, v/v)[1], diazomethane[1] and methanolic boron trifluoride[9]. The last is preferred (see below).

Separation of esters from the free pigments

In the following methods for separation of bile pigment esters on Silica Gel G, free bile pigments remain at the origin. This may be used for separating esters from unesterified pigments. Esters must be removed in advance of the separation of free bile pigments on polyamide TLC. This is done by dissolving the total pigments in chloroform and extracting unesterified pigments with 10% ammonia. The latter is acidified to Congo red paper with 7.5 *N* hydrochloric acid and extracted with chloroform. This is washed with water, dried by filtration and after suitable concentration is ready for chromatography.

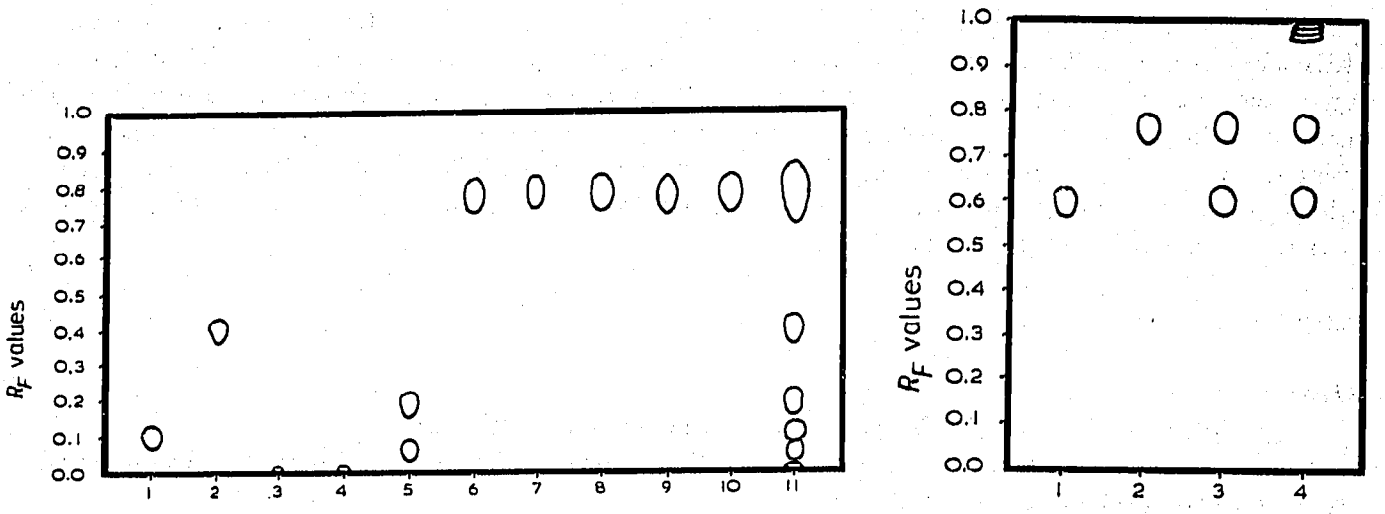

Fig. 1. TLC on polyamide layer developed with methanol–water (3:1, v/v). Spots correspond to: (1) Biliverdin; (2) mesobiliverdin; (3) bilirubin; (4) mesobilirubin; (5) mesobiliviolin[17], (a) stable, (b) labile; (6) *i*-urobilin, synth; (7) *d*-urobilin; (8) half-stercobilin; (9) stercobilin, nat.; (10) stercobilin, synth.; (11) mixture of 1–10.

Fig. 2. TLC on polyamide layer developed with methanol–10% ammonia–water (9:1:2, v/v/v), to 7 cm high. Spots correspond to: (1) Bilirubin; (2) mesobilirubin; (3) mixture of 1 and 2; (4) mesobiliviolins, mesobiliverdin, urobilin, mesobilirubin and bilirubin (from above downward).

Application

Ten μl of chloroform solution were applied to a spot. The total amount of pigment applied should vary from 0.1–5 μg; smaller amounts are undetectable without a densitometer, larger quantities cause tailing. For mixtures about 2 μg of each pigment were applied.

Separation of free bile pigments on polyamide layer

Unesterified bile pigments were separated on polyamide ITLC plates in Brinkmann 22 × 22 × 10 cm wide chromatography jars (15 × 15 cm plates, Fig. 1), or in cylindrical (2 in. I.D.) chromatography jars (5 × 15 cm plates, Fig. 2). The pigments were applied, the sheets were saturated for 45 min and ascending chromatograms were developed for about 2 h (until the solvent front moved 10 cm from the origin). The solvent was a mixture of methanol–water (3:1, v/v). Reference pigments and their mixtures were spotted 2 cm from the bottom of the sheet. For identification each plate with the unknown pigment was spotted in parallel and in mixture with the appropriate authentic reference compounds. In this system bile pigments forming hydrochlorides, *i.e.*, bilenes, bilitrienes and some bilidienes, were separated. Bilirubin and mesobilirubin are left at the origin. The runs required considerable time but the separation of the commonly occurring pigments, such as urobilins, violins, purpurins, verdins and rubins, was very satisfactory (see below). After development, the sheets were dried and the colored spots located. The bile pigments separated are shown in Fig. 1. The saturation period greatly influenced the separation. A comparison was made of saturation for 0, 15, 30, 45, 60 and 75 min. The best results corresponded to the longest saturation period. However, 45 min saturation gave satisfactory results and even shorter periods can be used for specific purposes, *e.g.*, mesobiliviolins could be separated satisfactorily without preliminary saturation.

