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Separation of porphyrin methyl esters by two-dimensional thin-layer chromatography

Qualitative[1,2], quantitative[3] and preparative[4] methods for the separation of porphyrin methyl esters by one-dimensional thin-layer chromatography (TLC) have been described. Porphyrin methyl esters with low R_F values are poorly differentiated in these systems and may be obscured by other pigments, often present in extracts from biological sources. Two-dimensional paper chromatography has been used to resolve mixtures of methyl esters of dicarboxylic porphyrins[5,6], separation being especially satisfactory with hydroxylated porphyrins. This paper describes a simple two-dimensional TLC method which has some advantages over existing techniques.

Materials and method

Solvents. Chloroform was washed three times with water and dried by shaking with anhydrous Na_2SO_4, and by filtration through a triple layer of chloroform moistened filter papers, before use. Kerosene (white) was obtained from Hopkin and Williams. All other solvents were of analytical reagent grade, except for butanone (special for chromatography), methyl acetate and propionate (laboratory reagent grade) and dichloromethane (redistilled) from British Drug Houses.

Porphyrins. Protoporphyrin IX dimethyl ester (Grade A) and coproporphyrin III tetramethyl ester were from Sigma; mesoporphyrin IX dimethyl ester from Koch-Light Laboratories. Synthetic harderoporphyrin trimethyl ester was a gift from Professor A. H. JACKSON, Department of Organic Chemistry, University of Wales, Cardiff. Uroporphyrin, hepta-, hexa-, and pentacarboxylic porphyrin methyl esters were isolated from porphyric urine. Haematoporphyrin IX dimethyl ester[7], isohaematoporphyrin IX dimethyl ester, bis-β-hydroxypropionic deuteroporphyrin IX tetramethyl ester[8], and mono-β-hydroxypropionic, monopropionic deuteroporphyrin IX tetramethyl ester were prepared from protoporphyrin IX dimethyl ester.

Chromatographic procedure. Glass plates (20 × 20 cm) were spread with silica gel (Camag D5) to a depth of 0.3 mm (Shandon Unoplan leveller with adjustable spreader), dried at 110°, and stored at room temperature exposed to the air for at least 18 h before use. Porphyrin methyl esters (up to 100 μg, depending on the composition of the mixture) in chloroform were applied as a compact spot at one corner of the plate, 3 cm from the margins. Development was carried out in the dark in tanks, previously saturated with solvent vapour, using the following solvent systems: (A) Carbon tetrachloride–dichloromethane–methyl acetate–methyl propionate (2:2:1:1)[9]. (B) Benzene–butanone (40:3). (C) Chloroform–kerosene–methanol (200:100:7), modified from the system of CHU AND CHU[2].

Development was carried out in system A until the solvent reached the top of the plate, which was then removed from the tank and dried in a stream of warm air. The plate was then turned through 90° developed similarly with system B and dried as before. The final development with system C was in the same direction as for system B, the solvent again being allowed to run to the top of the plate. After drying, the chromatogram was examined in visible and UV light (Wood's filter) to reveal porphyrins.

