# A THIN-LAYER CHROMATOGRAPHIC METHOD FOR THE RAPID SEPARATION OF PORPHYRIN DIMETHYL ESTERS

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## INTRODUCTION

Separation of the dimethyl esters of deutero-, haemato-, meso- and protoporphyrin may be achieved by the two-dimensional paper chromatographic procedure of CHU AND CHU[1]. This procedure is however rather time-consuming. Thin-layer techniques have been reported[2–5] for the chromatography of porphyrin esters but to date it has been necessary to use more than one solvent system for the separation of a mixture of the above esters. The present communication describes a method for their rapid separation in one dimension using a single solvent system.

## MATERIALS AND METHODS

### *Preparation of plates*

Plates were prepared in the usual way[6] by spreading a slurry of one part by weight Silica Gel G to two parts by volume water on 10 × 20 cm glass plates by means of the Desaga apparatus at a thickness of usually 0.25 mm; these are called "non-iron" plates. "Iron" plates were prepared by making the slurry with the same volume of 3 % (w/v) aqueous $FeSO_4 \cdot 7H_2O$ instead of water. Plates of each type were air-dried for 1 h, then strips of the gel layer, 8 mm on the sides and 4 mm at the bottom, were cleanly removed by an edge-stripping device[6]. The plates were then activated horizontally 1 h at 110° and allowed to cool in a desiccator for not more than 1 h before being used. Upon activation the "iron" plates became a pronounced yellow colour.

### *Porphyrins*

*Deuteroporphyrin dimethyl ester.* Deuteroporphyrin was prepared from protohaemin by the method of SCHUMM[7,8]. The crude product was esterified in methanol, containing 1 % (v/v) sulphuric acid, for 48 h at 20° in the dark. This was followed by chromatography on a column of alumina using benzene–chloroform, 10:1 (v/v) and crystallization from chloroform–methanol[8].

*Haematoporphyrin dimethyl ester.* Haematoporphyrin was prepared from protohaemin by the method of FISCHER AND ORTH[8,9]. The pigment was then esterified[8] using diazomethane and the ester purified by streaking from solution in benzene onto a 0.5 mm plate of "non-iron" Silica Gel G followed by development with benzene–methanol, 100:7.5 (v/v) and elution with the same solvent mixture.

*Mesoporphyrin dimethyl ester.* Mesoporphyrin was prepared from protohaemin by the method of FISCHER AND MÜLLER[10]. Diazomethane was used to esterify the product[8] after which the ester was purified by streaking from solution in benzene onto 0.5 mm "non-iron" Silica Gel G plates followed by development in benzene–methanol, 100:5 (v/v) and elution with the same solvent mixture.

*Protoporphyrin dimethyl ester.* Protoporphyrin dimethyl ester was prepared from protohaemin by Grinstein's method as modified by FALK[8]. The crude pigment was purified by batchwise absorption of impurities onto alumina from solution in benzene followed by recrystallisation from chloroform–methanol[8].

*Reagents*

All reagents used were "analytical-reagent" grade. Ether was washed free from peroxides and distilled prior to use. Benzene was used without pre-treatment. Chloroform was washed with distilled water, dried over anhydrous sodium sulphate and distilled prior to use. Methanol was dried and purified by the method of LUND AND BJERRUM[11,12].

*Spotting and development of plates*

1–5 $\mu$g of porphyrin ester was applied to the plate in 10 $\mu$l benzene from a capillary pipette which was held in the tube of a microscope from which the objective and eyepiece had been removed; this device enabled precise control over the raising and lowering of the pipette. The plates were developed in cylindrical glass tanks 11 cm diam. by 22 cm in benzene–methanol, 100:5 (v/v). It was found that best resolution was achieved by omitting the equilibration of the plate in an atmosphere of the solvent mixture. Sufficient solvent mixture for one run (60 ml) was poured into the tank after which the plate was immediately lowered carefully into the solvent and the lid placed in position. Development was carried out for about 70 min at 20°; this allowed the solvent front to travel about 17 cm. After development the plates were air-dried.

*Spectrophotometry*

Absorption spectra were obtained by means of a Cary model 14 recording spectrophotometer and a Zeiss model PMQ II spectrophotometer. A Zeiss microspectroscope was also used for measurement of wave-lengths of absorption bands of porphyrin esters both on the plates and in solution.