The development time or origin–front distance may vary according to the aim. In special cases when separation of any two pigments is desired, *e.g.*, mesobiliviolin and mesobiliverdin, the front might be allowed to run further than 100 mm. When the objective was a separation of urobilinoids from violins or glaucobilin, runs of 60 mm were sufficient.

Bilirubin and mesobilirubin remained at the origin in the above system but were readily separated on the same polyamide ITLC sheet with methanol–10% ammonia–water (9:1:2, v/v/v). Other conditions were the same as already given. The results are shown in Fig. 2. The quality of separation depends on the distance from origin to front at the end of the run. Studies were made as to effect of the origin–front distance, 50, 55, 60, 75 and 100 mm, the best separation being observed at 55 mm. Other pigments such as violins, verdins and urobilinoids do not interfere with separation of mesobilirubin and bilirubin; all of the former group move almost with the solvent front (see run 4 in Fig. 2).

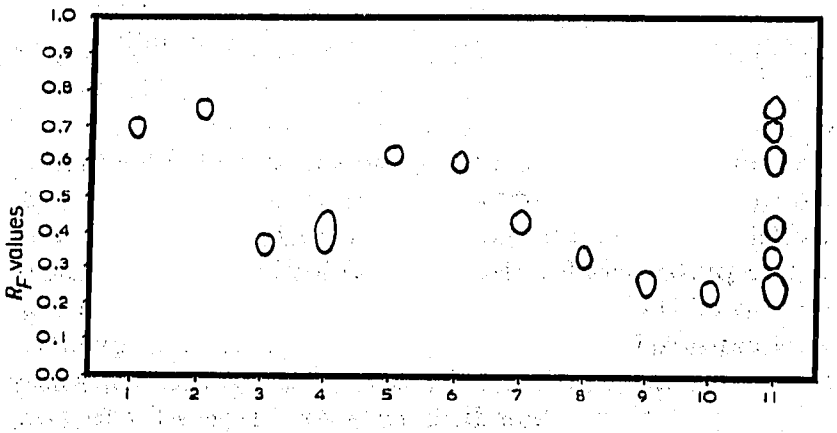

Fig. 3. TLC on Silica Gel G developed with benzene–ethanol (100:8, v/v), to 10 cm high. Spots correspond to: (1) Biliverdin; (2) mesobiliverdin; (3) bilirubin; (4) mesobilirubin; (5) bilipurpurin; (6) mesobilipurpurin; (7) labile mesobiliviolin; (8) stable mesobiliviolin; (9) urobilin; (10) stercobilin; (11) mixture of 1, 2, 5, 6, 7, 8, 9, 10.

TLC separation of dimethyl esters of bile pigments

This separation was achieved on a thin layer of Silica Gel G with solvent system benzene–abs. ethanol, (100:8, v/v) (Fig. 3). Other ratios of benzene–ethanol, 100:1, 100:4, 100:6, 100:8, 100:10, 100:12, 100:14 and 100:20 were compared and 100:8 was found to be the best for the general separation of bile pigments. The separating quality also depends greatly on the layer thickness and in this presentation 0.25 mm was used unless otherwise stated. For preparative separation with the layer 1 mm thick, better results were achieved with a slightly higher proportion of ethanol in the mixture, *i.e.*, 100:10 to 100:14. All bile pigments are separable in this system except bilenes, *e.g.*, urobilins and stercobilins, which move as one spot. TLC plates were developed in Brinkmann 22 × 22 × 10 cm wide chromatography jars. They were saturated for 30 min and developed until the solvent front reached 10 cm from the origins (these were 2 cm from the plate bottom), on an average of about 30 min. Drawings of developed chromatograms are shown in Fig. 3.