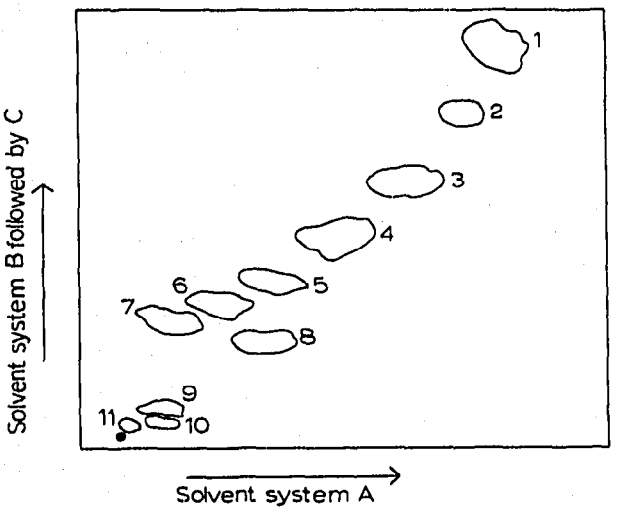

Fig. 1. Separation of a mixture of porphyrin methyl esters by two-dimensional TLC. 1 = mesoporphyrin IX, 2 = harderoporphyrin, 3 = coproporphyrin, 4,5,6 = penta-, hexa- and heptacarboxylic porphyrins, respectively, 7 = uroporphyrin, 8 = mono-β-hydroxypropionic monopropionic deuteroporphyrin IX, 9 = bis-β-hydroxypropionic deuteroporphyrin IX, 10 = haematoporphyrin IX, 11 = isohaematoporphyrin IX (all as their methyl esters).

Porphyrin methyl esters were eluted from the plates as described by CARDINAL *et al.*[4], and estimated by spectrophotometry, using correction factors[10].

Results and discussion

The separation of a mixture of porphyrin methyl esters is shown in Fig. 1. Improved resolution of the higher R_F porphyrins is obtained if development with system B is repeated, and of hydroxylated porphyrins by increasing the volume of methanol in system C (chloroform–kerosene–methanol, 200:100:15). Neither isomers of the I and III series, nor mixtures of protoporphyrin and mesoporphyrin IX dimethyl esters are separated. Porphyrin methyl esters with free carboxyl groups do not remain at the origin, but confusion with other low R_F porphyrin methyl esters can be prevented by comparing chromatograms obtained before and after re-esterification and acetylation[11] of the sample.

Although separations are reasonably reproducible, there is sufficient variation between chromatograms to make standardisation by internal markers desirable. Recoveries of porphyrin methyl esters are given in Table I. Destruction of proto-

TABLE I

RECOVERIES OF PORPHYRIN METHYL ESTERS AFTER TWO DIMENSIONAL TLC

	No. of estimations	*Range (%)*	*Mean (%)*
Coproporphyrin III tetramethyl ester	8	78–87	80
Mesoporphyrin IX dimethyl ester	4	88–96	92
Protoporphyrin IX dimethyl ester	4	32–59	49

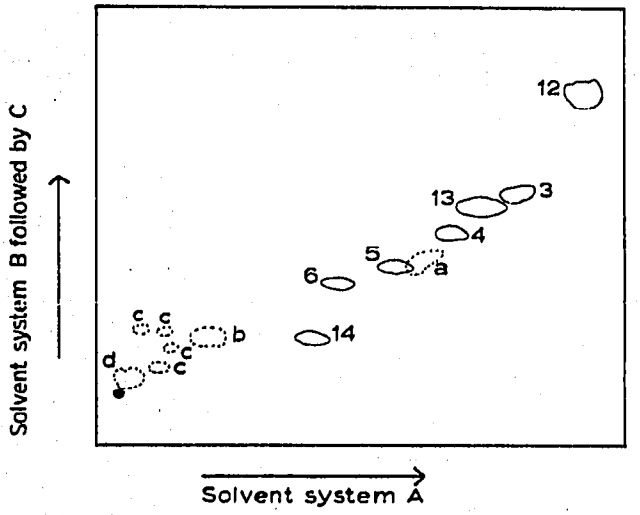

Fig. 2. Separation by two-dimensional TLC of the major porphyrins (continuous lines) and other pigments (dotted lines) extracted from the faeces of a patient with symptomatic porphyria. Pigments were esterified during extraction. 12 = dicarboxylic porphyrin, 13 = an unidentified tetracarboxylic porphyrin[15], 14 = "hydroxycoproporphyrin"[15], a = verdin, b = major violin, c = minor violins, d = brown pigment. Other porphyrins, see legend to Fig. 1.

porphyrin dimethyl ester on TLC in a solvent system containing benzene has been reported previously[3], and may partly account for the low recovery found.

This method has been used extensively to analyse faecal porphyrin excretion patterns (Fig. 2), and should be useful in the investigation of other material, for example bile[12], and normal and neoplastic liver tissue[13,14], where porphyrins with OH groups may otherwise be obscured by non-porphyrin pigments.

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