*pH tests on activated gel*

pH values were determined directly on slurries made from samples of activated silica gel scraped from the plates and mixed with water in the ratio 1 part by weight of gel to 4 parts by volume of water.

## EXPERIMENTAL AND RESULTS

*"Non-iron" plate chromatography*

No separation of the dimethyl esters of deutero-, meso- and protoporphyrin was obtained on Silica Gel G when run in benzene–methanol, 100:5 (v/v). Haematoporphyrin dimethyl ester was however easily separated from the other three in this

TABLE I

$R_F$ VALUES OF PORPHYRIN DIMETHYL ESTERS ON GEL LAYERS[a] WITH AND WITHOUT ADDITIVES

| *Porphyrin ester* | *Additive* | | |
|---|---|---|---|
| | *Nil* | *Iron*[b] | *Acid*[c] |
| Proto- | 0.92 | 0.55 | 0.26 |
| Deutero- | 0.88 | 0.33 | 0.21 |
| Meso- | 0.92 | 0.24 | 0.19 |
| Haemato- | 0.14 | 0.03 | |

[a] 0.25 mm layer of Silica Gel G; development in benzene–methanol, 100:5 (v/v).
[b] Gel slurried in 3 % (w/v) $FeSO_4 \cdot 7H_2O$.
[c] Gel slurried in 0.3 % (v/v) sulphuric acid.

system as its $R_F$ value is only about one seventh the value of the other esters (Table I). These results are similar to those reported by DEMOLE[2].

*"Iron" plate chromatography*

The addition of iron to Silica Gel G brought about a remarkable difference in the chromatographic behaviour of the porphyrin esters investigated and enabled

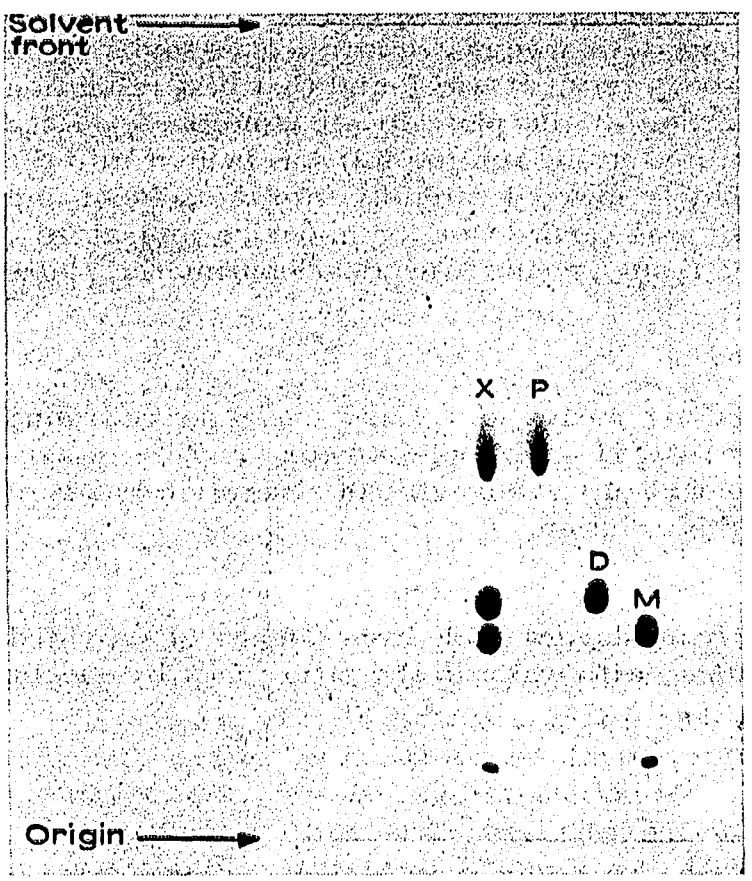


Fig. 1. Thin-layer (0.25 mm) chromatography on an "iron" plate of Silica Gel G slurried in 0.3 % (w/v) $FeSO_4 \cdot 7H_2O$ of the dimethyl esters of protoporphyrin (P), mesoporphyrin (M), deuteroporphyrin (D) and a mixture of these three (X); development in benzene–methanol, 100:5 (v/v) 55 min, 20°.