Urobilin and stercobilin were separated on Silica Gel G on 20 × 20 cm plates

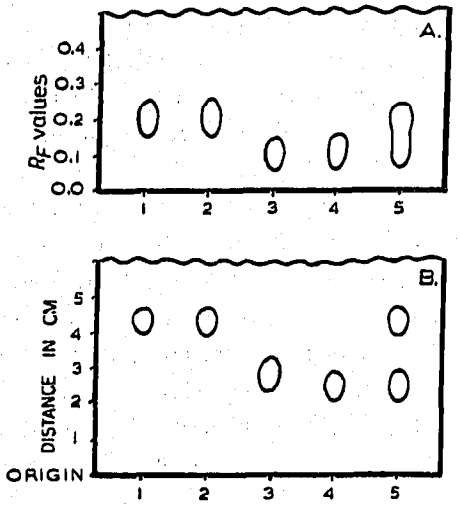

Fig. 4. TLC on Silica Gel G, developed with chloroform–ethanol (100:2, v/v). (A) Developed to 10 cm high, taken out, dried and developed again 5 times. (B) Developed to 10 cm high and allowed to run for another 3 h. Spots correspond to: (1) *i*-Urobilin, synth.; (2) *i*-urobilin, nat.; (3) half-stercobilin; (4) stercobilin, nat.; (5) mixture of 2 and 4.

with chloroform–ethanol (100:2, v/v) as the solvent. Plates were saturated for 30 min, then run until the front reached 10 cm above the origin. They were then taken out and dried. This process was repeated five times. The separation is shown in Fig. 4A. Better results were obtained when the plate was left in the developing chamber and the front was allowed to run to the top of the plate and then left in the chamber for another 3 h. In this case R_F values cannot be calculated but separation is greatly improved (Fig. 4B); the numerals on the axis indicate distances from the origin in centimeters. Both methods limit the separation according to saturation of the end rings, the di-pyrrolinones, with both unsaturated, including the natural *i*- and *d*-urobilins being separated from the di-pyrrolidones, both end rings saturated (natural and synthetic stercobilin), and the pyrrolinone-pyrrolidones with one end ring saturated, represented here by half-stercobilin[15]. Stercobilin and half-stercobilin travel as a single spot in this system in contrast to the dipyrrolinones.

Visualization of the pigments

Bile pigments are strongly colored compounds and they are visible on the thin layer plates without special treatment. The amount of the pigment which can be detected with the naked eye varies according to pigment color and lies in the region of 1–5 μg. Smaller quantities (0.1–1.0 μg) can be detected under U.V. light since most bile pigments fluoresce. Those not fluorescing have fluorescing metal complexes, thus after spraying with zinc acetate in ethanol, they can be detected under U.V. light. With these methods of visualization the bile pigments are readily detected.

DISCUSSION

With certain exceptions the above described chromatographic methods permit separation and identification of the components of mixtures of bile pigments. Separation is very convenient for most of the free (unesterified) bile pigments and by using this

method, loss incident to esterification is avoided. If preparation of the pigments excluded their esterification they can be applied directly; however, with any possibility of esterification, esters have to be removed or erroneous results will be observed. Urobilins and stercobilins (bilenes) run on polyamide layer as one spot. They can be separated from all other bile pigments but are not differentiated. This is not surprising since the number of carboxylic groups and pyrrolenine-pyrroline nitrogens are the same for both compounds. Rubins, *i.e.*, mesobilirubin and bilirubin, are not separated with methanol–water (3:1) and must be removed before chromatography in order to avoid tailing. They are easily separated with methanol–10% ammonia–water (9:1:2). Esters of bile pigments often allow better separation since the predominant carboxylic groups are blocked. Consequently the separation depends on whether the nitrogens are —N= or —N—. These differ between various pigments because the variation in this respect relates to the number of conjugated double bonds. This influence is much more important than the strongly polar carboxyl groups which are identical in all bile pigments.

There is only one group of pigments, *i.e.*, bilenes, which are not separated on Silica Gel G with benzene–ethanol (100:8). Bilenes consist of both natural and synthetic urobilins, stercobilins, half-stercobilin and their stereoisomers. Dipyrrolinones, such as the *d*- and *i*-urobilins, are separated from di-pyrrolidones, such as stercobilin, or pyrrolinone-pyrrolidone, such as half-stercobilin (see above) with chloroform–ethanol (100:2); however, *d*- and *i*-urobilins are indistinguishable in this system; the same is true for stercobilin and half-stercobilin. Further modification of this method is being studied and shows promise of separating the stereoisomers of the urobilins and stercobilins[16].

The benzene–ethanol (100:8) system can be changed according to the objective. For thicker layers of Silica Gel G, more polar, *i.e.*, more ethanol containing systems should be used. For example, this may be necessary in cases of marked predominance of a single pigment. Separation of methyl esters is advantageous except for adverse effect of esterification on the pigments. The most commonly used method of esterification with methanol–hydrochloric acid or sulphuric acid is too strenuous for bile pigments and some oxidation products are formed during the procedure[14]. Diazomethane is better since no oxidation products are formed; however, small amounts of unidentified products have been noted. Boron trifluoride is preferred since it is more protective.

A word of caution must be added. Esters of bile pigments tend to change on standing in chloroform. The proportion of pigment remaining at the origin increases and additional spots appear. For example, biliverdin and mesobiliverdin exhibit spots corresponding to purpurins; urobilins give spots corresponding to violins and glaucobilin. In order to prevent this change before chromatography it is necessary to protect the chloroform solution and use it as soon as possible after preparation. If dissolved at once before TLC and the remaining solution evaporated to dryness, the pigment can be stored for months without noticeable change.

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