their rapid separation as shown in Fig. 1. Haematoporphyrin dimethyl ester (not shown) again had a very much lower $R_F$ value than any of the other three (Table I). The concentration of iron sulphate (3 % w/v) used in making the 0.25 mm gel layer was that which was found to be optimal. As the concentration was lowered below this value, the resolution decreased and the result approached that obtained with a "non-iron" plate. Increases in concentration of up to five-fold that quoted did not provide any improvement in the result. It was observed that an approximate inverse relationship existed between the optimal concentration of iron sulphate used for making the gel layer and the layer thickness. Thus for 0.5 mm layers, 1 % (w/v) $FeSO_4 \cdot 7H_2O$ solutions gave satisfactory results.

*Absorption spectra of porphyrins on the gel layers*

Absorption spectra of the porphyrin spots after development were examined on the plates by means of a hand spectroscope and a microspectroscope using reflected and transmitted light. The porphyrins on the "non-iron" plates exhibited the typical spectra of neutral porphyrins whereas those on the "iron" plates exhibited the spectra of the dicationic porphyrins. This was to be expected as slurries made from silica gel removed from activated plates gave pH values of 6.9 and 2.6 for the "non-iron" and "iron" plates, respectively.

*Resolution after addition of acid*

In order to test the effect of a similar acidity to that of the "iron" plates but in the absence of iron, plates were spread using a slurry of Silica Gel G made up as before but in 0.3 % (v/v) sulphuric acid. After activation in the usual way and suspension in water of a similar quantity of gel as was used for the above pH tests, this material was found to give a pH value of 2.6. It was found with plates made in this way, spotted and run under the usual conditions, that deutero- and meso- did not separate and that there was incomplete separation of the protoporphyrin dimethyl ester from these two; the $R_F$ values which were obtained are listed in Table I. All of the porphyrin esters investigated on this type of plate exhibited typical dicationic absorption spectra.

*Fluorescence of spots in ultra-violet light*

All of the porphyrin spots on the above plates fluoresced under ultra-violet light. The spots on "non-iron" plates to which acid had not been added did not however fluoresce as strongly as either the spots on plates to which acid had been added or those on the "iron" plates.

*Tests on porphyrins after development on "iron" plates*

Deutero-, haemato-, meso- and protoporphyrin dimethyl esters were streaked separately onto "iron" plates and run under the usual conditions. After drying in air, the gel containing the spot was scraped off and the porphyrin eluted with the developing solvent. In each case part of the eluted pigment was used for spectral studies and part was respotted but this time onto a "non-iron" plate and run in comparison with a sample of the original pigment not previously run on an "iron" plate. No differences were observed with respect to either light absorption or to chromatographic behaviour between the pigments run on "iron" plates and those untreated.

*Tests with other metals incorporated into Silica Gel G*

Plates (0.5 mm) were spread with slurries made in 1 % (w/v) solutions of $CuSO_4 \cdot 5H_2O$, $MnSO_4 \cdot 4H_2O$ and $MgSO_4 \cdot 7H_2O$, dried, activated, spotted and run as usual. In each case the results were similar to those obtained on the "non-iron" plates. Slurries of the activated gels in water were found to have the following pH values: "Cu-gel", 5.1; "Mn-gel", 6.5; "Mg-gel", 6.7. Plates were prepared with 0.25 mm thick layers of Silica Gel G which had been slurried in 2.7% (w/v) $CuSO_4 \cdot 5H_2O$ dissolved in 0.3% (v/v) sulphuric acid. This solution was equimolar with the $FeSO_4 \cdot 7H_2O$ used for the same thickness "iron" plate and the pH value of 2.4 of a slurry of the gel in water after activation was close to that found for the "iron" plate material. Separation of the porphyrin esters on this plate was no better than on the "non-iron" plates to which acid had been added. It was observed however that with each of the porphyrin esters investigated there was a diffuse, fast-running spot which did not fluoresce in ultra-violet light. These spots were examined *in situ* with a hand spectroscope and were found in each case to possess two bands in the visible region of the spectrum.

In order to test the possibility of formation of copper-porphyrin derivatives under the above conditions, protoporphyrin dimethyl ester was chromatographed on columns of copper-impregnated and activated gel both with and without added acid and using the usual solvent system. In the case of the "acid-copper" columns two fractions were observed, a fore-running fraction which did not fluoresce under ultra-violet light and the absorption spectrum of which was identical with that reported for copper protoporphyrin dimethyl ester[8], and a slow-running fraction which fluoresced strongly under ultra-violet light and which exhibited a spectrum identical with that of the neutral porphyrin ester[8]. The single component which was obtained from copper columns to which no acid had been added did not fluoresce and exhibited the spectrum of the copper derivative.

*Other methods of incorporation of iron to the plate*

Iron was introduced onto a plate already spread and air-dried by spraying it with a solution of ferric chloride in acetone. It was found however that the iron remained largely on the surface of the plate and when run in the usual way the porphyrin esters moved preferentially to the lower layer where there was less iron and the chromatography then followed essentially the pattern of the "non-iron" plates. It was found possible to introduce the iron from solution in acetone by placing the end of a plate in the solution and allowing the solution to run up the plate. The iron was not spread uniformly over the plate however and formed successive bands of varying concentration along the length of the plate. Nevertheless successful separations of the porphyrin esters were achieved by this method and it did allow the possibility of utilisation on the one plate of both "iron" and "non-iron" regions.

*Impregnation of paper with iron*

Whatman No. 1 chromatography paper was impregnated with solutions of ferric chloride varying in concentration between 1.8 and 5.4 % (w/v)—calculated as $FeCl_3 \cdot 6H_2O$, and dried at 110° for 6 min. Deutero-, haemato-, meso- and proto-porphyrin dimethyl esters were spotted onto the papers which were then developed in the same solvent system as that used for the thin-layer chromatography. Chromato-

graphy of the esters was also carried out on untreated paper. There was a striking difference between the two types of paper; in the absence of iron, all but the haematoporphyrin ester ran at the solvent front whereas in the presence of iron, the four esters showed distinctly different $R_F$ values. However, the spots possessed long, diffuse fronts in contrast to the concise spots obtained on the "iron" gel plates. The optimal concentration of iron calculated as above was found to be 1.8%; $R_F$ values are listed in Table II. It is noteworthy that the order of $R_F$ values of meso- and deuteroporphyrin esters was reversed from that obtained on the "iron" plates.

TABLE II

$R_F$ VALUES OF PORPHYRIN DIMETHYL ESTERS ON IRON-IMPREGNATED PAPER[a]

| *Porphyrin ester* | *Proto-* | *Meso-* | *Deutero-* | *Haemato-* |
|---|---|---|---|---|
| $R_F$[b] | 0.8 | 0.6 | 0.4 | 0 |

[a] Whatman No. 1 paper "for chromatography" impregnated with 1.8% (w/v) ferric chloride calculated as $FeCl_3 \cdot 6H_2O$; development in benzene–methanol, 100:5 (v/v).
[b] $R_F$ values estimated from areas of greatest density as spots showed long diffuse fronts.

In order to test the effect of acid in the absence of iron, on the running of the porphyrin esters on paper, Whatman No. 1 paper strips were impregnated with sulphuric acid at the following concentrations: 0.001 *N*, 0.01 *N* and 0.1 *N*, after which they were air-dried. The four porphyrin esters used in this series were then spotted on to each of the papers and run under the same conditions as those used throughout. The results with 0.001 *N* sulphuric acid impregnated paper were similar to those obtained with untreated paper; in contrast, with the 0.1 *N* sulphuric acid impregnated paper the four porphyrin esters remained at the origin. On the 0.01 *N* sulphuric acid impregnated paper however the behaviour of the spots paralleled the results which were obtained with the acid "non-iron" gel plates; there was no separation of meso- from deutero- and there was incomplete separation of the proto- from these two; haemato- which travelled slowest was satisfactorily separated.

*Fluorescence in ultra-violet light and visible light absorption, of porphyrin esters on paper*

The porphyrin esters investigated fluoresced on both acid-treated and untreated paper whereas they exhibited no fluorescence on iron-treated paper. Neutral absorption spectra were displayed by the esters on untreated paper and on 0.001 *N* sulphuric acid impregnated paper whereas on paper treated with the more concentrated acid solutions and also on iron-treated paper, the spectrum appeared in each case to be that of the dicationic porphyrin with some small contribution from the neutral porphyrin spectrum.

## DISCUSSION

That a change in the acidity of the medium should cause some change in the chromatographic behaviour of the dicarboxylic acid porphyrins, dependent to an extent on the particular peripheral substituent groups, is only to be expected from a

consideration of the p$K$ values of the pyrrol nitrogens of these pigments[8]. It is clear however from the results both on layers of Silica Gel G and also on paper that the presence of iron exercises some special effect. If the result were restricted to Silica Gel G it would be reasonable to suppose that some change had been brought about in the gel structure, even though, as far as examination with the light microscope allowed, no change could be detected either in gel structure or in degree of aggregation between the iron-treated and untreated gels. This was not so in the case of the gel to which copper alone had been added; here a marked increase in aggregation occurred but which was without effect on the porphyrin separation. Also the importance which might be attached to changes in gel structure due to the presence of iron, would appear to be offset by the occurrence of the phenomenon on paper and might rather point to a chemical effect involving the porphyrin and a component of the stationary phase. It is interesting that combination was observed between copper and the porphyrins at room temperature under the conditions of chromatography. WANG AND FLEISCHER[13] have reported a similar combination in acetone solutions. It is tempting to postulate a weak combination of the porphyrins with the iron of the plate or the paper, such combination being dependent upon the peripheral substituent groups of the porphyrins and the acidity of the medium. A weak binding of iron to porphyrin was claimed to have been obtained by WANG AND FLEISCHER[13]; it should be pointed out however that PHILLIPS[14] has disputed this claim.

SUMMARY

The addition of iron salts to thin-layer plates of Silica Gel G was found to facilitate the separation of porphyrin dimethyl esters so greatly that rapid separation in one dimension and in one solvent system could be carried out. A somewhat similar result, although not so marked, was also observed with iron-impregnated paper. It was shown that pH adjustment of the medium by addition of mineral acid alone, to the value resulting from iron-salt hydrolysis in the medium, will not duplicate the effect. Addition of Cu, Mn or Mg to Silica Gel G was ineffective.

REFERENCES

1 T. C. CHU AND EDITH JU-HWA CHU, *J. Biol. Chem.*, 208 (1954) 537.
2 E. DEMOLE, *J. Chromatog.*, 1 (1958) 24.
3 R. W. BALEK AND A. SZUTKA, *J. Chromatog.*, 17 (1965) 127.
4 J. JENSEN, *J. Chromatog.*, 10 (1963) 236.
5 T. C. CHU AND EDITH JU-HWA CHU, *J. Chromatog.*, 21 (1966) 46.
6 H. R. BOLLIGER, M. BRENNER, H. GÄNSHIRT, H. K. MANGOLD, H. SELLER, E. STAHL AND D. WALDI, in E. STAHL (Editor), *Thin-Layer Chromatography*, Springer, Berlin and Academic Press, New York, 1965.
7 O. SCHUMM, *Z. Physiol. Chem.*, 178 (1928) 1.
8 J. E. FALK, *Porphyrins and Metalloporphyrins*, Elsevier, Amsterdam, 1964.
9 H. FISCHER AND H. ORTH, *Die Chemie des Pyrrols*, Vol. 2, Part 1, Akad. Verlagsgesellschaft, Leipzig, 1937.
10 H. FISCHER AND R. MÜLLER, *Z. Physiol. Chem.*, 142 (1925) 120, 155.
11 H. LUND AND J. BJERRUM, *Ber. deut. chem. Ges.*, 64 (1931) 210.
12 J. A. RIDDICK AND E. E. TOOPS, in A. WEISSBERGER (Editor), *Techniques of Organic Chemistry*, Vol. 7, Interscience, New York, 1955, p. 334.
13 J. H. WANG AND E. B FLEISCHER, in J. E. FALK, R. LEMBERG AND R. K. MORTON (Editors), *Haematin Enzymes*, Pergamon, London, 1961, part 1, p. 38.
14 J. N. PHILLIPS, discussion in ref. 13.